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Drug accumulation in melanin: an affinity chromatographic study

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Abstract

The affinity of several drugs to melanin has been indirectly assessed using an affinity chromatographic approach based on immobilized melanin. Plots of the retention of the drugs on the affinity column versus the number of molecules applied were fitted best by nonlinear, exponential curves characteristic for each drug. These curves reflect the complexity of the binding behaviour, consisting of a variety of hydrogen bonding, hydrophobic or ionic interactions as well as cooperative or anti-cooperative interactions between the drug molecules and melanin. The nonlinear fitting procedure was based on a descriptive function and allowed to discriminate the binding behaviour according to parameter estimates which specified the investigated drugs.

Keywords: Melanin; Chloroquine; Haloperidol; Zotepin; Trimipramine; Desipramine; Sulpiride; Clonazepam; Flunitrazepam; Benzodiazepine metabolites; Benzophenones

1. Introduction

Melanins are biopolymers mainly composed of indole-5,6-quinone units with several dopachrome and 5,6-dihydroxyindole carboxylic acid moieties present in the molecule. Melanin pigments are commonly classified into sulfur-containing pheomelanins and the non-sulfur-containing eumelanins [1]. One biological precursor of both forms is L-DOPA which yields eumelanin after oxidation, subsequent cyclization and polymerization. In the formation of pheomelanin, the addition of cysteine results in cysteinyldopa which then undergoes polymerization. Synthetic melanins prepared enzymatically or chemically from L-DOPA contain more carboxyl functions than natural melanins.

The binding of substances to melanin is of broad biological and pharmacological interest. Melanins are present in external and internal tissues (skin, hair, ear, eye, and brain). Thus, their capacity to bind and release (exogeneous and endogeneous) substances in a dynamic fashion may result in various, possibly pathogenetic effects onto the organism involved [2–4]. These conditions seem important in the pathogenesis of disease states associated with long-term therapy with a number of drugs. Toxic effects have been best recognized in the case of chlorpromazine and chloroquine, both inducing chorioretinopathy by binding to and subsequent release from retina melanin [5].

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The occurrence of tardive dyskinesia, a serious movement disorder associated with the chronic administration of antipsychotic drugs like haloperidol or tricyclic neuroleptics, is closely correlated to the neuromelanin affinity of these drugs [6]. A successful and promising causual therapy of tardive dyskinesia may consist in the displacement of the melanin-bound neuroleptic by other substances with high melanin affinity, but without any affinity to dopamine receptors [7].

More recently, research has focused on the role of melanin binding in some forms of cancer and cancer therapy. Various carcinogenic substances have been shown to accumulate selectively in pigmented cells of laboratory animals [8]. The use of melanin–affine medicines, accumulating in pigmented tissues, might be valuable in the treatment of diseases with hyperpigmentation. For instance, a melanin–affine anticancer drug for the selective treatment of malignant melanoma seems conceivable.

Pathophysiologically, melanin or the binding of substances to melanin is suspected to play an important role in Parkinson's disease (PD). This suggestion results from the observation that heavily pigmented dopaminergic nigrostriatal neurons are preferentially lost in PD. A selective destruction of pigmented neurons in the substantia nigra is observed after the application of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Its active metabolite methylphenylpyridine (MPP^+) accumulates intraneuronally by the catecholamine uptake system and binds with high affinity to neuromelanin [9]. In addition to this condition, the binding of Fe(II) ions to melanin might be involved in the pathogenesis of PD [10]. Fe(II) ions bound to the redox polymer melanin might cause cell damage by reactive oxygen species like the hydroxyl radical synthesized in the Fenton reaction.

The molecular nature of drug binding to melanin is rather complex. Several parameters, like ionic and aromatic interactions, van der Waals attraction or the formation of charge-transfer complexes determine the affinity of substances to melanin. Both natural and synthetic melanins have been used in binding studies using radiolabelled ligands, and no significant differences have been observed [11]. The use of native instead of radioactively labelled ligands seems preferential since a metabolization of the drug under investigation can not be excluded by measuring radioactivity only. A spectroscopic investigation of the binding to melanin of non-radioactively labelled ligands is difficult since melanins are completely insoluble in water and organic solvents and their molecular weights are not known. In addition, the strong absorption of melanins in the whole spectral range prohibits a spectroscopic detection of free ligand concentrations.

In this study, we investigated the binding of several drugs to melanin using an affinity chromatography method. We employed a stationary phase of immobilized synthetic melanin. The advantages of this procedure are the use of native, non-isotope labelled drugs and the easy spectroscopic determination of the ligands due to the immobilization of melanin.

2. Experimental

Aminopropyl silica (APS, Polygosil 60-5-NH_2) was obtained from Macherey–Nagel, Düren, Germany. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide methiodide (EDC methiodide), 5,6-dihydroxy phenylalanine (L-DOPA), sodium maleate, chloroquine, haloperidol, sulpiride, and desipramine were purchased from Sigma, Deisenhofen, Germany. Flunitrazepam was a gift of Merckle, Blaubeuren, Germany; desmethylflunitrazepam was a gift of Hoffmann–La Roche, Basel, Switzerland, clonazepam was a gift of Arzneimittelwerk Dresden, Dresden, Germany, zotepin and trimipramine were a gift of Rhone–Poulenc Rorer, Köln, Germany. The benzophenone derivatives 2-methylamino-5-nitro-2′-fluorobenzophenone from flunitrazepam (benzophenone I), 2-amino-5-nitro-2′-fluorobenzophenone from desmethylflunitrazepam (benzophenone II) and 2-amino-5-nitro-2′-chlorobenzophenone from clonazepam (benzophenone III) were synthesized by acidic hydrolysis of benzodiazepines according to the procedure of Cano et al. [12]. All other chemicals were obtained from Merck, Darmstadt, Germany and were of analytical grade. Water was purified using a Milli Q UF water purification system (Millipore, Eschborn, Germany).

2.1. Synthesis of the stationary phase

A synthesis of a melanin-based stationary phase for affinity chromatographic studies was described by Ibrahim and Aubry [13]. Nevertheless, this procedure had to be modified since some details were found to be not reproducible in our laboratory. The initial solution of lyophilized melanin in dimethylsulfoxide (DMSO) described by Ibrahim and Aubry is not possible according to our experimental findings. The resulting non-solubilized melanin particles will lead to a great inhomogeneity in the affinity gel and thus to highly variable results in the chromatographic resolution between columns. The use of colloidally solved L-DOPA melanin instead of melanin particles in our synthesis led to an affinity gel with a high homogeneity in particle size.

L-DOPA (150 mg) was dissolved in 60 ml 100 m*M* potassium phosphate buffer pH 8.0 and was allowed to stand at room temperature and daylight for 7 days. The resulting colloidal solution of autoxidized L-DOPA melanin was diluted with 240 ml water. After addition of 3 g APS, coupling of melanin to APS was started by adding a solution of 75 mg EDC in 6 ml water. The reaction mixture was shaken for 12 h in the dark. The resulting stationary phase was filtered off and washed three times with 20 ml DMSO–water (50:50, v/v), nine times with 20 ml 1 m*M* NH_3, and six times with 20 ml water. Immobilization was quantitative as could be judged by the colourless supernatant after the coupling reaction. The immobilized melanin was suspended in 100 m*M* potassium phosphate pH 7.0 containing 10% 1-propanol (v/v) and stored at 4°C.

2.2. Chromatographic setup

Columns (75×4.6 mm) constructed in our laboratory were slurry-packed using 100 m*M* potassium phosphate pH 7.0 containing 10% 1-propanol (v/v) at a flow-rate of 4 ml min^{-1} resulting in a pressure of approximately 4 MPa. The chromatographic experiments were carried out using isocratic elution with this eluent and a flow-rate of 2 ml min^{-1}. The chromatographic system consisted of two Knauer HPLC pumps 64 (Knauer Wissenschaftliche Geräte KG, Berlin, Germany), one delivering the eluent, the other the solution of drug to be investigated. Each pump was continuously purged with the respective solution. A Rheodyne 9725i sample injector was installed allowing to switch between the two pumps. Detection was performed using a Pharmacia VW 2251 variable wavelength detector (Pharmacia Biotech, Freiburg, Germany) at the absorbance maximum of the drugs. All drugs were investigated as solutions in the mobile phase.

The column was equilibrated with eluent before each run. After application of 2 ml of drug solution to the column (approximately five times the void volume), elution was started immediately.

3. Results

3.1. Binding to APS

In order to characterize the retention of the drugs on the HPLC column as a measure of the true affinity to melanin, the binding behaviour of selected drugs with different affinities to melanin was investigated on non-modified APS. Each drug eluted with the void volume of the column and revealed no affinity to this melanin-free stationary phase (Table 1). Thus, the retention on the affinity column may represent the interactions of the drug with immobilized melanin.

3.2. Binding to L-DOPA melanin

Chromatograms of various amounts of desipramine applied to the affinity column are shown in Fig. 1. HPLC retention parameters on immobilized L-DOPA melanin were calculated for several drugs. Retention parameters were dependent on the amount of drug applied to the columns. When more drug was applied to the column, the retention parameters decreased (Table 1). Melanin binding of all drugs investigated was reversible as shown in Fig. 2. Correlation coefficients r between the amount of drug applied and the area under the peak detected ranged from 0.904 for haloperidol to 1.000 for desipramine. Even the highly melanin affine drugs zotepin ($r=0.999$) and chloroquine ($r=0.992$) were eluted completely from the affinity column.

Table 1
Comparison of the retention parameters k of various amounts of drugs on APS and melanin–silica

		Haloperidol	Desipramine	Trimipramine	Flunitrazepam
APS	20 nmol	1.43	0.61	1.00	0.00
	100 nmol	0.70	0.53	0.84	0.00
Melanin	20 nmol	56.9	16.6	15.8	2.1
	100 nmol	20.0	9.4	9.3	1.7

Retention parameters of the drugs were calculated according to the equation $k=(t_R-t_0)/t_0$, where t_R and t_0 are the elution times of the compound of interest and a nonretained compound, i.e. sodium maleate.

3.3. Reproducibility of results

Each column was tested for quality and reproducibility of results by recording binding isotherms for desipramine. The variability of k-values and the parameters c and D (see below) between four different columns of different batches are shown in Table 2.

3.4. Scatchard analysis

Despite of the complexity of the binding of drugs to melanin, a classical Scatchard analysis of the binding isotherms was performed. $V-V_0$ was plotted versus N_0 $(V-V_0)$ to yield a Scatchard plot based on equations derived from enzyme kinetics [14]. The Scatchard plots were arbitrarily divided into two

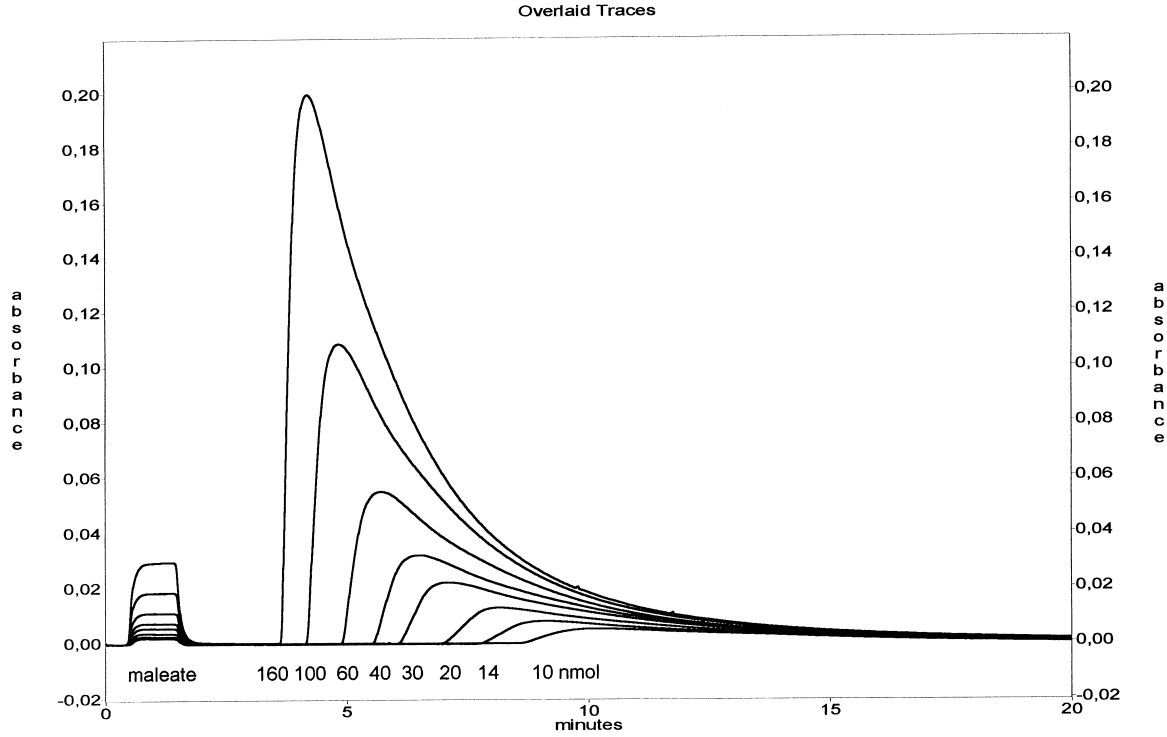

Fig. 1. Chromatograms of various concentrations of desipramine on immobilized synthetic L-DOPA melanin.

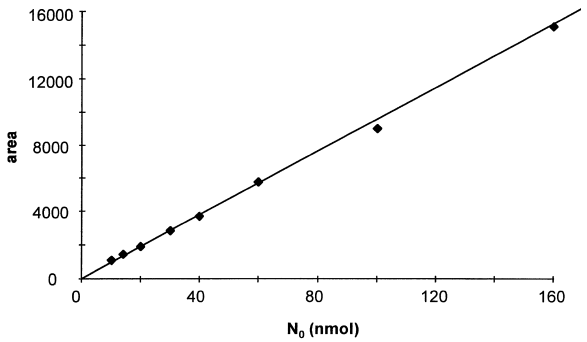

Fig. 2. Correlation between the amount of drug applied to the affinity column and the area under the peak detected for the example of sulpiride. The figure shows a regression line with a correlation coefficient $r=0.999$.

linear parts, one representing low, the other high drug concentrations. V is the elution volume of the drug at a given concentration, V_0 is the void volume of the system and N_0 is the number of drug molecules applied to the column. The analysis resulted in similar affinity constants for each drug, but in a large variability of the total number of binding sites (Table 3).

3.5. Binding isotherms

Due to the shortcomings of the Scatchard analysis, another approach for the determination of the binding behaviour of drugs to melanin was chosen. The

Table 2
Reproducibility of chromatographic parameters between various batches of the stationary phase for desipramine

	k (20 nmol)	k (100 nmol)	c	D
Column 1	15.3	9.3	0.18 [0.17, 0.18]	−11.9 [−14.6, −9.0]
Column 2	16.3	8.5	0.18 [0.17, 0.18]	−10.8 [−12.3, −9.3]
Column 3	13.7	7.6	0.17 [0.17, 0.18]	−9.7 [−11.2, −8.3]
Column 4	16.6	9.4	0.18 [0.17, 0.18]	−10.0 [−10.5, −9.6]

Table 3
Affinity constants K and number of binding sites n for several drugs on immobilized synthetic L-DOPA melanin determined by Scatchard analysis

Drug	Number of binding sites n [10^{-9} mol mg^{-1}]	Affinity constant K [10^{-6} mol l^{-1}]
Chloroquine	375	21
	560	76
Haloperidol	98	38
	270	339
Zotepin	45	13
	78	7
Desipramine	7.8	16
	22	74
Trimipramine	16	41
	31	122
Sulpiride	14	42
	36	137
Clonazepam	0.5	7
	4.3	95
Flunitrazepam	0.8	12
	7.8	155

The first value given for each drug represents the ‘high affinity’ binding sites, the second value represents the ‘low affinity’ binding sites.

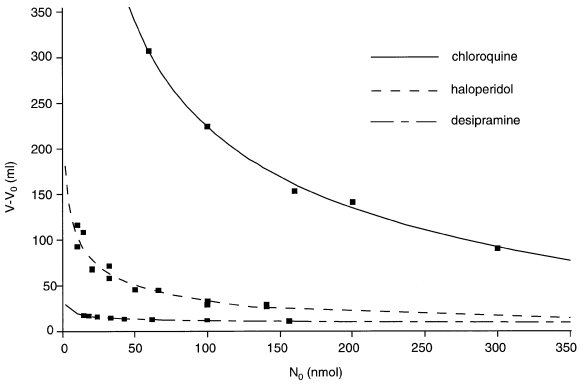

Fig. 3. Binding isotherms of chloroquine, haloperidol, and desipramine on synthetic L-DOPA melanin.

Table 4
Affinity parameters c and D and the respective CI_{95}s for the binding of drugs and benzophenone metabolites of benzodiazepines to synthetic L-DOPA melanin

Drug	c	D
Chloroquine	0.37 [0.36, 0.38]	−171.3 [−198.3, −144.1]
Haloperidol	0.27 [0.26, 0.28]	−51.6 [−69.2, −33.5]
Zotepin	0.27 [0.26, 0.27]	−40.8 [−52.4, −29.0]
Desipramine	0.18 [0.17, 0.18]	−10.0 [−10.5, −9.6]
Trimipramine	0.17 [0.16, 0.17]	− 7.5 [−8.3, −6.7]
Sulpiride	0.15 [0.15, 0.16]	−4.1 [−4.8, −3.3]
Clonazepam	0.08 [0.07, 0.08]	−2.1 [−2.4, −1.8]
Flunitrazepam	0.08 [0.07, 0.08]	−2.0 [−2.4, −1.7]
Benzophenone I	0.15 [0.11, 0.16]	2.8 [−2.1, 8.3]
Benzophenone II	0.15 [0.15, 0.16]	−4.0 [−5.6, −2.4]
Benzophenone III	0.15 [0.14, 0.16]	−5.6 [−7.0, −4.3]

measured retention ($V-V_0$) was plotted versus the applied particule number N_0. The void volume of the system was determined as the elution volume of non-melanin affine sodium maleate. Typical values were about 0.8 ml. We fitted the data points using Freundlich's isotherm $(V-V_0)=k\ N_0^{\ m}$ [15]. The obtained curve did not represent an optimal fit to the data points. Therefore, another approach, the descriptive function $(V-V_0)=D+1/N_0^{\ c}$ was chosen to be fitted to the data points. For each drug, the parameters c and D were estimated by nonlinear regression analysis. This fitting procedure led to markedly better approximations (Sum of Standard Errors (SSE) for chloroquine: Freundlich: 232, our isotherm: 106, SSE for desipramine: Freundlich: 0.17, our isotherm: 0.06). Please note that the number of parameters of both approaches was two (k and m or c and D).

Binding isotherms of several drugs are shown in Fig. 3. The parameters obtained from nonlinear fitting of the data points are listed in Table 4 together with the respective 95% confidence intervals (CI_{95}).

3.6. Competition studies

Competition studies were carried out between haloperidol and desipramine. The column was equilibrated with elution buffer containing 1 μM haloperidol. Plots of $(V-V_0)$ versus N_0 for desipramine in the absence and in the presence of 1 μM haloperidol are presented in Fig. 4. Scatchard analysis revealed a loss of possible binding sites n for desipramine (5.6 instead of 7.8×10^{-9} mol mg^{-1} for high affinity sites and 17 instead of 22×10^{-9} mol mg^{-1} for low affinity sites) as well as higher values for the affinity constant K (21 instead of 16×10^{-6} mol l^{-1} and 85 instead of 74×10^{-6} mol l^{-1}, respectively) in the presence of haloperidol. Nonlinear fitting resulted in parameters of $c=0.15$, $CI_{95}=[0.14, 0.15]$ and $D=-5.9$, $CI_{95}=[-7.0, -4.7]$ for the melanin binding of desipramine in the presence of haloperidol. These two parameters are significantly different from those obtained for desipramine in the absence of haloperidol (see Table 4).

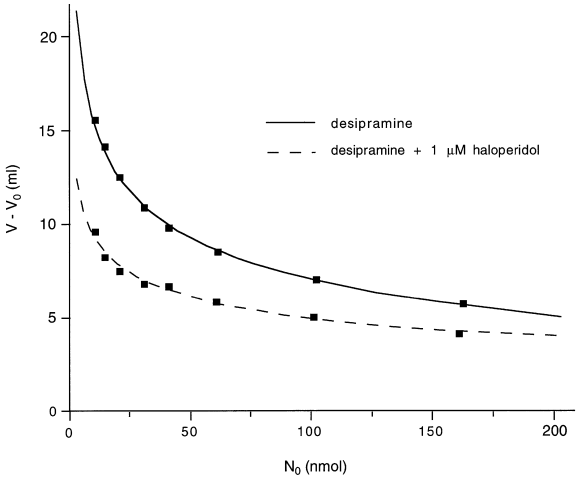

Fig. 4. Binding isotherms of desipramine on synthetic L-DOPA melanin in absence and presence of 1 μM haloperidol in the elution buffer.

4. Discussion

In this study we investigated the binding of drugs to synthetic L-DOPA melanin. We have developed an affinity chromatographic method based on a stationary phase of melanin covalently coupled to aminopropyl silica via amide bonds between the carboxyl functions of melanin and the amino functions of APS. Drug doses were applied to the column to simulate the actual binding behaviour to melanin stores in vivo after administration of a drug bolus to a patient.

Studies on the melanin affinity of several drugs have been carried out earlier, employing classical methods for the investigation of ligand binding to biopolymers like equilibrium dialysis [16,17] or ultrafiltration methods [18]. Shortcomings of these methods are for instance the unspecific binding of the drug molecules to the dialysis membrane or ultrafiltration membrane, leading to large uncertainties in the determination of drug–melanin interactions.

In affinity chromatography, the volume necessary to elute the drug applied to the column depends on the total number of binding sites available, the concentration of the drug and its affinity [19]. Thus, the difference between the volume V at which the front of the ligand appears and the void volume of the column V_0 reflects the affinity of a drug to melanin. Large elution volumes are associated with high melanin affinities and a large number of binding sites. Multiple binding sites for each drug may be supposed due to the variability of the covalent structure of melanin. Each drug thus may use several specific binding sites expressed by the melanin macromolecule.

The binding behaviour of small molecules to biological macromolecules is determined by a variety of interactions involving hydrogen bonding and hydrophobic or ionic interactions. In addition, cooperative or anticooperative binding behaviour must be considered in the analysis of binding isotherms [20]. An (anti)cooperative influence on the binding strength of free ligands is induced by ligands bound somewhere on the macromolecule. For large ligands, a mutual exclusion of binding sites also must be considered [21].

Especially binding to a multitude of different sites provides a complex thermodynamic profile due to their variety and variability. Rather non-specific binding must be assumed to occur in the case of interactions with melanin, in contrast to a highly specific ligand binding to receptors or enzymes. This non-specific character renders the interpretation of the multiform binding behaviour seen with melanin difficult.

Scatchard analysis of the linear parts of the binding isotherms allowed to discriminate between the different drugs. The elution behaviour of all drugs investigated correlated exactly with the total number of binding sites obtained from Scatchard analysis. No correlation, however, was found between the elution behaviour and the affinity constants. The similar values of K for all drugs result from the similar concentrations investigated. The investigation of other concentrations was prohibited by the detection limits of our chromatographic method. A variation of the number of binding sites with similar values of K has been reported by Ibrahim and Aubry [13] for the binding of promethazine and chlorpromazine to melanin. Our Scatchard analysis only reflected two arbitrarily chosen classes of binding sites. In addition, the correct application of this binding analysis would require the exact knowledge of all interactions occuring in the binding process [20]. Especially for the binding of ligands to melanin, a process which is not completely understood, the application of classical binding studies may therefore result in erroneous results.

Another classical approach for the calculation of binding isotherms data which do not obey the Langmuir expression is based on an empirical formula, the Freundlich isotherm. This equation, empirical at the time of its proposal, is widely applied for the analysis of unspecific ligand binding like the adsorption of toxic substances on activated charcoal [22]. We found that the Freundlich isotherm did not fit our data points satisfactorily. Therefore, we used another exponential function for nonlinear regression analysis of the binding of drugs to melanin. This function was fitted to all data points and the resulting parameters c and D allowed to discriminate the affinities of several drugs to melanin. Although these parameters are not based on thermodynamic or kinetic considerations, they characterize the data points rather exactly, as can be judged from the

narrow CI_{95}s. Therefore, *c* and *D* reflect the binding of the drugs to all binding sites in the biopolymer, in contrast to the two classes of binding sites in Scatchard analysis which were arbitrarily chosen. Information on dissociation constants of the drug–melanin complexes, interactions between drug molecules and the number of binding sites from these parameters, i.e. the mechanistic interpretation of our parameters *c* and *D*, requires further work which is in progress in our laboratory.

The assumption of a multitude of various binding sites with different affinity constants might finally lead to a similar functional graph as $(V-V_0)=D+1/N_0{}^c$, describing the binding isotherm, but this approach will be complicated by interactions between the bound and the free drug molecules. None of the classical methods for the analysis of binding isotherms utilizes a function which fits to the data points with similar quality as this, although merely descriptive, exponential function. (Quality in this context means goodness-of-fit with a minimum of parameters to be estimated.)

As can be seen by a comparison of Table 1 and Table 2, the retention parameters *k* and the values of the parameters *c* and *D* closely correlate with each other; *c* is a parameter describing the inflection of the resulting nonlinear curve, and, hypothetically, the competitions between the bound and free drug molecules for their binding sites. *D*, an asymptote, may reflect the affinity of high drug doses to melanin.

High melanin affinities of chloroquin and haloperidol are well known [5,6]. For the neuroleptic zotepin, an affinity to melanin has not been evaluated so far. According to the literature, an investigation of the binding of desipramine, trimipramine, and sulpiride to melanin has not been carried out as well.

In the case of flunitrazepam, we did not observe the affinity to melanin of 9-[^{3}H]-flunitrazepam described by Kuhn after repeated administration [23]. Another study described binding of [*N-methyl*-^{3}H] flunitrazepam to melanoma cells which, however, was independent on the melanin content of these cells [24]. Both benzodiazepines, flunitrazepam and clonazepam, have been shown to induce melanogenesis due to binding to B16/C3 mouse melanoma cells [25]. In that study, a lack of correlation between the binding and the extent of melanogenesis was observed. Therefore, the authors hypothesized that the benzodiazepines would have to be metabolized or internalized to affect melanogenesis.

Searching for the origin of the purported melanin affinities of benzodiazepines, we investigated the binding of desmethylflunitrazepam, a main metabolite of flunitrazepam, to melanin. This substance even revealed less affinity to synthetic L-DOPA melanin than flunitrazepam, i.e. desmethylflunitrazepam eluted with the void volume of the column (for this reason, a binding isotherm was not recorded). In addition, when a solution of flunitrazepam in eluent was allowed to stand at room temperature for several weeks, a spontaneously formed melanin–affine metabolite was observed. Thus, we supposed hydrolysis of the parent drug yielding metabolites with higher affinity to melanin.

Acid hydrolysis of flunitrazepam, desmethylflunitrazepam and the chemically related benzodiazepine clonazepam led to benzophenones which revealed a significantly higher affinity to melanin than their parent drugs. These benzophenone derivatives might be the melanin–affine substances observed in autoradiographic studies with radiolabelled flunitrazepam [23].

In addition to the investigation of the binding behaviour of single drugs, the affinity chromatographic approach for the determination of melanin binding is also suitable for the detection of interactions between two drugs competing for their binding to melanin. The binding of desipramine to melanin was notedly reduced in the presence of haloperidol. This indicates that desipramine and haloperidol use at least some identical binding sites. A more detailed characterization of the interactions between the two drugs in analogy to the classical methods for the determination of enzyme inhibition seems difficult or even impossible due the large amount of different binding sites in melanin. Nevertheless, the alterations in the binding behaviour allow to investigate the interactions between two (or more) drugs with our method. This may be useful for the identification of substances which may be used to displace drugs bound to melanin, e.g. in the therapy of tardive dyskinesia [7].

In summary, our affinity chromatographic method provides a tool for the rapid and accurate determi-

nation of drug affinities to melanin. Knowledge about melanin affinities enables a prediction of (beneficial or adverse) pharmacological effects of drugs resulting from their binding to melanin. In addition, it allows the characterization of highly melanin affine substances in order to evaluate them as possible therapeutics for a targeted therapy of melanin-associated diseases, like hyperpigmentation conditions, tardive dyskinesia or Parkinson's disease.

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