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Review

Applications of affinity chromatography to the study of drug–melanin binding interactions

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Abstract

This short review reports on progresses in the study of drug–melanin interactions using the technique of affinity chromatography. Melanins are natural or synthetic pigments derived from the oxidation and polymerization of various precursors including L-dopa, tyrosine and cystein. Accumulation of toxic compounds, drugs, and metal ions in pigmented tissues through reversible binding to melanin has been linked to chronic toxicity. Affinity chromatography using chromatographic stationary phases based on physically adsorbed or chemically bonded melanin provides a useful tool for studying the interactions of small molecules and metal ions with melanin © 2002 Bristol Myers Squibb Company. Published by Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Drug-binding; Melanins

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1. Introduction

Melanins are a family of pigmented biopolymers that occur widely in nature and in particular in the skin, hair and eyes of animals. Their main function is to protect internal tissues against UV radiations. In man, melanins are also present in the ear and in certain brain structures like the Substantia Nigra. However, the role of melanins in the brain (neuromelanins) is still mostly unknown.

Observations of drug binding to pigmented tissues have been reported since the 1950s and the pharmacological and toxicological implications of the accumulation of drugs in pigmented tissues are widely acknowledged albeit not fully understood [1–3]. The phenomenon has found its major application in

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forensic science where hair analysis for drugs of abuse is now current practice.

The physico-chemical characteristics of melanins make them quite difficult to study by standard methods. Despite having been studied for so long, the exact structure of melanins has not been fully elucidated. An excellent review of the chemistry of melanins was published in 1988 [1]. Melanins are large irregularly structured polymers of pyrrol units containing carboxyl and phenolic hydroxyl groups, often associated with proteins. They are classified in sulfur-containing melanins (pheomelanins) and non sulfur-containing melanins (eumelanins) based on the results of elemental analysis. Their structure, size and building blocks are infinitely varied. The large, dark eumelanin polymers are practically insoluble. Pheomelanin, which are responsible for colors ranging from carrot red to brown are soluble in basic solutions. Another kind of melanin with a structure similar to eumelanin, is made from the enzymatic or chemical polymerization and oxidation of L-dopa. This synthetic form of melanin is readily available, soluble in base and DMSO and can be synthesized quite easily. For this reason, most studies of drug interactions and all affinity chromatographic applications have been using synthetic melanin, based on results of an early study indicating that synthetic melanin did not differ significantly from natural melanin extracted from bovine eyes [2].

Affinity chromatography using melanin-based stationary phases is still in early stages of development. Only three independent research groups have reported work related to the study of melanin binding by affinity chromatography. Early research was aimed at proving that the approach was valid and possessed some unique features that could facilitate the elucidation of the nature of drug–melanin interactions [4–8]. For this reason, researchers mainly used model drugs for which melanin binding had been well established in vivo or in vitro. One article however, presented work on novel drug entities and opened up the application of the model to the study of drug–drug interactions in the development of new drug candidates [6]. Another study based on affinity chromatography, used columns made of powdered human hair [9]. Although the purpose of the study was not to study binding to melanin but to keratine, it was interesting to note that significant differences were observed between untreated hair and bleached hair, differences that could be in part attributed to interactions with the hair pigment.

This review attempts to give an overview of the advantages and problems of using affinity chromatography to study drug–melanin interactions.

2. Pharmacological relevance of drug binding to melanin

A wide number of drugs and other organic or inorganic substances have been shown to have an affinity for melanins. A non-exhaustive list is presented in Table 1. The capacity of melanin to store and release endogenous and exogenous compounds in a dynamic fashion may result in various, possibly toxic, effects on pigmented tissues. Toxic effects have been best documented for chloroquine and chlorpromazine, which both induce chorioretinopathy by being slowly released into the eye from their melanin binding sites [1,3]. The extrapyramidal

Table 1
Drugs of abuse, metals and pharmaceuticals with an affinity for melanins

Compound name	Ref.s
Chlorpromazine and other phenothiazines	[4,8,5,19]
Clembuterol	[4]
Salbutamol	[4]
Nor-testosterone	[4]
Trenbolone	[4]
Diethylsilbestrol	[4]
Chloroquine	[6,18]
Haloperidol	[6,5]
Zotepin	[6]
Trimipramine	[6]
Desipramine	[6]
Sulpiride	[6]
Benzophenone	[6]
Cocaine	[11]
Opioides	[11]
Imipramine	[8]
Clomipramine	[8]
Amitryptiline	[24]
Methipremazine	[24]
Quinine	[24]
Iron	[18]
Nickel	[18]
Lead	[18]
Copper	[18]

disorders and degeneration of the pigmented neurones, often associated with chronic administration of phenothiazines in psychiatric patients, are believed to be related to the specific affinity of neuroleptics for melanins. Melanin binding could also play a role in cancer therapy as compounds related to thiouracil have been shown to be incorporated into melanin during its biosynthesis, with the potential of treating melanoma. Many drugs of abuse, cocaine in particular also get incorporated in melanocytes and can be detected in hair samples long after the drug has cleared the blood stream [10,11]. Finally, interest in melanin may rebound after some recent findings that synthetic melanin may have some antiviral properties which could be due in part to its ability to bind a viral protein, GP 120, produced by the HIV virus [12–14].

Melanins, like many other biopolymers are chiral molecules. Enantiospecific binding has been reported for other chiral biopolymers like proteins (enzymes, plasma proteins, receptors) and polysaccharides (cellulose, amylose) both in vivo and in vitro with sometimes several degree of magnitude differences in the affinities of enantiomers. Stereospecific interactions with melanins have been reported for cocaine [15] with a ten-fold difference in the affinity of the naturally occurring (−)-cocaine and its synthetic enantiomer (+)-cocaine.

3. Preparation of immobilized melanin

Several approaches have been evaluated for preparing chromatographic supports based on immobilized melanin suitable for affinity chromatography. These attempts go from physically adsorbed to chemically bonded melanin resulting in phases of various degree of stability. Noncovalent immobilization of melanin was originally reported by Kaliszan et al. [7]. L-Dopa melanin, dissolved in alkali, was adsorbed on an immobilized artificial membrane (IAM) stationary phase. The major disadvantage of using a non-covalently bound melanin phase was the continuous leaching of melanin from the stationary phase so that experiments had to be carried out in close circuit, reducing the reusability of the support and complicating the interpretation of the results. The first attempt of making a covalently bound melanin phase was by Howells et al. [4]. Using an activated diol bonded silica phase, L-dopa was immobilized on the silica particle by a Schiff's base reaction. Synthetic melanin was subsequently formed on the silica particles by enzymatic polymerization of L-dopa. Although the authors recognized the potential of this new phase for chromatographic applications, they did not prepare columns but rather used the stationary phase for in vitro incubation as an alternative to natural melanin granules from fish ink. In 1995, Ibrahim and Aubry reported the first stable melanin chromatographic stationary phases [5]. Synthetic melanin was covalently bound to aminopropyl silica via the formation of an amide bond between one carboxyl function of the melanin and the amino group of the stationary phase. The resulting phase, after being washed with ammonia to remove any unbound melanin, did not leach melanin under the experimental conditions of the binding experiments. Several phases were made using this procedure with silica of various pore sizes. The amount of melanin immobilized was directly related to the amount of aminopropyl present at the surface of the silica. A covalently bound melanin phase was prepared independently by a variation of the chemical reaction described above [6]. Four batches were prepared of the same phase and results showed that the procedure was very reproducible with less than 10% variation in the calculated k' for desipramin, a drug with high affinity for melanin. These results indicate that the synthesis of a stable affinity-chromatography phase is relatively straightforward. Another stable phase was also prepared by in situ polymerization of L-dopa into the pores of an aminopropyl silica [5]. This very simple procedure produced columns of equivalent properties to the chemically bound melanin phase but with significantly less melanin immobilized (8–10 mg/g as opposed to up to 25 mg/g). It must be noted, however, that the efficiency of the columns, regardless of the process, was rather poor, less than 1000 plates/m, probably because of the coexistence of multiple types of interactions. Adsorption kinetics on immobilized melanin was found to be rapid compared to adsorption to melanin granules [4]. Fig. 1 presents the adsorption of clenbuterol on sepia melanin granules and immobilized L-dopa melanin as a function of time.

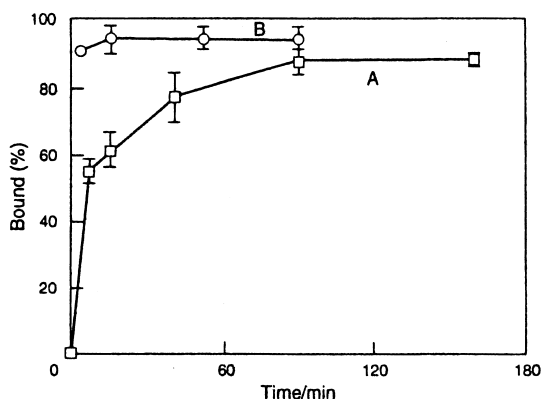

Fig. 1. Effect of incubation time on the adsorption of clenbuterol on natural melanin and on L-Dopa melanin covalently bound to silica. (A) Binding to Sepia melanin; (B) binding to immobilized synthetic melanin. Reproduced from Analyst, Vol. 119, L. Howells et al., Melanin as an adsorbant for drug residues, pp. 2691–2693, copyright 1994, with permission of the Royal Society of Chemistry.

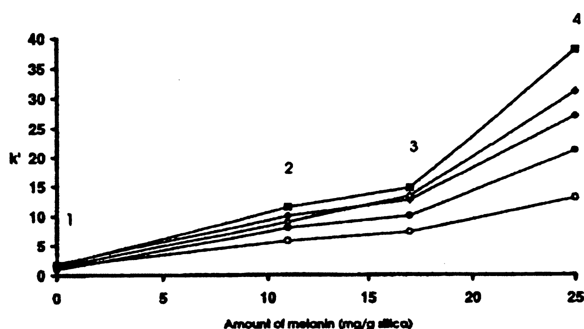

Fig. 2. Influence of the amount of melanin immobilized on the retention of five phenothiazines on melanin-based stationary phases. Column identification: 1: aminopropyl silica; 2 and 3: stationary phase prepared by in situ polymerization of L-dopa within an aminopropyl silica of pore size 100 and 300 Å respectively; 4: stationary phase prepared by covalent binding of L-dopa melanin on aminopropyl silica of pore size 300 Å. Analytes: ■: thioridazine; ♦: chlorpromazine; ●: promazine; ◇: flufenazine; ○: promethazine. Reproduced from Anal. Biochem., 229, H. Ibrahim and A.F. Aubry, pp. copyright 1995 with permission of Academic Press.

4. Experimental approach to studying drug binding to melanin by affinity chromatography

Frontal analysis seems to be the most appropriate technique, mainly because the low column efficiency limits the use of elution chromatography [5]. Nevertheless, elution chromatography proved useful in collecting retention data for the generation of quantitative structure retention relationships (QSRRs) [7,16]. Knörle et al. used elution chromatography with a large injection volume. Samples were infused in the column as a 2-min infusion at 1 ml/min followed by elution with a suitable mobile phase [6]. Retention on melanin-based stationary phases is related to the amount of melanin immobilized on the silica as presented in Fig. 2 for a series of five phenothiazines. The silica support yields negligible retention of the test analytes.

In all cases, data analysis was done by variations of the Scatchard plot, even though it is recognized that the assumptions made in this model may lead to erroneous conclusions regarding the number of sites. Using this approach, the number of sites (expressed as number/per mass unit, since the molecular mass is not known) and the affinity of the drug for each type of site is calculated. An alternative to Scatchard analysis was also proposed [6] using the non-linear descriptive function:

$$(V - V_0) = D + 1/N_0^C$$

where V is the elution volume of the compound of interest, V_0 is the void volume of the system, N is the number of binding sites in nmol and C and D are constants.

Although the fit to the experimental data was much improved, the biological significance of the parameters C and D remain elusive, which limits the general interest of the approach considerably. The binding isotherms of chloroquine, haloperidol and desipramine fitted to the above equation are presented in Fig. 3.

Competitive binding is the area where affinity chromatography has truly proven itself as a superior technique among in vitro experimental approaches [17]. The study of drug–drug interactions is of foremost importance in drug safety. Displacement of one drug by another from its binding sites on a biopolymer provokes an increase in the free concentration of the drug with sometimes grave toxicological consequences [17]. Affinity chromatog-

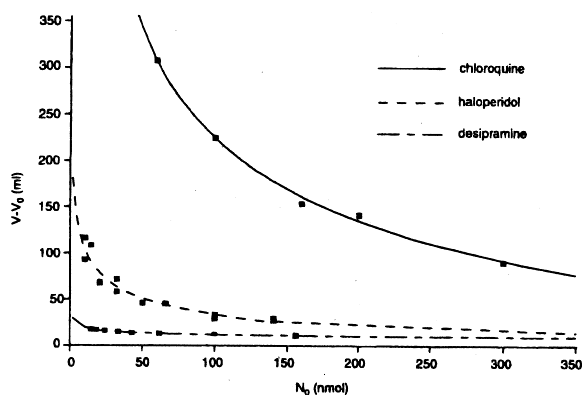

Fig. 3. Binding isotherms of chloroquine, haloperidol, and desipramine on synthetic L-Dopa melanin. Reproduced from J. Chromatogr. B, 714, R. Knörle et al., Drug accumulation in melanin: an affinity chromatographic study, pp. 171–179, copyright 1998 with permission of Elsevier Science.

raphy has proven its superiority over other techniques for the study of drug–drug interactions. Using chromatography simplifies competition studies as the effects on retention are readily observed without having to quantify the two drugs separately [17]. In addition it can discriminate between different types of interactions (direct competition, cooperative binding, inhibition). Melanin is no exception and all reports of melanin based chromatographic stationary phases included experiments on displacement of one drug by another. Interestingly, the competitor selected was always haloperidol due to its high affinity for melanin. Drugs displaced from immobilized melanin by haloperidol included chlorpromazine [5] and desipramine [6]. The displacement of chlorpromazine from its binding site by haloperidol at two different concentrations is presented in Fig. 4.

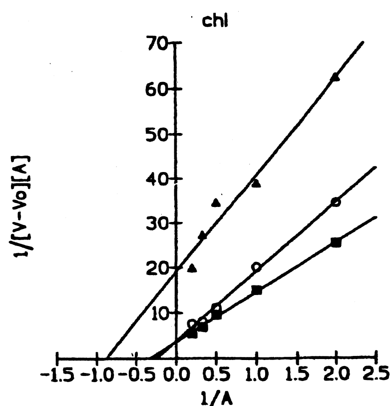

Fig. 4. Competition between haloperidol and chlorpromazine for binding to immobilized L-dopa melanin. ■: Chlorpromazine; ○: Chlorpromazine in the presence of 2×10^{-6} M haloperidol; ▲: Chlorpromazine in the presence of 3×10^{-6} M haloperidol. Reproduced from Anal. Biochem., 229, H. Ibrahim and A.F. Aubry, Development of a melanin-based HPLC stationary phase and its use in the study of drug–melanin binding interactions, pp. 272–277 copyright 1995, with permission of Academic Press.

5. Comparison of affinity chromatography with other methods

All methods for the determination of drug–melanin binding other than affinity chromatographic include an incubation step followed by a detection step. Detection must often be done after separation of the free fraction from the bound fraction using centrifugation or filtration. The free fraction is measured by UV spectroscopy or liquid scintillation counting [2,3,18,19]. Alternatively, electron spin resonance spectroscopy [20,21] and infrared spectroscopy [22] have been used to study the binding of metal ions to melanins. Because of the poor solubility of melanins, drug binding studies on natural pigments are often conducted in non-homogenous conditions in which melanin granules are in suspension in the medium. When the experiment is conducted in solution, the high background UV absorption makes it difficult to detect and accurately measure free ligand concentration. Radiolabeled ligands are often used to overcome these limitations and improve detectability, but their availability then becomes the limiting factor.

Electron spin resonance experiments are based on the observation of a stable free radical in natural [20,21] or synthetic melanins [19]. No other biological polymer contains a stable free radical, which makes the measurements very specific. The ESR signal of melanin is modified by binding of metal ions. The number of binding sites at the surface of the pigment and affinity for melanin can be estimated by plotting the amplitude of the signal as a function of ion concentration. This technique is also used to measure competition of ions for binding sites on

melanin and competition of melanin with metal chelators such as EDTA for binding to metal ions [21]. Accumulation of metal ions was found to be different for melanins of various sources and increased in the following order: synthetic melanin, hair, retina, melanoma, lentigo and nevus of the skin [20]. All published work in this area deals with metal binding to melanin, although the same approach could possibly be applied to small molecules such as pharmaceuticals.

Finally, infrared spectroscopy has been used to study the structure of melanin and melanin complexes with metal ions or other chemicals and provided some insight on the chemical groups involved in binding sites for various ligands [22].

In all of the experiments described above, each experimental point is obtained by incubating melanin with the ligand or a mixture of the ligand and competitor, at each concentration of ligand or competitor. In affinity chromatography, the experiments are faster and easier. Since the polymer is immobilized, only solutes eluting from the column are detected. In addition, the support is reusable for as long as the stationary phase is physically stable (several hundreds of injections) making it a useful tool for screening large series of molecules. In the case of melanin, an added advantage is that the experiment can be conducted at physiological pH and in aqueous medium as opposed to incubation experiments, which may be done in basic conditions to keep the melanin in solution. One potential drawback of affinity chromatography is that the immobilization procedure is susceptible to affect the binding properties of the biopolymer. How much the chemical binding to a chromatographic phase affects the results of binding experiment is evaluated by comparing results obtained by affinity chromatography with results obtained by other methods.

Unfortunately, directly comparable results for melanin are scarce and not very convincing. A comparison of percentage bound, binding affinities and number of sites for chlorpromazine determined by affinity chromatography and by incubation methods is presented in Table 2. There is no agreement of results even within the same methodology. Melanin binding depends on the nature and origin of the polymer used for the experiment. It is also very sensitive to the ionic environment in which the experiment is conducted. Binding of chlorpromazine, chloroquine, paraquat and Ni^{2+} was found to decrease as the concentration of Na^{+} or even H^{+} increased [18]. Presence of small amounts of metal ions in the medium also affects the binding. Although it may not be directly comparable to other

Table 2
Affinity and binding parameters of chlorpromazine on melanin of various types as determined by in vitro incubation or by affinity chromatography

Method	Melanin type	Medium	Number of sites (μmol/mg)	Affinity M^{-1}	Percentage bound	Ref.
Incubation	Ocular	Water	0.14 0.38 0.25 0.015	7.3×10^{6} 8.8×10^{4} 2.2×10^{3} 2.7×10^{8}	85.7	[18]
Incubation	Ocular	NaCl 0.1 M			71.0	[18]
Incubation	L-Dopa	Buffer 0.1 *M*, pH 7.4, 37°C	0.015	2.7×10^{8}		[19]
Affinity chromatography	L-Dopa	Buffer 0.1 *M*, pH 7, ambient	0.016	3.6×10^{5}		[5]
Incubation	Ocular	Buffered medium. Incomplete information			52	[2]

methods at this time, affinity chromatography is still useful in providing comparative data for a series of compounds.

6. Progress in the elucidation of binding interactions with melanin

The most important progress in the study of interactions with biopolymers in recent years has been the use of chemometrics. Quantitative structure activity relationships can correlate structural descriptors of the ligand with a measure of their ''biological activity''. In the case of interactions with a biopolymer, the activity can be the percentage bound, for example. Affinity chromatography provides an excellent means of producing accurate and reproducible data for QSRR derivations. A recent review by R. Kaliszan presents a good summary of the work to date on the subject, looking at biopolymers ranging from plasma proteins to polysaccharides, including melanins [16].

Retention data obtained on melanin-based stationary phases have been used to generate quantitative structure retention relationships with the objective to better understand the molecular interactions of small molecules with melanin [7,8,16]. The following equation was derived for a series of neuroleptic drugs and related inactive phenothiazines:

$$\log k_{\mathrm{MEL}} = -0.2247(\pm 0.0730)\log k_{\mathrm{IAM}} - 0.3256(\pm 0.0760)\,E_{\mathrm{LUMO}} + 0.696(\pm 0.010)$$
$$n = 13, R = 0.933, s = 0056, F = 34, P \leq 0.0001 \quad (1)$$

where k_{MEL} is the capacity factor determined on the melanin column, k_{IAM} is a measure of hydrophobicity determined chromatographically on an immobilized artificial membrane column and E_{LUMO} is the energy of the lowest unoccupied molecular orbital. A graphical representation of the data is presented in Fig. 5. This equation indicates that melanin interactions are hydrophobic in nature. The presence of E_{LUMO} suggests that charge–transfer interactions are significant in the formation of the drug–melanin complex. It must be noted that two

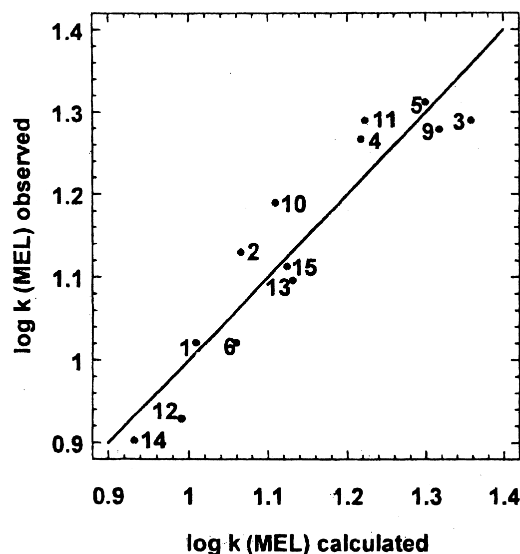

Fig. 5. Plot of the logarithms of the capacity factor, determined for a series of neuroleptic drug and related inactive phenothiazines on an immobilized melanin column, against the corresponding data calculated using Eq. (1). Reproduced from J. Chromatogr. B, 715, R. Kaliszan, Retention data from affinity HPLC in view of chemometrics, pp. 229–244, copyright 1998 with permission of Elsevier Science.

fluorinated compounds did not fit the equation and were excluded from the experimental set. These conclusions were in agreement with two other molecular modeling studies on melanin. In both studies [23,24], the affinity of a series of structurally diverse compounds, calculated from results previously reported in an article by Potts [2] was correlated to molecular descriptors. One study identified the hydrogen donor and acceptor properties (which E_{LUMO} is a measure of) of melanin as the main source of interaction with drug compounds.

7. Conclusions

Like other biopolymers, melanins can selectively bind certain classes of drugs and other toxic substances such as metal ions. Affinity chromatography using stable phases based on immobilized melanin provides a useful tool for rapidly screening compounds. The research so far has been aimed at

developing and validating systems using model compounds but the concept is applicable to the screening of new drug candidates. Efforts should be directed towards validating this approach by comparing results obtained with different techniques within the same laboratory and with extreme care being taken in controlling the source and composition of the melanin and the experimental conditions.

Future developments in the area may include the immobilization of other synthetic melanins mimicking pheomelanin and or natural melanins for a direct comparison of their binding properties, or short-chained oligomers of L-dopa that may improve column efficiency. Another area where affinity chromatography should be useful is in the evaluation of enantioselective binding to melanin. Preliminary tests conducted in the author's laboratory on chloroquine [unpublished results] were inconclusive as to whether immobilized melanin was indeed able to steroselectively bind enantiomers but further investigation would certainly confirm that it is.

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