Journal of Chromatography, 615 (1993) 281–288
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6791

Prediction of drug binding to melanin using a melanin-based high-performance liquid chromatographic stationary phase and chemometric analysis of the chromatographic data

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(First received November 10th, 1992; revised manuscript received February 22nd, 1993)

ABSTRACT

The high-performance liquid chromatographic retention parameters (k) have been determined for a series of 29 phenothiazines and related drugs. The k values were obtained on a hydrocarbon-bound silica stationary phase, an aminopropyl stationary phase and an aminopropyl phase coated with melanin. Polycratic retention data determined on a hydrocarbonaceous column were extrapolated to 0% of organic modifier in binary aqueous eluent yielding the chromatographic hydrophobicity parameter, log k'_w. Logarithms of capacity factors determined isocratically on the aminopropyl column were subtracted from analogous values obtained with the same column loaded with melanin. The resulting parameter, log k'_{m-a}, in combination with log k'_w produced a regression equation (correlation coefficient $r = 0.9531$, significance level $p = 10^{-6}$) which could be used to describe drug–melanin binding efficiency, E_B. Theoretical E_B values were calculated by means of the derived equation for the whole series of 29 drugs chromatographed. The efficiency of binding, E_B to synthetic melanin was also determined by an ultrafiltration method for fifteen members of the series. No statistically significant differences were observed between the E_B values calculated using the chromatographic and ultrafiltration approaches. The results indicate that chemometric analysis of the appropriate chromatographic data is a practical method for the evaluation of melanin binding.

INTRODUCTION

Melanin is a biopolymer which is widely distributed throughout living organisms. The biopolymer is composed mainly of indole-5,6-quinone units (Fig. 1) with several dopachrome and 5,6-dihydroxyindole carboxylic acid moieties present in the pigment [1]. This ubiquitous biopolymer has the capacity to accumulate a wide range of chemically unrelated compounds [1–6] resulting in a variety of pharmacological effects; for example, it has been demonstrated that there is a correlation between ocular toxicity and high affinity for melanin [6] and the melanin binding has been

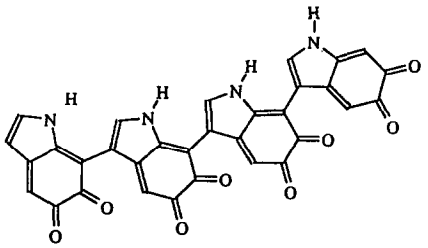

Fig. 1. Structure of melanin tetramer.

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0378-4347/93/$06.00

implicated in ototoxicity and pigment disturbances of the skin and hair [7].

In addition, it has been postulated that the ability of a drug to evoke extrapyramidal disorders is related to its affinity for melanin binding. This is based upon the observation that in Parkinson's disease, the number of pigmented neurons in the substantia nigra and locus coeruleus are decreased and the melanin granules in these brainstem nuclei show a decrease in the dense component of melanin before the complete breakdown of the substructure of the neuron itself [8].

The possible existence of a relationship between some aspects of a drug's toxicity and its affinity for melanin creates the need to rapidly and accurately measure drug–melanin binding. However, the standard experimental techniques used to measure ligand–biopolymer binding such as ultrafiltration are difficult to readily adapt to melanin binding studies. This is due in part to the relative insolubility of the pigment.

One alternative approach to the determination of drug–melanin binding utilized a correlation between the percentage of melanin binding and the binding energy of the drug–melanin tetramer complex as calculated by molecular modelling [9]. However, as the authors indicated, this method is of limited reliability because (1) the energy calculations do not take into account solvation and entropy factors involved in binding and (2) the method does not take into account the ability of the compounds to reach melanin-containing structures *in vivo*. In addition, the affinity data used to derive quantitative structure–activity relationships (QSAR) were based on a single concentration and were considered as qualitative rather than quantitative [6].

The dissociation constants (K_D) and stoichiometry of the interaction between soluble (−)-DOPA melanin and the enantiomers of ephedrine and pseudoephedrine have been investigated by NMR spectroscopy [10]. NMR spectroscopy was used since the method was able to detect and measure the interactions between the test compounds and the indole units of the synthetic pigment. In this study, the observed K_D for (−)-ephedrine was about three times as large as the K_D for (+)-ephedrine, (−)-pseudoephedrine and (+)-pseudoephedrine. The stoichiometric relationship between the test drugs and melanin indole units was approximately 2:1 for (−)-ephedrine and 1:1 for the other isomers. These results indicate that melanin binding is a stereospecific process which may take place at specific sites on the biopolymer.

The results from the NMR studies are interesting and informative, but the technique cannot be readily used in QSAR studies which require data from a large series of structurally related compounds. Another approach to the determination of drug–melanin binding involves the use of high performance liquid chromatography (HPLC) on a melanin-based HPLC stationary phase (MEL-SP). Previous work in this laboratory has demonstrated that chromatographic studies utilizing a human serum albumin-based HPLC stationary phase can accurately determine the extent of drug-protein binding [11,12], the sites at which the binding takes place [12–14] and provide data for QSAR studies and computational modelling of the binding mechanisms [15]. In order to determine if a MEL-SP can be used to rapidly and accurately assess the extent of melanin binding, we have ionically coated an aminopropyl HPLC stationary phase and chromatographed a series of phenothiazines and related compounds on the resulting MEL-SP. This paper reports the initial studies with this new phase and the results indicate that the MEL-SP can indeed be used to measure the extent of drug-melanin binding.

EXPERIMENTAL

Materials

2-Acetylphenothiazine, 2-methoxyphenothiazine and 2-(trifluoromethyl)phenothiazine were obtained from Chemical Dynamics (South Plainfield, NJ, USA). The following agents were purchased from Sigma (St. Louis, MO, USA): acetopromazine maleate, chlorpromazine hydrochloride, ethopropazine hydrochloride, fluphenazine dihydrochloride, perphenazine, phenothiazine, prochlorperazine edisylate, promazine

hydrochloride, promethazine hydrochloride, propiomazine maleate, thioridazine hydrochloride, trifluoperazine dihydrochloride, triflupromazine hydrochloride, trimeprazine tartrate, *cis*-thiothixene, chlorprothixene hydrochloride, carbamazepine, clomipramine hydrochloride, desipramine hydrochloride, imipramine hydrochloride, chlorpheniramine maleate, diphenhydramine hydrochloride, pheniramine maleate, pyrilamine maleate, tripelenamine citrate and triprolidine hydrochloride.

Melanin prepared by oxidation of tyrosine with hydrogen peroxide was also purchased from Sigma. Other substances used were commercially available analytical reagent grade chemicals.

Determination of melanin–drug binding efficiency

Melanin, 20 mg, was dissolved in 0.5 ml of dimethylsulfoxide (DMSO) and the solution diluted with 50 ml of sodium phosphate buffer (0.1 *M*, pH 7.00). A series of five buffer solutions was prepared for each of the test drugs with concentrations ranging from $6.25 \cdot 10^{-9}$ *M* to 10^{-7} *M*. A 1-ml portion of a test solution was added to 1 ml of melanin solution and the mixture was left to equilibrate at room temperature and in the dark. Thus, 0.4 mg of melanin was left to interact with 3.125, 6.25, 12.5, 25 and 50 nmol of a drug. After 2 h, 1 ml of the mixture was transferred to a Centrifree membrane ultrafilter cone (Amicon Division, W.R. Grace & Co., Beverley, MA, USA) and centrifuged at 1800 *g* for 15 min. The concentration of the drug in the filtrate was determined by HPLC employing a Suplex pKb-100 column and a mobile phase composed of sodium phosphate buffer (0.05 *M*, pH 7.00)–methanol (70:30, v/v).

Apparatus

The chromatographic system consisted of a Spectroflow 400 pump, a 480 injector module equipped with 20-μl loop, a 783 programmable absorbance detector (all from ABI Analytical, Ramsey, NJ, USA) and a Shimadzu C-R6A integrator (Shimadzu, Kyoto, Japan).

Chromatographic conditions

The chromatographic experiments were carried out at a flow-rate of 1 ml/min at ambient temperature.

Hydrocarbonaceous silica column, Suplex pKb-100

The Suplex pKb-100 deactivated reversed-phase hydrocarbonaceous silica column (15 cm × 4.6 mm I.D.; 5 μm particle size) was purchased from Supelco (Bellefonte, PA, USA).

Chromatography was carried out polycratically using eluents composed of the following proportions (v/v) of methanol to buffer: 90:10, 80:20, 70:30, 60:40 and 50:50. The buffer of pH 7.00 was prepared by adding sodium hydroxide (0.1 *M*) to a solution of acetic acid (0.02 *M*), phosphoric acid (0.02 *M* and boric acid (0.02 *M*).

Capacity factors were calculated assuming constant dead volume of the column. The dead volume was determined by measuring signals of deuteromethanol or deuterium oxide chromatographed with neat methanol or water eluents, respectively [16]. Logarithms of capacity factors, log k', for individual solutes were regressed against volume fraction of methanol in the eluent. Linearity of the relationship in the whole eluent composition range studied ($r > 0.995$ for all solutes but desipramine for which $r = 0.983$) allowed for extrapolation of log k' to 0% methanol (100% buffer). The normalized to pure buffer retention parameters, log k'_w, were used in further analysis.

Rexchrom S5-100-NH_2 amino column

The aminopropyl stationary phase packed into a stainless-steel column (25 cm × 4.6 mm I.D.; 5 μm particle size) was obtained from Regis (Morton Grove, IL, USA). The chromatography was carried out isocratically using a mobile phase composed of sodium phosphate buffer (0.1 *M*, pH 7.00)–acetonitrile (20:80, v/v). Logarithms of capacity factors, log k'_{amino}, were calculated assuming that the column dead volume was the value obtained when deuteromethanol was chromatographed with neat methanol eluent.

Rexchrom S5-100-NH_2 saturated with melanin

The same column which was used to generate log k'_{amino} data was loaded with melanin and retention data, log $k'_{melanin}$, were determined for the solutes under the same chromatographic conditions used to obtain the log k'_{amino} values, *i.e.* isocratic conditions using a mobile phase composed of sodium phosphate buffer (0.1 *M*, pH 7.00)–acetonitrile (20:80, v/v).

The aminopropyl stationary phase was coated with melanin using the following procedure: (1) a solution of 40 mg of melanin dissolved in 2 ml of DMSO was diluted with 100 ml of sodium phosphate buffer (0.1 *M*, pH 7.00); (2) the melanin-containing solution was recirculated for 36 h through the column at a flow-rate of 1 ml/min; (3) the column was washed with 300 ml of sodium phosphate buffer (0.1 *M*, pH 7.00)–acetonitrile (20:80, v/v); (4) an additional 700 ml of the buffer–acetonitrile eluent was recirculated through the column for 36 h and this mobile phase was used throughout the study. The solutes were dissolved in the mobile phase and injected onto the column.

Reference hydrophobicity parameters

Where possible, logarithms of *n*-octanol–water partition coefficients, log *P*, determined experimentally by the slow-equilibration "shake-flask" procedure, were taken from a recent compilation [17].

Chemometric analysis

The melanin–drug binding efficiencies, E_B, determined by ultrafiltration techniques for fifteen of the solutes studied were related to chromatographic (fifteen solutes) and log *P* (thirteen solutes) data by means of multiparameter regression analysis using the CSS package (StatSoft, Tulsa, OK, USA) run on a personal computer. The relationships derived were tested according to the requirements of a meaningful correlation analysis [18].

RESULTS AND DISCUSSION

Scatchard plots of bound-to-free ligand ratio *versus* bound ligand obtained from the fifteen compounds used in the ultrafiltrate studies were curvilinear. This is in agreement with results reported for chlorpromazine [3]. Instead of questionable [19] attempts to resolve the plots into two or more linear components we decided to analyze linear relationships between amount of ligand bound against ligand added [20]. Excellent correlations ($r > 0.99$) based on five experimental data points observed in case of every agent tested prove reliability of the slope of the plot of ligand bound *versus* added as a quantitative measure of binding efficiency, E_B. The E_B values obtained in this manner are presented in Table I.

The subgroup of fifteen test agents selected for the ultrafiltration studies included eleven phenothiazines and four non-phenothiazine-based drugs with E_B values ranging from 0.4658 to 0.8471. These data are well suited for use in QSAR analysis since, from the view point of meaningful QSAR analysis, it is important that the experimental E_B data cover a relatively wide range of parameter value and are evenly distributed over the range of results.

The HPLC retention parameters log k'_w and $\Delta\log k'_{m-a}$ determined in this study are presented in Table I. The variables log k'_w and log k'_{m-a} were selected among several chromatographic parameters tested as those which best described E_B. The n-octanol–water partition parameters, log *P* [17], commonly recognized as reference hydrophobicity parameters, are listed in Table I for the sake of comparison.

The QSAR equation describing E_B in terms of log k'_w and $\Delta\log k'_{m-a}$ has the following form:

$$E_B = 0.1319(\pm 0.0138)\log k'_w + 0.0962(\pm 0.0381)\,\Delta\log k'_{m-a} + 0.1544 \quad (1)$$
$$t = 9.56;\ p \leqslant 10^{-5}\ (\log k'_w)$$
$$t = 2.52;\ p \leqslant 0.027\ (\log k'_{m-a})$$
$$n = 15,\ r = 0.9531,\ F = 59.5,\ p = 10^{-6}$$

where the figures in parentheses are standard deviations of regression coefficients, *n* is the number of data points used to derive regression, *r* is multiple correlation coefficient, *F* is the *f*-test value, *t* is the *t*-test value and *p* is significance level of individual variables and of the whole equation.

TABLE I

EFFICIENCY OF MELANIN BINDING, E_B, FOR A SET OF PHENOTHIAZINES AND OTHER BASIC DRUGS DETERMINED FROM ULTRAFILTRATION STUDIES (OBSERVED) AND CALCULATED USING EQN. 1 AND THE CHROMATOGRAPHIC RETENTION PARAMETERS OBTAINED ON HYDROCARBON-BOUND SILICA, AMINOPROPYL SILICA AND IMMOBILIZED MELANIN STATIONARY PHASES

The logarithms of the *n*-octanol–water partition coefficients, log *P*, are also presented.

Compound	Efficiency of melanin binding, E_B [a]		log k'_w [b]	log k'_m [c]	Δlog k'_{m-a} [d]	log P [e]
	Observed	Calculated				
Fluphenazine	0.8042	0.8378	4.79	0.8855	0.5352	4.36
Propiomazine	0.7082	0.7353	3.94	0.9368	0.6356	
Ethopropazine	0.7305	0.7566	3.64	0.7330	1.2675	3.48
Triflupromazine	0.8087	0.7704	4.37	0.6894	0.4110	5.19
Promazine	0.6766	0.6544	3.37	0.9445	0.5758	4.55
Trimeprazine	0.7486	0.6690	3.54	0.7295	0.4944	
Chlorpromazine	0.6750	0.7192	4.02	0.6610	0.3584	5.35
Trifluoperazine	0.8472	0.8374	4.86	1.1354	0.4355	5.03
Perphenazine	0.6921	0.7169	4.12	0.6610	0.1977	4.20
Thioridazine	0.8254	0.7808	4.18	1.2961	0.7791	5.90
Prochlorperazine	0.8234	0.8266	4.66	1.2950	0.5974	
Imipramine	0.5824	0.6076	3.15	0.7058	0.3912	4.80
Clomipramine	0.7039	0.7024	3.87	0.8375	0.3891	5.19
Triprolidine	0.4990	0.4821	2.42	0.5805	0.0884	3.92
Diphenhydramine	0.4658	0.4948	2.37	0.3558	0.2880	3.27

[a] Slope of the linear relationship of amount of ligand removed from solution against ligand added.
[b] Intercept of the relationship of log k' against percent of organic modifier in mobile phase. Capacity factors were determined on deactivated hydrocarbonaceous silica column.
[c] Retention parameters determined on melanin-coated aminopropyl column.
[d] Difference in log k' determined on melanin-coated minus melanin-free aminopropyl column.
[e] Logarithm of *n*-octanol–water partition coefficient obtained from ref. 17.

The predictive potency of eqn. 1 regarding drug–melanin binding is illustrated in Fig. 2. It can be noted that one agent (trimeprazine) deviates from the regression, *i.e.* its predicted binding efficiency is slightly lower than the value determined in this study. The E_B values calculated from the chromatographic parameters and eqn. 1 were compared with the values from the ultrafiltration studies using a paired *t*-test, and no statistically significant difference was detected between the two data sets, two-tailed p value = 0.9983.

The analysis of significance of individual terms of eqn. 1 proves a prevailing importance of log k'_w for description of E_B. However, the term Δlog k'_{m-a} which is significant at the 97.3 significance level, appears a valuable correction factor in binding prediction. The two independent variables of eqn. 1, log k'_w and Δlog k'_{m-a}, are practically orthogonal (intercorrelation r = 0.26) and can be safely combined in a multiparameter regression equation. It can be presumed that log k'_w quantifies abilities of solutes to interact with melanin in a less structurally specific but more molecular size ("bulkiness") dependent manner. The parameter Δlog k'_{m-a} seems to account for more structurally specific drug–melanin interactions.

Replacement of log k'_w in eqn. 1 by the standard reference hydrophobicity parameter, log P, leads to the deterioration of the relationship. Multiple correlation coefficient, r, and significance level, p, of the equation E_B = f(Δlog k'_{m-a},

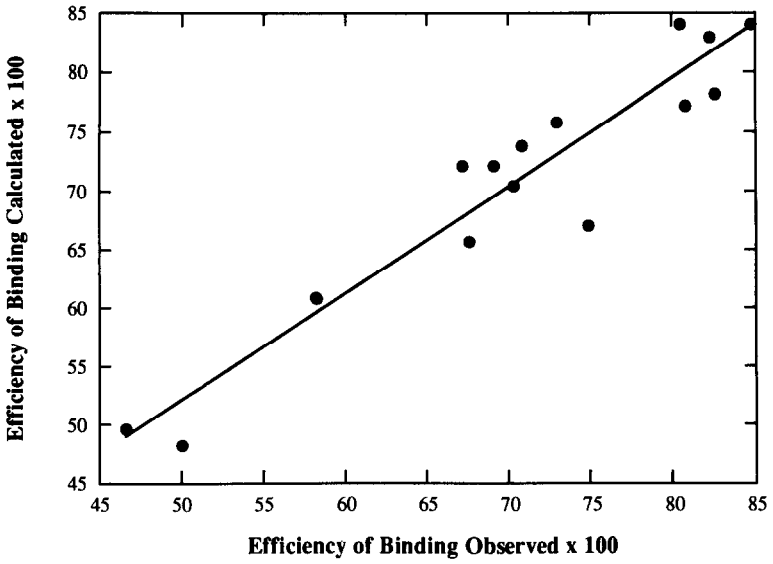

Fig. 2. Relationship between efficiency of binding to melanin determined experimentally and calculated by eqn.1 for a subseries of fifteen test drugs.

TABLE II

EFFICIENCY OF MELANIN BINDING, E_B, FOR A SET OF PHENOTHIAZINES AND OTHER BASIC DRUGS CALCULATED USING EQN. 1 AND THE CHROMATOGRAPHIC RETENTION PARAMETERS OBTAINED ON HYDROCARBON-BOUND SILICA, AMINOPROPYL SILICA AND IMMOBILIZED MELANIN STATIONARY PHASES

Compound	Calculated efficiency of melanin binding, E_B [a]	log k'_w [b]	Δlog k'_{m-a} [c]	log P [d]
Promethazine	0.5231	3.66	0.4187	
2-Acetylphenothiazine	0.6056	3.80	1.0841	
2-Trifluoromethylphenothiazine	0.7692	5.22	0.8373	
Phenothiazine	0.5647	3.86	0.5765	4.15
2-Methoxyphenothiazine	0.6441	3.98	1.2370	
Acetopromazine	0.4691	3.20	0.4880	
Carbamazepine	0.2851	2.15	0.0154	2.45
cis-Thiothixene	0.5351	3.70	0.4884	3.78
Chlorprothixene	0.6231	4.22	0.6898	5.18
Desipramine	0.3182	2.00	0.5645	4.90
Pyrilamine	0.3460	2.18	0.6075	3.27
Pheniramine	0.2360	1.60	0.2589	
Chlorpheniramine	0.3745	2.57	0.3690	3.39
Tripelenamine	0.3318	2.19	0.4464	

[a] Slope of the linear relationship of amount of ligand removed from solution against ligand added.

[b] Intercept of the relationship of log k' against percent of organic modifier in mobile phase. Capacity factors were determined on deactivated hydrocarbonaceous silica column.

[c] Difference in log k' determined on melanin-coated minus melanin-free aminopropyl column.

[d] Logarithm of *n*-octanol–water partition coefficient obtained from ref. 17.

log P) decreases to 0.7582 and 0.02, respectively.

The parameter log k'_w obtained by extrapolation of the reversed-phase HPLC retention data to neat water eluent is considered a convenient measure of solute hydrophobicity [21]. This parameter cannot be assumed identical with log P from the n-octanol–water partition system, however. There is a low correlation between log k'_w and log P (r = 0.63 for nineteen solutes from Table I for which experimental log P data were available). In case of the Suplex pKb-100 HPLC column used here we have partitioning of solutes between alkane of the stationary phase material and aqueous eluent. In case of log P the partition is of an alkanol–water type. Although both hydrophobicity measuring systems reflect some "phobia" of solutes towards aqueous environment, different aspects of the complex phenomenon of hydrophobicity manifest themselves in individual systems [22]. Nonetheless, when interpreting the log k'_w *versus* log P relationship, one must be cognizant of the fact that the log P data are uncorrected for ionization which may actually take place at the pH at which the chromatographic experiments were performed (pH 7.00).

The theoretical binding efficiencies for the remaining fourteen solutes were calculated using the chromatographic data and eqn. 1. The resulting E_B values are presented in Table II and appear reasonable for these agents. However, no relevant quantitative melanin binding data are available for comparison with the calculated values.

It is of interest to explore the possible relationships between the predicted efficiency of melanin binding of compounds (E_B) and biological activity. While there are little data available, there is some information on categorization of phenothiazines regarding their Parkinsonian side effects.

Based on the observation of 1500 patients with intensive phenothiazine medication, Forrest [23] noted that "...chlorpromazine medication produced less dyskinetic side effects than treatment with pyrazine- and piperidine-linked drugs. The most severe symptoms among our patients were produced by the administration of fluphenazine (calculated E_B = 0.8378), perphenazine (0.7169), thioridazine (0.7808) and especially trifluoperazine (0.8374)...". Seeman [24] categorized chlorpromazine (0.7192), chlorprothixene (0.6231) and thioridazine as producing less Parkinsonian signs in contradiction to trifluoperazine and fluphenazine. As a first approximation, the E_B values calculated by eqn. 1 appear to be in agreement with the clinical observations. In addition, the low E_B values calculated for the non-neuroleptic drugs studied in this project are consistent with their observed lack of Parkinsonian-like side effects.

In conclusion we wish to emphasize after Raghavan *et al.* [9] that the ability to predict strongly melanin binding compounds or those lacking significant binding based on theoretical calculations or on simple chromatographic measurements offers a convenient alternative to both the pharmaceutical industry and various drug regulatory agencies when questions arise concerning the safety in human of a new drug entity. The melanin-based HPLC column appears to be a useful tool in this area.

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