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Affinity Chromatography Method for Determination of Binding of Drugs to Melanin and Evaluation of Side Effect Potential of Antipsychotic Agents

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Abstract: The extrapyramidal side effect parameters of typical and atypical antypsychotics were correlated with affinity chromatographic data determined on the melanin-based column. The chromatographic study was performed according to the hypothesis that extrapyramidal symptoms (EPS) as side effects of the use of antipsychotic drugs at clinically effective doses are correlated to the affinity of these drugs to neuromelanin. For that aim the polymerization product of L-DOPA (melanin) was immobilized onto aminopropyl silica and the binding efficiency of melanin towards antipsychotics has been determined. The results indicate that melanin based-column can be used to evaluate the risk of EPS of drug candidates to antipsychotic drug therapy.

Keywords: Affinity chromatography, antipsychotics, drug-binding, extrapyramidal symptoms, melanin binding.

INTRODUCTION

Antipsychotics drugs had a revolutionary, beneficial impact on medical and psychiatric practice [1]. Unfortunately, the conventional drugs can cause extrapyramidal symptoms (EPS), likely to appear even with clinically effective doses. Because EPS have become an unavoidable consequence of the use of antipsychotics, the researchers for many years focused on reducing their frequency [2]. Because of the risk of extrapyramidal adverse actions, antipsychotics were divided into two basic groups: first generation – formerly known as typical, and second – newer generation, so-called atypical antipsychotics with lower risk of adverse extrapyramidal symptoms [3]. This classification relates the EPS to substance-specific pharmacological profiles but does not consider the built-in-anticholinergic effect of some antipsychotics drugs. Hence, the classification is not much useful in clinical practice.

The antipsychotics-neuromelanin binding interaction has received considerable attention as a potentially co-responsible for extrapyramidal side effects [4, 5]. Numerous chromatography based methods were used in evaluating the binding of drugs to melanin [6-8]. Most recently, the new magnetic beads method for the estimation of potential of bioactive agents to evoke adverse effects due to melanin binding was proposed [9]. A significant linear correlation (R=0.8905) between the melanin binding efficiency data of seven antipsychotics and their risk of extrapyramidal side effects (EPS) assessed semiquantitatively, has been presented and proposed for further evaluation. In this study the affinity HPLC method with melanin immobilized on silica as a stationary phase was used as a potential tool for high-throughput screening (HTS) in preselection of drug candidates hopefully devoid of EPS side effects that can be useful in drug research and development process. The detailed analysis of typical and atypical psychotropic drugs was performed and described here.

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EXPERIMENTAL

Chemicals

Amisulpride, clozapine, 3,4-dihydroxy-L-phenyl-alanine (L-DOPA), N-(3-dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC), cis-(Z)-flupenthixol dihydrochloride, fluphenazine hydrochloride, haloperidol, N-hydroxysulfosuccinimide sodium salt, perphenazine, pimozide, risperidone, sertindole, sodium phosphate dibasic were purchased from Sigma-Aldrich (Stainhaim, Germany). Benperidol, bromperidol, as a European Pharmacopoeia Reference Standards, olanzapine, as a United States Pharmacopoeia Reference Standard and zuclopentixol hydrochloride, as a British Pharmacopoeia Chemical Reference Substance were purchased from LGC Promochem (Wesel, Germany). Perazine dimalonate was from LGC GmbH Biotechnologiepark Luckenwalde (Germany) and quetiapine was from Chemicals Inc. (Toronto, Canada). The structural formulae of the studied drugs are given in Fig. (**1**).

Methanol, orthophosphoric acid and 2-propanol of chromatographic purity were all purchased from POCh (Gliwice, Poland). Water used in the study was prepared using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). The aminopropyl silica (APS) stationary phase with pore size 300 Å and particle size 7μm (Nucleosil 300-7 NH_2) was purchased from Macherey-Nagel (Düren, Germany).

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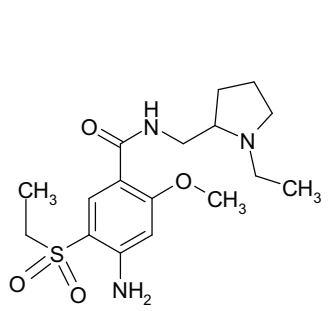
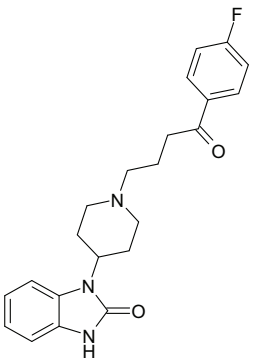
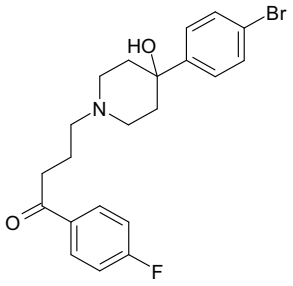


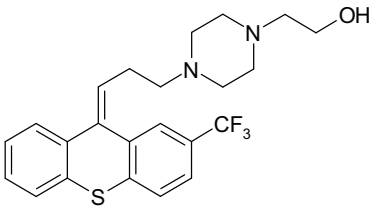
Amisulpride



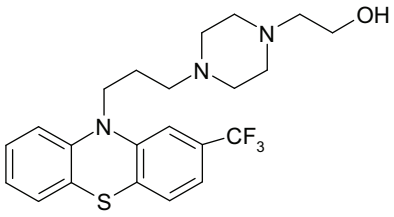
Benperidol



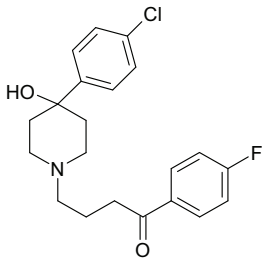
Bromperidol



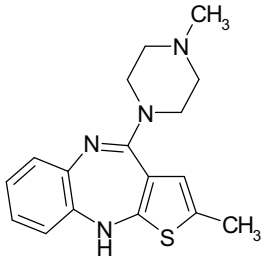
Flupenthixol



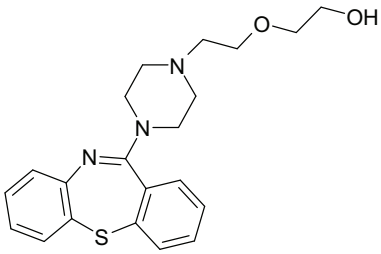
Fluphenazine



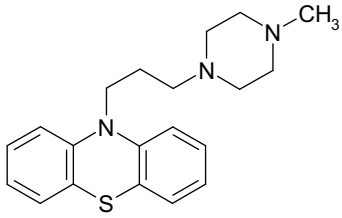
Haloperidol



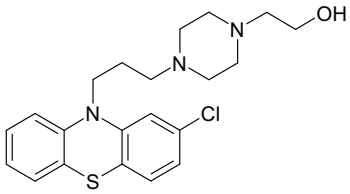
Olanzapine



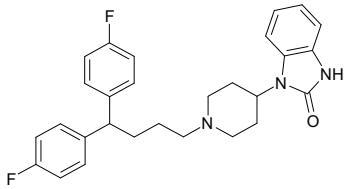
Quetiapine



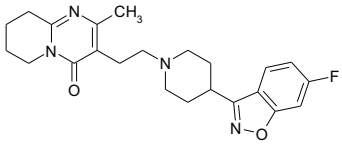
Perazine



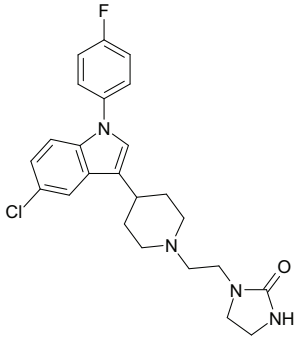
Perphenazine



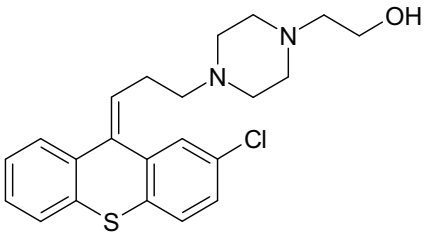
Pimozide



Risperidone



Sertindole



Zuclopenthixol

Fig. (1). The structural formulae of the studied typical and atypical antypsychotics.

Preparation of Melanin-based Stationary Phase

The polymerization product of L-DOPA was immobilized onto aminopropyl silica (APS) particles as follows. A 15 mg sample of L-DOPA was dissolved in 3 ml of 25 mM potassium phosphate buffer (pH 6) and left to stand at ambient temperature for 7 days. A 150-mg portion of APS was rinsed three times with 5 ml of 25 mM potassium phosphate buffer (pH 6) and centrifuged. Next, 1 ml of the melanin solution (2.5 mg/ml of polymerization product of L-DOPA) was diluted in 2 ml of 25 mM potassium phosphate buffer (pH 6) with addition of 250 µl of solution of EDC and NHS (1 mg/ml). The resulting solution was stirred for 10 min and then added to a centrifuge tube with the previously activated APS. The mixture was then rotated at 250 rpm in an orbital shaker for 24 h at ambient temperature. Next, the mixture was centrifuged at 1500 rpm for 5 min and the supernatant was discarded. Then, 20 ml of 1M hydroxylamine was added and shaken for 30 min at 4C. The resulted end-capped melanin-coated silica was rinsed three times with 5 ml of 15% methanol solution. Then, the suspension of melanin-APS was placed in a glass column with inner diameter of 5 mm and length of 20 mm (5/20 Tricorn, GE Healthcare Bio-Science AB/Amersham Biosciences, Freiburg, Germany) and allowed to settle. The fittings on the Mel-APS column were tightened and the column was washed with methanol:water 60:40 (v/v) for 3 h and with 2-propanol:water 20:80 (v/v) for 1 h using a chromatographic pump providing solvent flow rate of 0.4 ml/min.

Chromatographic Binding Studies to Melanin-based Stationary Phase

The retention times, t_R, of the studied antipsychotics drugs by the melanin column were determined in isocratic mode with mobile phase consisting of 2-propanol:25 mM sodium phosphate buffer (pH 6.8) 13:87 (v/v), with flow rate of 0.6 mL/min. All the measurements were done in triplicate with UV multiwave-length detection and sample injection volume of 5 µL. The Shimadzu (Kyoto, Japan) chromatographic system was composed of two solvent pumps (LC-20AD), autosampler (SIL-20A), diode array detector (SPD-M20A), column oven (CTO-20AC) and degasser DGU-20A3.

RESULTS AND DISCUSSION

The physical and chemical interpretation of the interaction that takes place inside the reversed-phase based column is relatively simple. The use a set of quantitatively comparable retention parameters from RPLC and structural descriptors allows for obtaining quantitative structure-retention relationships (QSRR) models that can be often successfully transformed into the quantitative structure-activity relationships (QSAR). However, this study showed some difficulties in correlating the affinity chromatographic retention data for a set of antipsychotic drugs determined by HPLC with a column packed with melanin immobilized on silica support and the reported extrapyramidal symptoms parameters. The correlations studied concerned the extrapyramidal motor side effects scale (EPS) and their number needed to harm (NNH). The calculated scales are relative to clozapine, which has the lowest EPS rate of all the studied antipychotics [10]. The EPS index was evaluated by co-medication with an anticholinergics - biperiden. NNH comprises the number of patients required to observe an additional case of EPS in relation to clozapine as a reference substance [10]. Also, the correlations between the retention data and odds ratio (ODS) of EPS, as a quantitative assessment of the EPS-inducing potency for each antipsychotic substance, were evaluated. The ODS have become widely used in medical practice as a convenient interpretation factor in case-control studies [11].

The meaningless correlation between the chromatographic retention of set of 14 antipsychotics, including five so-called 'atypical' drugs (amisulpride, olanzapine, quetiapine, risperidone, sertindole) and 9 'typical' agents (benperidol, bromperidol, flupentixol, fluphenazine, haloperidol, perazine, perphenazine, pimozide, zuclopentixol) and their side effects associated with the prescription of the drugs were observed (Fig. **2**). The best, but still unsatisfactory relationships were found for chromatographic retention data determined on the melanin-based column and NNH ($R = 0.5459$). The low correlations are certainly due to diverse structures of antipsychotic drugs and their physical and chemical properties, which are directly related to their chromatographic behavior.

The low significance correlations are useless. Thus**,** we continued our research to find the best fitting of chromatographic data to the EPS, ODS or NNH parameters. The analysis of relationships between the chromatographic retention time and extrapyramidal indexes were of particular interest. The further study was divided into five parts for different derivatives.

Aromatic Heterocycles Containing Piperazine Moiety

Overall, the aromatic heterocycles have the potential to interact with binding sites of biomolecules through a variety of bonding forces. They can interact through hydrophobic and van der Waals interactions, while the heteroatoms, such as nitrogen or sulphur, present in the structure could interact by hydrogen or ionic bonding. The performed relationships between the chromatographic retention data of a set of heterocyclic neuroleptics and either EPS, ODS or NNH showed the influence of position of the heteroatoms in the aromatic ring onto the interaction with immobilized melanin as the stationary phase. However, the much better correlation between the retention time and the EPS was observed for aromatic heterocycles containing piperazine moiety (Table **1**). The reported structure-activity relationships (SAR) study proved that the distance between nitrogen from both piperazine and aromatic ring is responsible for neuroleptic activity [1]. The optimal distance for that activity is determined by three carbon atoms chain. Unfortunately, the high antipsychotic activity is correlated with high values of EPS and ODS parameters (Table **2**). Except of that, the piperazine-1-ethanol moiety has the crucial role for the high EPS value (flupenthixol, fluphenazine, perphenazine and zuclopenthixol), as opposed to methyl substituent in perazine. However, the highest extrapyramidal side effects were observed for tricyclic neuroleptic drugs, such as flupentixol and zuclopentixol. Based on their structures, one can assume that the double bound in carbon chain between the piperazine and tricyclic structure is probably responsible for

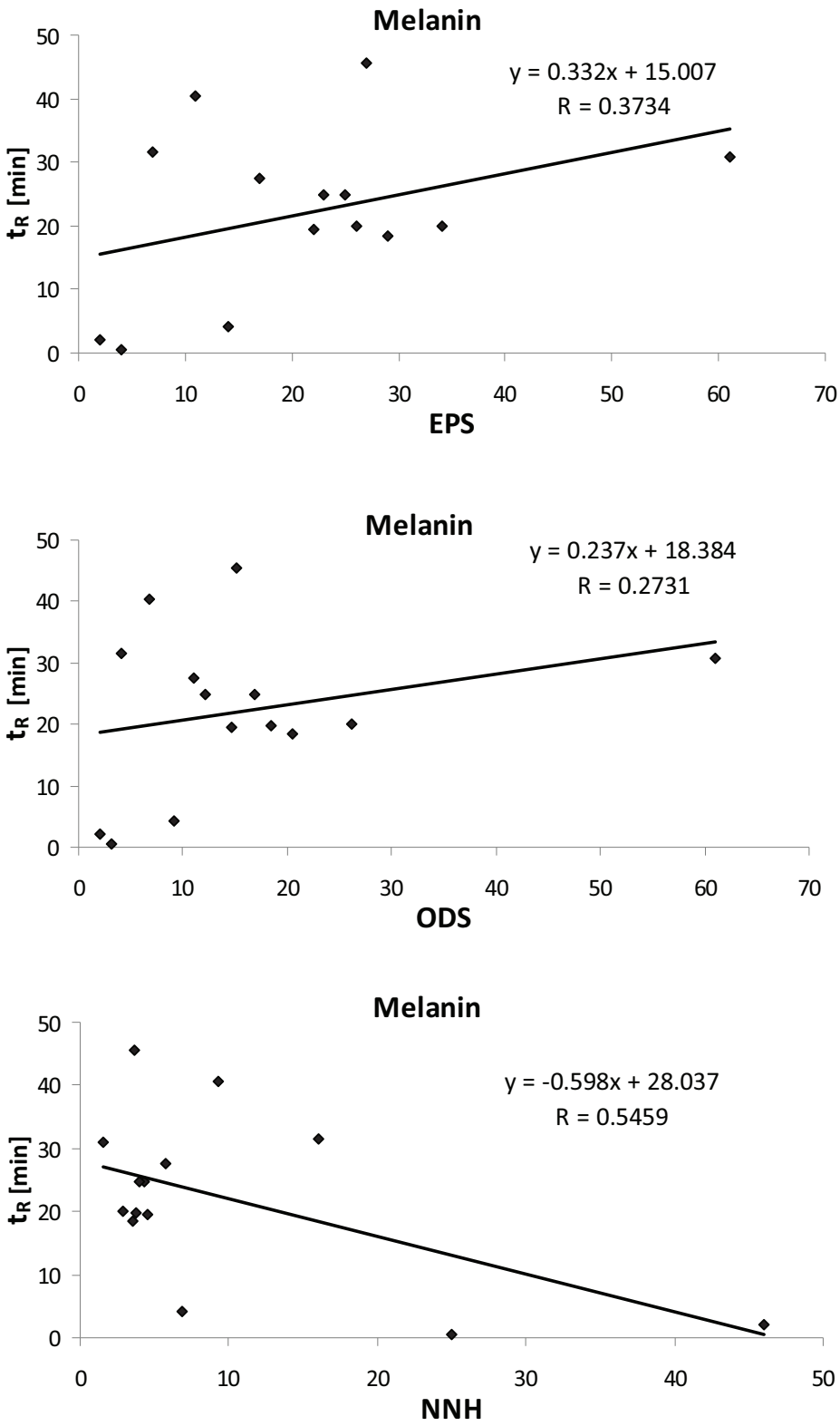


Fig. (2). Relationships between the chromatographic retention (t_R) of antypsychotic drugs determined **on** immobilized melanin column and the risk of extrapyramidal symptoms (EPS) with reference to clozapine, odds ratio (ODS) of EPS relative to treatment with clozapine and NNH - the number of patients required to observe an additional case of EPS in relation to the reference drug clozapine.

Table 1. The equations and correlation coefficients of the relatioships between the chromatographic retention time of studied antypsychotics developed on the melanin column and their extrapyramidal side effect parameters: risk of extrapyramidal symptoms (EPS) with reference to clozapine, odds ratio (ODS) of EPS relative to treatment with clozapine and NNH- the number of patients required to observe an additional case of EPS in relation to the reference drug – clozapine

Aromatic heterocycles containing piperazine moiety; n=7	
EPS	y=0.917x+5.903 R=0.6622
ODS	y=1.190x+7.917 R=0.5390
NNH	y=-0.692x+30.761 R=0.7021
Non-flurinated antypsychotics; n=6	
EPS	y=1.214x+2.138 R=0.7014
ODS	y=1.811x+2.921 R=0.6204
NNH	y=-0.685x+29.731 R=0.6054
Fluor-containing antypsychotics; n=8	
EPS	y=-0.056x+2.676 R=0.1112
ODS	y=0.031x+24.458 R=0.0959
NNH	y=2.118x+15.750 R=0.6428
Non-sulfur-containing antypsychotics; n=6	
EPS	y=-0.095x+29.787 R=0.2073
ODS	y=0.004x+26.921 R=0.0141
NNH	y=2.026x+17.776 R=0.6821
Sulfur-containing antypsychotics; n=8	
EPS	y=0.956x+3.345 R=0.6336
ODS	y=1.253x+5.442 R=0.5295
NNH	y=-0.599x+15.750 R=0.5748

Table 2. The extrapyramidal side effect parameters of studied drugs: risk of extrapyramidal symptoms (EPS) with reference to clozapine, odds ratio (ODS) of EPS relative to treatment with clozapine and NNH - the number of patients required to observe an additional case of EPS in relation to the reference drug – clozapine [1] and retention times measured on the melanin HPLC column. The retention time of clozapine – 1.96 min

Antipsychotic Drug	EPS	Odds Ratio	NNH	t_R [min]
Quetiapine	2	2.1	46	2.15
Olanzapine	4	3.1	25	0.57
Perazine	7	4.1	16	31.54
Sertindole	11	6.8	9.3	40.50
Amisulpride	14	9.1	6.9	4.19
Risperidone	17	11.0	5.8	27.52
Pimozide	23	12.2	4.3	24.77
Fluphenazine	22	14.7	4.5	19.54
Zuclopenthixol	27	15.2	3.7	45.55
Perphenazine	25	16.8	4.0	24.77
Flupentixol	26	18.4	3.8	19.90
Haloperidol	29	20.5	3.5	18.49
Bromperidol	34	26.2	2.9	20.00
Benperidol	61	79.1	1.6	30.87

the high intensity of the side effect. Also, the substitution of an electron-withdrawing group (-F, $-CF_3$) at position 2 increases both antipsychotic efficacy and extrapyramidal side effects [1].

Non-flurinated and Fluor-containing Antipsychotics

Certain chemical groups of bioactive compounds, which are susceptible to metabolic enzymes, can be replaced by a halogen substituent. Of the four halogens, fluorine drugs have a prominent position. The electronegative nature of fluorine can generate hydrogen bonds from H-bond donors [12]. Hence, the fluorination of certain drugs can alter their physical, chemical, electronic and conformational properties, which finally can change the pharmacological and also the side effects of drugs. Based on data in Table **2**, one can notice that high EPS values have the fluor-containing 'typical' antipsychotics**,** with exception of perazine.

The non-halogen-containing drugs, mostly 'atypical', reduce psychotic symptoms while producing few EPS [10]. These non-fluor-containing-drugs give the similar correlations (in the range of 0.605-0.701) between chromatographic retention determined on studied columns and extrapyramidal side effect parameters (Table **2**). A poor correlation of chromatographic data with EPS values was obtained for fluor-containing-antipsychotics. It must be noted here that benperidol with the highest EPS value and average retention time appeared to be an outlier in the correlation analysis. After the exclusion of that drug from further analysis, much better correlations of the data were obtained for the melanin column (Fig. **3**). There is a nearly perfect correlation of retention time and NNH and a worse but still acceptable for EPS and ODS parameters. Curiously, the relationships between retention data determined on melanin column and EPS and ODS have negative value of the slope -0.878 and -0.945 respectively. The results demonstrate that the fluor-containing-antipsychotics, unlike others, have a reverse relationship between affinity to the melanin and their extrapyramidal side effects. This contrary effect might probably be due to steric effects of fluorine atoms that can alter drug metabolism or enzyme substrate recognition [13]. Hence, the fluorine substitution into structure of a biologically active compound can cause unexpected changes in the biological activity due to the ability of these bulky atoms to occupy the binding site of molecular targets [12]. Moreover, the surprising reverse relationship after exclusion of benperidol can be explained by 'polar hydrophobic' properties of fluorine-containing compounds which can lead to changed affinity to natural receptors. The fluorine substitution on the aromatic ring reduces its polarizability and increases the hydrophobic surface area of the molecule. Such dual nature of fluorine-containing drugs can lead to their unexpected chromatographic behavior.

Non-sulfur-containing and Sulfur Containing Antipsychotics

Most of the tricyclic antipsychotics have two benzene rings which are linked by sulfur and nitrogen atom. The nature of sulfur at position 5 of phenothiazines and thioxanthenes

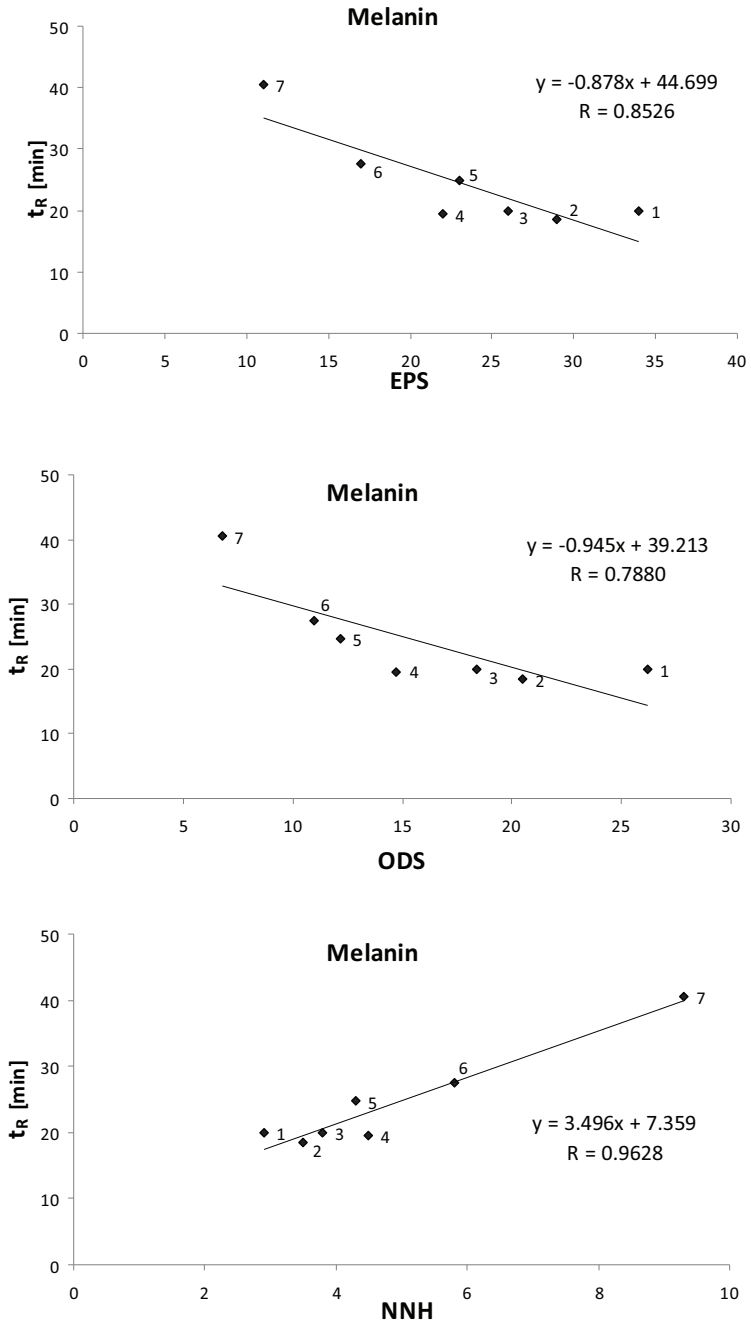


Fig. (3). Relationships between the chromatographic retention (t_R) of fluor-containing antipsychotic drugs (excepting benperidol) determined on the melanin-based column and the risk of extrapyramidal symptoms (EPS) with reference to clozapine, odds ratio (ODS) of EPS relative to treatment with clozapine and NNH - the number of patients required to observe an additional case of EPS in relation to the reference drug clozapine. Analytes: bromperidol (1), haloperidol (2), flupenthixol (3), fluphenazine (4), pimozide (5), risperidone (6), sertindole (7).

influences their antipsychotic activity [1]. Also, the presence of sulfur causes a better correlation between the chromatographic retention of the sulfur-containing antipsychotics and EPS than that for the non-sulfur containing drugs.

CONCLUSION

For the new antipsychotics drug design it is important to evaluate the risk of side effects of drug candidates. Hence, the analytical methods determining the binding of drugs to melanin should be evaluated according to hypotheses that extrapyramidal symptoms are correlated to neuromelanin affinity of these drugs.

Compared to older conventional antipsychotics, the newer 'atypical' drugs are characterized by decreased incidence of EPS or other movement disorders at doses producing antipsychotic effects. However, the importance of EPS in the context of treatment of psychosis tends to be underestimated [3]. Moreover, the dichotomous classification as a 'typical' and 'atypical' antipsychotics appears to be dispensable in clinical practice. As often reported, the commonly

used EPS parameter is appropriate because it does not provides clinically useful distinction among the currently used antipsychotics [10]. The new NNH parameter describing the number needed to harm, instead of EPS, may be of help in risk consideration for antipsychotic treatment.

Based on our result, the following conclusions can be drawn. First, there are evident relationships between the antipsychotics binding to melanin and their extrapyramidal motor side effects. The weak and sometimes meaningless correlations observed were probably due to non-homo-geneous structurally sets of compounds considered, comprising of benzepines, phenothiazines, thioxanthenes, butyrophenone and other heterocyclic antipsychotics. Hence, the significant differences in chromatographic behavior were unavoidable. Surprisingly enough, regardless of that, the NHH proved to be the most 'robust' side effect index for miscellaneous structures of studied drugs, with correlation coefficient (0.575-0.702). Hence, it can appear to be more effective not only in optimizing the treating of psychotic disorders but also in predicting affinity to melanin to evaluate the risk of adverse effects of antipsychotic drugs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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