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Journal of Chromatography B, 819 (2005) 169–174
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Qualitative assessment of IC_{50} values of inhibitors of the neuronal nicotinic acetylcholine receptor using a single chromatographic experiment and multivariate cluster analysis

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Received 28 October 2004; accepted 13 January 2005
Available online 19 February 2005

Abstract

It has been widely demonstrated that affinity chromatography can be used to derive binding affinities, and that these affinities can be correlated to data obtained using standard techniques such as membrane binding, ultrafiltration and equilibrium dialysis. The purpose of this study is to evaluate the use of immobilized nicotinic acetylcholine receptor stationary phase in chromatographic experiments to assess the functional activity of series of noncompetitive inhibitors (NCIs) as reflected in their IC_{50} values. Chromatographically determined retention values and computer generated molecular descriptors were obtained for 29 compounds and the data were analyzed by cluster analysis. The approach qualitatively ranked the test compounds as efficient NCIs (low IC_{50} values) or poor NCIs (high IC_{50} values). The data obtained with the 29 compounds used in this study demonstrate that the experimental approach had been able to place 25 of these compounds in the correct IC_{50} clusters. To our knowledge, this is the first relationship established between chromatographic retention and IC_{50} for membrane-bound receptors. These results suggest that the chromatographic approach may be useful in development of lead drug candidates including the determination of off-target binding.

Keywords: Affinity chromatography; Immobilized receptors; Nicotinic acetylcholine receptor stationary phase; Drug discovery

1. Introduction

Numerous works demonstrated that affinity chromatography technique can be used to derive binding affinities, and that these affinities can be correlated to data obtained using standard techniques such as membrane binding, ultrafiltration and equilibrium dialysis [1–6]. The problem occurs, however, with the application of affinity chromatography to the direct determination of functional activities, such as IC_{50} or EC_{50} values. This may be partially explained by the fact that the binding affinity of the drug towards the receptor is not necessarily directly related with the efficacy of this drug acting on the pharmacological target [7], although in the case of competitive agonists and antagonists these properties can be related using the Cheng-Prusoff relationship, Eq. (1) [8].

$$K_i = \frac{IC_{50}}{1 + (S/K_m)} \quad (1)$$

where K_i is the binding affinity of the inhibitor, IC_{50} is the functional strength of the inhibitor, S is substrate concentration and K_m is the affinity of the substrate for the enzyme.

Recently, Moaddel et al. [9] have demonstrated that the Cheng-Prusoff approach can be used with affinity chro-

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doi:10.1016/j.jchromb.2005.01.043

matography techniques to directly assess agonistic activity. In these studies, an immobilized nicotinic acetylcholine receptor (nAChR) stationary phase and displacement chromatography were used to determine the relative agonistic activities of a series of drug candidates. A single experiment was used to rank the affinities of the compounds for the nAChR as reflected by their ability to displace the marker ligand. Since the concentration of the the marker ligand was a thousand fold lower than the displacer, Eq. (1) reduced to Affinity (K_d), expressed as Δml, is equivalent to activity (EC_{50}). The chromatographically determined ranking of the EC_{50} values were confirmed using standard nicotine stimulated $^{86}Rb^+$ efflux assay.

Another interesting work was presented by Andrisano et al. [10], where the chromatographic column containing immobilized acetylcholinesterase (AChE) stationary phase was used as an enzymatic reactor for the substrate acetylthiocholine. The authors observed that the enzymatic reaction occurring on the column was inhibited by AChE inhibitors injected simultaneously with the substrate. The inhibition was concentrate dependent and allowed to calculate the IC_{50} values which were found to be well correlated with the respective values determined in reference experiments for tested inhibitors.

The Cheng-Prusoff relationship cannot be used with non-competitive inhibitors (NCIs) where the allosteric type of interaction occurs. The present work describes a method of estimation of the relative antagonistic activities, expressed as IC_{50} values, of a series of NCIs towards the nicotinic acetylcholine receptor using data obtained from affinity chromatography on an immobilized nAChR stationary phase. This approach utilizes the non-direct method of multivariate analysis for assessment of IC_{50} values.

The nAChRs are a family of ligand gated ion channels found in the central and peripheral nervous systems. The receptor contains multiple binding domains including a luminal high affinity binding domain at which NCIs have been shown to bind [11]. A number of marketed drugs and their metabolites bind at this site, and this property may be responsible for many of the side effects attributed to these compounds [12–18]. For example, the impairment of cardiovascular function observed during ketamine anesthesia has been associated with the inhibitory action of ketamine on ganglionic nAChRs [12].

The identification of an inhibitor of the nAChR has been primarily accomplished using labor-intensive functional studies such as nicotine-stimulated $^{86}Rb^+$ efflux assays [13,17,18] or electrophysiological approaches [14]. However, we have recently demonstrated that affinity chromatography on a stationary phase containing an immobilized nAChRs can be used as an alternative approach to the identification of NCIs [19,20]. Using this technique and non-linear modeling of chromatographic process, the interactions between NCIs and the immobilized α3β4 nAChR subtype have been characterized including determination of the thermodynamic retention factors, *k*, association and disassociation rate constants, k_a and k_d, respectively, and the association equilibrium constant, K_a [19].

In a subsequent study, kinetic parameters of the chromatographic process were obtained for dextromethrophan and levomethorphan and compared with their ability to inhibit nicotine-induced $^{86}Rb^+$ efflux in the same cell line used to create the α3β4 nAChR stationary phase [20]. The results demonstrated that non-linear chromatography on an immobilized nAChR column could be used to directly measure the relative length of the effect of a NCI on the function of the receptor. However, no relationship was established between the strength of the inhibitory effect, i.e. the IC_{50} value, and any of the non-linear chromatographic parameters.

In this study, a liquid chromatographic column containing immobilized α3β4 nChR has been used to directly assess the IC_{50} values of a series of NCIs of this receptor. Chromatographically determined retention values and computer generated molecular descriptors were obtained for 29 compounds and the data were analyzed by 3-D cluster analysis. The approach correctly ranked 25 of the 29 test compounds as efficient NCIs (low IC_{50} values) or poor NCIs (high IC_{50} values) on the basis of a single chromatographic experiment. To our knowledge, this is the first relationship established between chromatographic retention and IC_{50} for membrane-bound receptors.

2. Materials and methods

The compounds used in this study are listed in Table 1. All of the compounds were purchased from Sigma-Aldrich Co. (St. Louis, MO) with the exception of dextrorphan, (+)-3-hydroxy-morphinan, (+)-3-methoxy-morphinan which were kindly donated by Hoffman-LaRoche (Nutley, NJ), levomethorphan which was purchased from Cerillant Co. (Round Rock, TX), D-620 which was kindly donated by G.D. Searle (Chicago, IL) and the metabolites of diltiazem: deacetyl- diltiazem (M1), *N*-demethyl-diltiazem (MA), *N*-demethyl-deacetyl-diltiazem (M2), *O*-demethyl-deacetyl-diltiazem (M4) and *N*,*O*-didemethyl-deacetyl-diltiazem (M6) were kindly provided by Marion Merrill Dow, Inc., (Kansas City, KS).

2.1. Preparation of the α3β4 nAChR column

The preparation of the α3β4 nAChR column has been previously described [21]. In brief, the KXα3β4R2 cell line with expressed α3β4 nAChR was used to create the column. The cell line was provided by K. Kellar (Georgetown University, Washington, DC). Membranes prepared from 10^6 cells were suspended in 30 ml of Tris-HCl (50 mM, pH 7.4), containing 5 mM EDTA, 3 mM benzamidine and 0.2 mM PMSF. The suspension was homogenized for 3 s × 10 s at the setting of 15 on a Model PT-2100 homogenizer (Kinematica AG, Luzern, Switzerland), intermittently placing the tissue in an ice bath. The mixture was centrifuged for 30 min at 4 °C at

Table 1
IC_{50} values, logarithm of thermodynamic retention factors (log k) and molecular descriptors (E_{HOMO} and S_{YZ}) and assigned Clusters for the compounds used in this study

	Compound	log k	E_{HOMO}	S_{YZ}	Cluster	IC_{50} (μM)
1	Methadone	1.65	−9.20	66.6	1	1.9 [13]
2	Diltiazem	1.64	−8.66	62.3	1	2.3
3	Norverapamil	1.99	−9.12	69.1	1	2.6
4	Verapamil	1.98	−9.04	69.4	1	3.0
5	Dextromethorphan	1.79	−8.72	51.1	2	10.1 [20]
6	Levomethorphan	1.55	−8.74	52.7	2	10.9 [20]
7	Dextrorphan	1.43	−8.77	50.9	2	29.6 [18]
8	(+)-3-Hydroxy-mophinan	1.75	−8.78	49.8	2	10.3
9	(+)-3-Methoxy-morphinan	1.42	−8.83	49.3	2	54.2
10	Clozapine	2.19	−7.69	44.5	2	28.0
11	Laudanosine	1.36	−8.54	50.1	2	139.7
12	Phencyclidine	1.38	−9.05	51.6	2	7.0 [18]
13	Adamantadine	0.95	−9.71	35.4	3	3.4 [15]
14	Bupropion	1.11	−9.51	34.2	3	1.4 [16]
15	Ketamine	0.92	−9.49	42.5	3	1.4 [12]
16	Mecamylamine	1.04	−9.22	40.2	3	1.0 [18]
17	Memantine	1.22	−9.71	42.3	3	6.6 [15]
18	Methamphetamine	0.92	−9.39	27.8	3	401.2
19	MK−801	1.28	−9.11	43.0	3	26.6 [18]
20	*N*-demethyl-diltiazem	1.61	−8.65	66.5	1	4.2
21	Deacetyl-diltiazem	1.60	−8.58	62.4	1	30.4
22	*N*-demethyl-deacetyl-diltiazem	1.61	−8.58	57.4	2	77.6
23	*O*-demethyl-deacetyl-diltiazem	1.45	−8.41	61.8	1	73.2
24	*N*,*O*-didemethyl-deacetyl-diltiazem	1.48	−8.65	58.1	2	63.1
25	Galapamil	1.88	−9.05	66.3	1	6.4
26	D-620 (verapamil metabolite)	1.25	−9.35	48.5	2	48.9
27	Nicardipine	2.33	−8.84	65.2	1	2.5
28	Amlodipine	2.00	−8.72	62.9	1	5.8
29	Nifedipine	1.27	−8.63	58.4	2	24.7

100,000 × g and the supernatant was discarded. The pellet was suspended in 10 ml of Tris-HCl (50 mM, pH 7.4) containing 100 mM NaCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 5 mM KCl, 2% sodium cholate and 10 μg/ml leupeptin. The resulting mixture was stirred for 18 h at 4 °C and centrifuged at 100,000 × g for 20 min, and the supernatant containing nAChR-cholate solution was collected.

The supernatant (nAChR-cholate suspension) was mixed with 200 mg of dried IAM-PC packing material (particle size 12 mM, 300 Å) (Regis Technologies, Morton Grove, IL) and stirred gently for 1 h at 25 °C, transferred into dialysis tubing and dialyzed for 24 h at 4 °C against 1 l of Tris-HCl (50 mM, pH 7.4) containing 5 mM EDTA, 100 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM PMSF. The resulting mixture was centrifuged for 3 min at 4 °C at 700 × g and the supernatant was discarded. The pellet (nAChR-IAM) was washed with 5 ml of Tris-HCl (50 mM, pH 7.4) and centrifuged. This process was repeated until the supernatant was clear.

The nAChR-IAM (200 mg) was packed into a HR 5/2 glass column (Amersham Pharmacia Biotech, Uppsala, Sweden) to yield a 150 mm × 5 mm (i.d.) chromatographic bed. Since no attempt was made to isolate and purify the nAChR used to create the immobilized nAChR columns, these columns are characterized through the determination of the amount of active binding sites [22]. The column used in this study contained ca. 350 fM of immobilized active receptor and was stable for >6 month.

2.2. Chromatographic studies

The studies were conducted using a LC–MS system composed of a LC10AD pump (Shimadzu, Columbia, MD), ESA 540 auto-injector (ESA, Inc, Chelmsford, MA) and Micromass Q-Tof mass-spectrometer (Micromass, Beverly, MA). The data were recorded and processed using MassLynx v. 3.5. (Micromass). The mobile phase was composed of ammonium acetate (0.01 M, pH 7.4) modified with methanol in a ratio of 85:15 (v/v) buffer:methanol. The mobile phase was delivered at flow rate of 0.2 ml/min at ambient temperature (20 °C ± 2 °C). A 20 μl aliquot of a 10 μM solution of each analyte was injected onto the system and the resulting chromatographic profiles were determined using selective monitoring for the MW + H ion of each compound.

2.3. Data analysis

log k values was determined using non-linear chromatography function implemented in PeakFit for Windows, and the details have been reported elsewhere [19].

Cluster analysis was carried out using the *K*-mean clustering algorithm within the Statistica v. 6.0 package. (StatSoft Inc., Tulsa, OK, USA).

2.4. Functional studies

The IC_{50} values for compounds 2-4, 8-11 and 18 and compounds 20–29 were determined using nicotine induced $^{86}Rb^{+}$ efflux from KXα3β4R2 cells as previously described [20].

3. Results

This paper reports the development of an approach to qualitatively assess IC_{50} values using capacity factors and molecular descriptors describing these retentions. The log *k* values for compounds 1,2, 5–19 have been previously reported [23] and compounds 3 and 4 were determined during this study. The data are presented in Table 1. This parameter has been described using Eq. (2) [23]:

$$\log k = 5.255(\pm 0.942) + 0.491(\pm 0.092)E_{HOMO} + 0.012(\pm 0.005)S_{YZ} \quad (2)$$
$$r = 0.984, \quad s = 0.168, \quad F = 27.929, \quad n = 17$$

The molecular descriptors E_{HOMO}, the energy of the highest occupied molecular orbital, and S_{YZ}, the surface area of the molecular projection onto the *YZ* plane, calculated for these compounds are presented in Table 1.

The IC_{50} values for compounds 1, 5–7, 12–17 and 19 have been previously reported and those for compounds 2–4, 8–11 and 18 were determined in this study. These values and their sources are reported in Table 1.

Linear regression analyses were used to compare IC_{50} values with log *k*, E_{HOMO} and S_{YZ}. No statistically significant relationships were observed (all *p* values >0.05). However, a visual examination of a 3-D scatterplot of the chromatographic and molecular parameters suggested that the NCI's could be subdivided into three separate clusters, Fig. 1. This was confirmed using multidimensional cluster analysis, which is designed to specifically identify patterns within multivariate datasets [24]. The object was to sort cases into groups, or clusters, such that the degree of association is strong between members of the same cluster and weak between members of different clusters. Each cluster thus describes the class to which its members belong; and this description may be abstracted through use from the particular to the general class.

The analysis identified three distinct clusters: Cluster 1 formed by compounds 1–4 and characterized by log *k* values ranging from 1.3 to 2.2, E_{HOMO} from −9.2 to −8.6 and S_{YZ} from 60 to 70; Cluster 2 formed by compounds 5–12 with log *k* ranging from 1.3 to 2.2, E_{HOMO} from −9.0 to −7.7 and S_{YZ} from 45 to 60; Cluster 3 formed by compounds 13–19 with log *k*, ranging from 0.9 to 1.3, E_{HOMO} from −9.8 to −9.1 and S_{YZ} from 25 to 45.

When the IC_{50} values were considered, the Clusters also appeared to represent a qualitative grouping of functional properties in which Cluster 1 contained compounds with IC_{50} values 1.9–3.0 μM, Cluster 2 contained compounds with IC_{50} values ranging from 7.0 to 139.7 μM, and Cluster 3 contained compounds with IC_{50} values ranging from 1.0 to 6.6 μM, Table 1.

Of the initial set, 17 of the 19 compounds were located within clusters that encompassed their IC_{50} values, including compounds 12 and 17, which were on the boundaries of Cluster 2 and Cluster 3, respectively. Compound 19 was incorrectly placed in Cluster 3, although it also lies close to the boundary between Clusters 2 and 3. Compound 18 was also incorrectly placed in Cluster 3 as it displayed no significant inhibitory activity towards the nAChR. Compound 18 lies on the outer limits of the cluster and may represent a fourth cluster containing small molecules with high IC_{50} values. The analysis of a larger cohort of compounds will be required to further define the limits of these clusters.

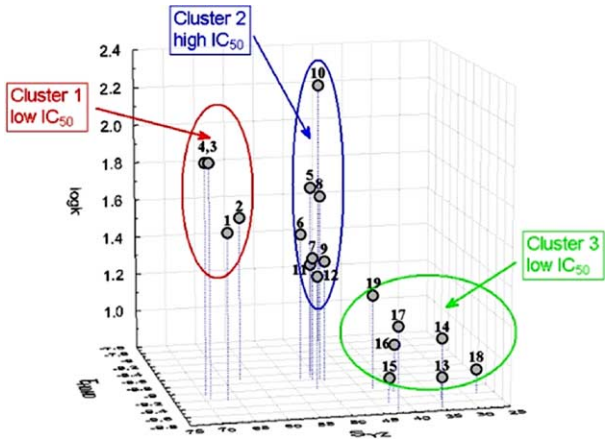

Fig. 1. The results from the cluster analysis of the compounds used in this study using the log *k*, E_{HOMO} and S_{YZ} parameters.

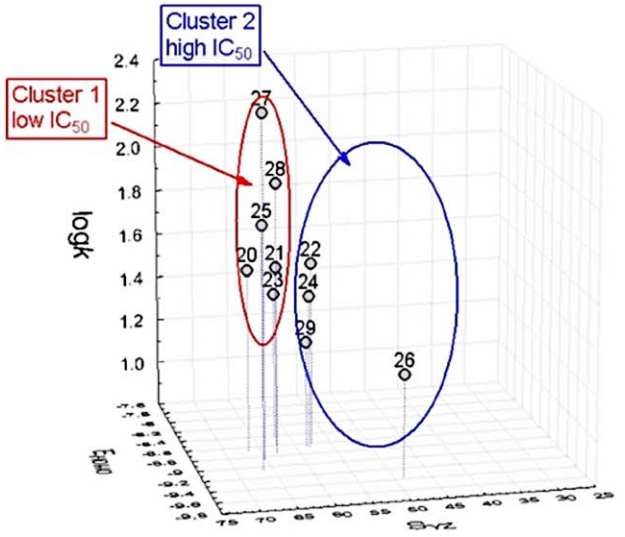

Fig. 2. The results of the application of the cluster analysis to the test cohort of 10 calcium channel blockers and their metabolites.

The initial 19 test compounds contained two calcium channel blockers, verapamil and diltiazem, and norverapamil, a primary metabolite of verapamil, and the data demonstrated that these compounds were efficient NCIs of the α3β4 nAChR (Table 1). In order to further explore this class of drugs and to validate the model, four additional calcium channel blockers (compounds 25, 27, 28, 29), five metabolites of diltiazem (compounds 20–24) and one additional metabolite of verapamil (compound 26) were chromatographed on the α3β4 nAChR. The log *k* and molecular descriptors were calculated for each compound, Table 1, and used in the cluster analysis. The cluster analysis placed compounds 20, 21, 23, 25, 27 and 28 in Cluster 1 and compounds 22, 23, 26 and 29 in Cluster 2, Fig. 2. The IC_{50} values for the compounds were then determined and 8 of the 10 compounds had been placed in the correct cluster, Table 1.

4. Discussion

Recent studies of the binding of NCIs to the inner lumen of the α3β4 nAChR have suggested that this interaction is a multi-step process [23]. The initial step involves an electrostatic interaction between a nitronium moiety on the NCI and a carboxylate moiety on the outer ring of the nAChR. A proton is then transferred from the nitronium moiety to the carboxylate moiety, followed by the entrance of the deprotonated NCI into the lumen and its eventual binding at a site near the hydrophobic gate in the transmembrane portion of the receptor.

The molecular descriptors identified in the previous nonlinear chromatographic study are consistent with this mechanism, where E_{HOMO} reflects the ability of the compound to transfer a proton to the carboxylate moiety and S_{YZ} is associated with the entrance of the compound into a defined steric environment [23]. The thermodynamic retention factor, *k*, reflects the equilibrium between the initial binding of the compound to the nAChR, k_a, and the stability of the final NCI-nAChR complex, k_d. All of these steps contribute to the observed IC_{50} value. Thus, it is not surprising that linear regression analyses between only one of these factors and the IC_{50} values did not work, while cluster analysis using all three of the variables was able to identify populations with qualitatively similar IC_{50} values.

The analysis appeared to segregate the compounds with low IC_{50} values into two clusters, Clusters 1 and 3, by size and charge, with the smaller, more electronegative compounds appearing in Cluster 3. One possible source of this division is the log *k* values used in the analysis. The *k* values were experimentally determined and reflect the total chromatographic process occurring on the column, i.e. both specific and non-specific interactions. Since lipophilicity plays a key role in non-specific membrane binding as well as chromatographic retention in a reversed phase system, the clog *P* (the logarithm of partition coefficient in *n*-octanol–water system) values for compounds 1–19, were calculated. The clog *P* values for the compounds in Cluster 1 (4.68 ± 1.11) were significantly higher than those calculated for the compounds in Cluster 3 (2.76 ± 0.54), $p = 0.0033$ (Student's *t*-test), and it appears that differences in lipophilicity were the source of the differentiation of compounds with low IC_{50} into Cluster 1 or Cluster 3. While this analysis is consistent with the data, it remains to be confirmed. Further studies using a wider set of Cluster 1 and Cluster 3 compound will be studied using columns derived from cell lines expressing or not expressing the α3β4 nAChR and other nAChR subtypes. The results from these studies will be reported elsewhere.

The clog *P* values calculated for the compounds in Cluster 2 (3.69 ± 0.39) were not significantly different from those calculated for the compounds in Cluster 1, and there was no significant difference between the log *k* values of the compounds. However, there were significant differences between the E_{HOMO} ($p = 0.0417$) and S_{YZ} ($p = 0.0001$) values, which are consistent with the assumption that the clustering analysis reflects the binding process associated with the inhibition of nAChR activity.

5. Conclusions

The application of affinity chromatography to the determination of functional properties, such as IC_{50} values, has not been previously established. This study presents an experimental approach that can be used to directly relate chromatographic retention to IC_{50} values. The results demonstrate that the method was able to place 25 tested compounds into the correct IC_{50} clusters. In addition, the data for compounds 20–29 took 2 days to complete using a single nAChR column, which was stable for >6 months, and an automated LC–MS system. This is in contrast to the 6 weeks required to complete the functional studies for 18 compounds. These results suggest that the chromatographic approach may be useful in development of lead drug candidates, in particular in the assessment of unexpected toxicities arising from off-target binding.

6. Nomenclature

NCI	noncompetitive inhibitor
nAChR	nicotinic acetylcholine receptor
AChE	acetylcholinesterase
IC_{50}	the concentration of ligand producing 50% inhibition of functional effect
k	thermodynamic capacity factor
k_a	association rate constant
k_d	dissociation rate constant
K_a	equilibrium constant for association
E_{HOMO}	the energy of the highest occupied molecular orbital
S_{YZ}	the surface area of the molecular projection onto the *YZ* plane,

clog P the logarithm of partition coefficient in *n*-octanol–water system (obtained in calculation method).

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