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Multidimensional on-line screening for ligands to the α3β4 neuronal nicotinic acetylcholine receptor using an immobilized nicotinic receptor liquid chromatographic stationary phase

Michael T. Baynham, Sharvil Patel, Ruin Moaddel, Irving W. Wainer*

Bioanalytical and Drug Discovery Unit, Gerontology Research Center, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, MD 21224-6825, USA

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Abstract

The α3β4 subtype of the neuronal nicotinic acetylcholine receptor (nAChR) subtype was immobilized on a liquid chromatographic support and the resulting column used for the rapid and direct on-line screening for nAChR ligands. A multidimensional chromatographic system was developed consisting of the immobilized receptor column (NR column) connected via a switching valve to a C_{18} column that was, in turn, connected to a single quadrupole mass spectrometer. A mixture of 18 compounds, containing α3β4 nAChR (7) and compounds that are not α3β4 nAChR ligands (11), was injected onto the NR column. The mobile phase consisted of ammonium acetate (10 m*M*, pH 7.4)–methanol (95:5, v/v) and the flow-rate was 0.2 ml/min. For the first 8 min the eluent was directed to waste. At $t = 8$ min, the switching valve was rotated and the NR column connected to the C_{18} column. The eluent from the NR column was directed to the C_{18} column for 12 min. At $t = 20$ min, the switching valve was rotated and the NR column was disconnected from the C_{18} column. The compounds trapped on the C_{18} column were separated and eluted onto the mass spectrometer using a mobile phase of ammonium acetate (10 m*M*, pH 7.4)–methanol (40:60, v/v) at a flow-rate of 1.0 ml/min. Detection was accomplished using total ion monitoring. The multidimensional system correctly isolated six of the seven α3β4 nAChR ligands and only one of the 11 non-ligands was found with the α3β4 nAChR ligands. The results indicate that the multidimensional liquid chromatographic system can be used for the on-line screening of chemical mixtures for α3β4 nAChR ligands. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: High-throughput screening; Immobilized receptors; Nicotinic receptor ligands

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are key targets for drug discovery [1,2]. The motivating factor behind these efforts are the possible application of these compounds to the treatment of a variety of diseases including Alzheimer's, Parkinson's, epilepsy and Tourette's Syndrome [1,2]. The drug discovery programs have been primarily aimed at two cholinergic agonist binding sites on the nAChR. These sites have been extensively studied and a wide variety of competitive agonist and antagonists have been identified [1,2]. nAChRs also contain other binding sites at which non-com-

*Corresponding author. Tel.: +1-410-558-8498; fax: +1-410-558-8409.

E-mail address: wainerir@grc.nia.nih.gov (I.W. Wainer).

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petitive inhibitors (NCIs) bind [2]. NCIs inhibit nAChR function by a rapid reversible channel blockade or a shortened channel opening time [1,2]. NCIs include mecamylamine [2], ketamine [3], and dextromethorphan [4]. In most cases, the non-competitive inhibitory properties of a compound at the nAChR are not its primary pharmacological function. Indeed, whether or not a compound functions as a NCI is usually determined after observation of unexpected pharmacological effects. For example, ketamine is an anesthetic that competitively blocks the *N*-methyl-D-aspartate receptor [3]. However, ketamine also acts as a NCI at nAChRs and this activity may be responsible for some of the drugs side-effects [3,5].

Drug discovery programs aimed at nAChR-mediated diseases require routine and rapid determination of a compound's agonist, antagonist and NCI properties. The standard approach to the high-throughput screening for competitive agonists and antagonists involves competitive binding affinity experiments using recombinant receptor systems and radiolabeled marker ligands [1]. NCIs are experimentally determined by measuring concentration-dependent effects on whole cell currents that yield IC_{50} or EC_{50} values [3,4]. An alternative method for the screening of compounds for competitive and non-competitive inhibitory properties is the determination of binding affinities to the nAChR. One approach to the direct measurement of absolute and relative binding affinities at the nAChR is affinity chromatography using nAChR affinity columns.

Liquid chromatographic columns containing immobilized α3β4-nAChR or α4β2-nAChR (NR columns) have been previously reported and used to determine the binding affinities of competitive agonists and antagonists [6,7]. The initial studies with these columns involved displacement chromatography with a single marker ligand and a single displacer. These experiments were conducted using frontal chromatographic techniques [6,7]. Zonal chromatography could also be employed [7], but the peak shapes and poor efficiency of the NR columns precluded the use of these columns in the chromatographic separation of complex mixtures.

In the current studies, a NR column containing an immobilized α3β4-nAChR stationary phase has been coupled to a C_{18} reversed-phase column and a mass spectrometer. This produced a multidimensional chromatographic system in which the NR column was used to initially sort a mixture of compounds through their affinity to the α3β4-nAChR, physicochemical properties were then used to resolve the compounds with an affinity to the α3β4-nAChR on the C_{18} and the mass spectrometer was used to identify these compounds. Chromatographic retention was used as the marker of affinity on the NR column and the eluent was directed to waste for the first 8 min of the run and then directed onto the C_{18} column. The compounds contained in the eluent were compressed at the head of the C_{18} column, then resolved using a step-gradient and ultimately identified using a mass spectrometer. The results of the study indicate that the multidimensional NR column–C_{18} column–mass spectrometer system can be used for the rapid on-line screening of mixtures for potential ligands to the α3β4-nAChR.

2. Materials and methods

2.1. Materials

Nicotine, epibatidine, nornicotine, anabasine, acetylcholine, benzamidine, butrylcholine, caffeine, glutamic acid, 3-hydroxytyramine, naltrexone, ketamine, norketamine, 2,3-dihydroxybenzoic acid, epinephrine, norepinephrine, cytisine and 4-dimethylaminopyridine were purchased from Sigma (St. Louis, MO, USA). Tris–HCl and cholic acid sodium salt were also purchased from Sigma. HPLC-grade methanol, ammonium acetate and 0.1 *M* ammonium hydroxide solution were purchased from Fisher Scientific (Pittsburgh, PA, USA). The water used in the study was obtained using a Nanopure reverse osmosis water purification system (Barnstead, Dubuque, IA, USA).

2.2. Chromatographic system

The coupled-column chromatographic system used in these studies is presented in Fig. 1. The system was composed of a HP-1100 chromatography system (Agilent Technologies, Palo Alto, CA, USA), which consisted of a binary gradient pump (P1), autosampler, column oven, switching valve and a diode-

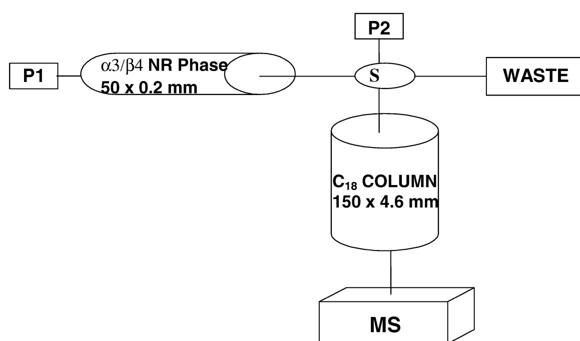

Fig. 1. The multidimensional system used in this study.

array detector. The column containing the immobilized nicotinic receptors was connected on-line to a reversed-phase Zorbax C_{18} column (150×4.6 mm I.D., 5 μm, obtained from Agilent). A Rheodyne LabPRO switching valve (Ronert Park, CA, USA) was used to connect the two columns. A second isocratic HP pump (P2) was used to supply the mobile phase to the C_{18} column. Mass spectrometry experiments were conducted using an Agilent Technologies Mass Selective Detector (MSD) operating in positive ion electrospray (ES+) mode.

2.3. Preparation of the α3β4 nicotinic receptor column

The nicotinic receptor (NR) column was prepared as previously described [6,7]. In brief, cells from the KXα3β4R2 cell line [8] were suspended in Tris–HCl (50 m*M*, pH 7.4) (buffer A), homogenized for 30 s with Brinkmann Polytron, and centrifuged at 35 000 *g* for 10 min at 4 °C. The pellet was resuspended in 6 ml of 2% cholate in buffer A and stirred for 2 h. The mixture was centrifuged at 35 000 *g* for 30 min, and the supernatant containing NR-cholate solution was collected.

The IAM liquid chromatographic stationary phase (200 mg, IAM-PC stationary phase, Regis Chemical, Morton Grove, IL, USA) was suspended in 4 ml of the obtained detergent solution containing proteins. The mixture of IAM–detergent–receptor was stirred for 1 h at room temperature. The suspension was dialyzed against 2×1 l buffer A for 24 h at 4 °C. The IAM liquid chromatographic support was then washed with buffer A, centrifuged and the solid collected.

The IAM liquid chromatographic support containing the NR was packed into a HR5/2 glass column (Pharmacia Biotech, Uppsala, Sweden) to form a chromatographic bed of 20 mm×5 mm I.D. The NR column was then placed in the chromatographic system and used as described below.

2.4. Chromatographic procedures

An ammonium acetate buffer (10 m*M*, pH 7.4) (Buffer) was used throughout the study. The pH of the Buffer was adjusted to 7.4 with 0.1 *M* ammonium hydroxide. The Buffer was used to prepare separate 1 μ*M* stock solutions for each compound used in the study. A 100 μl aliquot of each stock solution was added to a 10-ml test tube and the Test Mixture was briefly vortex-mixed. The stock solutions and Test Mixture were stored at 4 °C until use.

Separations on the NR column were achieved using a mobile phase consisting of Buffer–methanol (95:5, v/v) at a flow-rate of 0.2 ml/min and a column temperature of 35 °C.

The retention times of each test compound were determined by injecting a 50 μl aliquot of each stock solution onto the chromatographic system. The retention times of the compounds were monitored by single-ion monitoring at the appropriate *m/z*.

When the Test Mixture was injected onto the chromatographic system, the eluent from the NR column was directed through the switching valve to waste for the first 8 min of the chromatographic run. At $t=8$ min, the switching valve was rotated and the eluent from the NR column was directed onto the C_{18} column. At $t=20$ min, the switching valve was rotated and the eluent from the NR column was again directed to waste.

At $t=20$ min, the second pump (P2) was engaged and a mobile phase consisting of Buffer–methanol (40:60, v/v) was pumped through the C_{18} column at a flow-rate of 1.0 ml/min. The eluent from the C_{18} column was directed into the mass spectrometer. At $t=30$ min, the mobile phase was changed to Buffer–methanol (95:5, v/v), the flow-rate reduced to 0.2 ml/min and the mass spectrometer turned off.

2.5. Mass spectrometry

Mass spectrometry experiments were performed using an Agilent MSD single quadrupole mass spectrometer in positive ion electrospray mode; data were recorded using Chem Station (version 6.3) software. The Capillary voltage was set at 2.5 kV and the fragmentor at 50 V. The mass spectrometer drying gas temperature was 350 °C, with nitrogen supplied as the nebulising gas (11 l/min). Single-ion monitoring was used when the mass spectrometer was directly coupled to the NR column and individual compounds were chromatographed. Total-ion monitoring was used in the experiments involving the multidimensional system and the test mixture.

3. Results and discussion

The retention times of the compounds used in this study were initially determined on the α3β4 nAChR using single-ion monitoring and the data are presented in Table 1. For compounds with no known affinity for the α3β4 nAChR the retention times ranged from 2.1 to 7.1 min, except for naltrexone, which eluted at 9.8 min. This result is most probably due to the compound's hydrophobicity. However, preliminary studies with naltrexone indicate that this compound may be a NCI of the α3β4 nAChR, albeit with an EC_{50} of >100 μ*M* (K. Kellar, personal communication). It is unclear if the NCI activity of naltrexone plays any role in the observed retention.

Cytisine is a marker ligand for the α4β2 nAChR and has displayed no significant affinity for the α3β4 nAChR [1]. Previous studies with NR columns composed of either the α3β4 nAChR or the α4β2 nAChR have demonstrated that NR columns can be used to distinguish between the activities of the two nAChR subtypes [7]. The fact that the retention time of cytisine was 4.8 min on a NR column containing immobilized α3β4 nAChR is consistent with the previously reported selectivity of immobilized nAChR subtypes.

The compounds with a known affinity for the α3β4 nAChR eluted with retention times between

Table 1
Compounds used in this study, their respective affinities for the α3β4 nAChR defined as yes if the compound is reported in Refs. [1,2,4,8] to bind to the receptor and no if it is not reported to bind to the receptor in Refs. [1,2,4,8], and the observed retention times on the column containing immobilized α3β4 nAChR

No.	Compound	Affinity for α3β4 nicotinic acetylcholine receptor	Retention time (min) $t<8.0$
1	Anabasine	No	2.1
2	Acetylcholine	Yes [1,2]	4.7
3	Benzamidine	No	6.3
4	Butyrlcholine	No	6.0
5	Caffeine	No	2.7
6	Cytisine	No (α2β4 ligand) [1]	4.8
7	2,3-Dihydroxybenzoic acid	No	3.2
8	Norepinephrine	No	2.1
9	Epinephrine	No	2.1
10	Glutamic acid	No	2.1
11	3-Hydroxytyramine	No	7.1
			$t\geq 8.0$
12	Ketamine	Yes (NCI) [3]	8.3
13	4-Dimethylaminopyridine	Yes (α1 ligand) [1,2]	9.5
14	Naltrexone	No	9.8
15	Nicotine	Yes [1,2]	10.3
16	Epibatidine	Yes [1,2]	13.1
17	Norketamine	Yes (NCI) [3]	8.5
18	Nornicotine	Yes [9]	8.8

8.3 and 13.1 min except for acetylcholine, which eluted at 4.7 min (Table 1). There was no attempt to relate retention on the NR with affinity for the α3β4 nAChR since this relationship had been previously established [6,7]. In this study, the individual retention times for compounds identified in the literature as α3β4 ligands were determined in order to ascertain when to switch the eluent flow to the C_{18} column.

Under the chromatographic conditions, epibatidine had the longest retention of the test compounds, 13.1 min, which is consistent with the fact that, of the compounds tested, it has the highest affinity for the α3β4 nAChR, $K_d = 0.30$ n*M* [7]. The affinity of nicotine for the α3β4 nAChR is 300-fold less than epibatidine, $K_d = 90$ n*M* [7], and the compound eluted 2.8 min before epibatidine at 10.3 min. Nornicotine has a 10-fold lower affinity than nicotine [9], and the compound eluted from the NR column at 8.8 min. In whole cell studies, the affinity of acetylcholine has been reported to be three-fold lower than nicotine [1]. However, there are no reported assays for the determination of the binding affinity of acetylcholine to the isolated α3β4 nAChR, and the reason for the short retention time of this compound on the NR column is unknown.

The results indicate that, in most cases, the magnitude of the chromatographic retention on the NR column is a reflection of binding affinity for the α3β4 nAChR. This suggests that the NR column could be used for the direct resolution of a mixture of compounds into α3β4 nAChR ligands and non-ligands. However, as the chromatogram of epibatidine illustrates, the chromatographic efficiency of the NR column is extremely poor (Fig. 2a). Indeed, co-injection of epibatidine and nicotine produced a chromatogram with no significant resolution of the two compounds even though their peak retention times were 2.8 min apart and their affinities differed by 300-fold (data not shown). In addition, the chromatogram of epibatidine presented in Fig. 2a was produced after the injection of a 1 μ*M* solution using SIM analysis at [M+H] 209.1. Due to the poor peak shape, this concentration was near the limit of detection of the mass spectrometer.

Clearly, the selectivity of the system was negated by the poor chromatographic efficiency. One way of overcoming the chromatographic inefficiency of the NR column is to couple it to a more efficient column. In the resulting multidimensional system, the NR column would separate nicotinic receptor ligands from non-ligands and the second column, in this case a C_{18} column, would separate the nicotinic receptor ligands from each other. The effect of the coupled NR–C_{18} column system on the chromatogram of epibatidine is presented in Fig. 2b. The overall retention time of epibatidine from injection onto the NR column to mass spectrometric detection was 23.7 min. In addition, peak compression on the C_{18} column increased the sensitivity of the method. The chromatogram in Fig. 2b was produced by a 200 n*M* solution of epibatidine and demonstrates that the sensitivity of the analysis has been significantly increased.

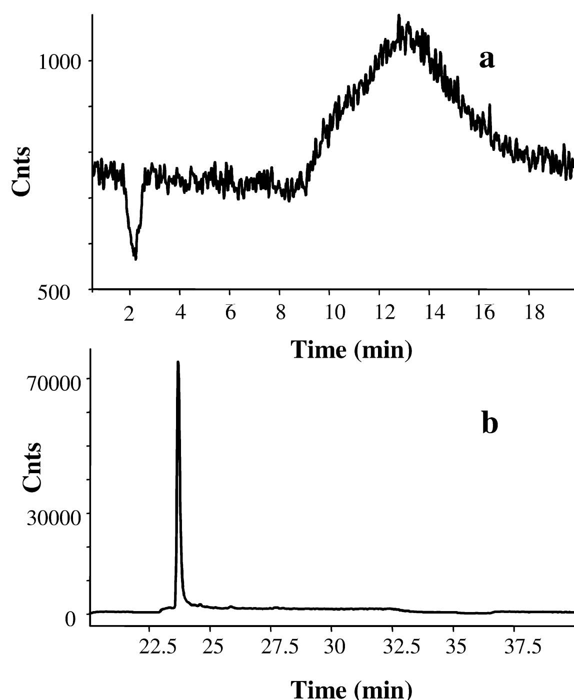

Fig. 2. A representative chromatogram from the chromatography of epibatidine on (a) the NR column alone, (b) the multidimensional system.

In order to test the hypothesis that the coupled NR–C_{18} system could be used to screen mixtures for ligands to the α3β4 nAChR, the compounds listed in Table 1 were combined to form a Test Mixture. Based on the individual chromatographic results, a

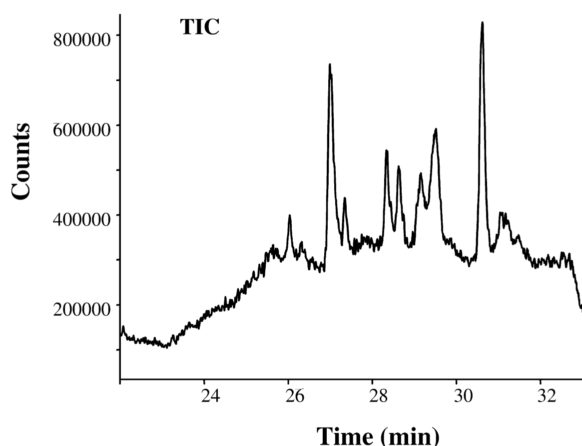

Fig. 3. Chromatogram from the chromatography of the Test Mixture on the multidimensional system.

cut-off time of 8 min was chosen, i.e. after injection onto the NR column, the eluent was directed to waste from 0 to 8 min and then, at the 8 min mark, the eluent was directed onto the C_{18} column. After 20 min, the C_{18} column was taken off-line from the NR column and the compounds compressed on the column were eluted into the mass spectrometer.

The Test Mixture was chromatographed using the conditions described above. The eluent from the C_{18} column was monitored using the total-ion current and the resulting chromatogram is presented in Fig. 3. The compounds in the eluent were identified from their [M+H] masses. The eluent from the C_{18} column contained seven of the 18 compounds from the Test Mixture (Table 1), six were known α3β4 nAChR ligands and the seventh was naltrexone, which may be a NCI.

The sensitivity and specificity of the multidimensional system was investigated by performing a Fisher's Exact Test using GraphPad Instat software (Version 5.05, GraphPad Software, San Diego, CA, USA). The question addressed was, "can the multidimensional system distinguish between ligands and non-ligands to the immobilized α3β4 nAChR using a retention time of 8 min as the selector?" The results of the test are presented in Table 2 and indicate that sensitivity, specificity, and positive and negative predictive values of the system are extremely significant.

Table 2
Fisher's Exact Test to determine, "Can the multidimensional system distinguish between ligands and non-ligands to the immobilized α3β4 nAChR using a retention time of 8 min as the selector?" The two-sided *P* value is 0.0025 (extremely significant) and the row/column association is statistically significant

Variable	Value	95% confidence interval
Sensitivity	0.9091	0.5874–0.9977
Specificity	0.8571	0.4210–0.9964
Positive predictive value	0.9091	0.5874–0.9977
Negative predictive value	0.8571	0.4210–0.9964
Likelihood ratio	6.364	

4. Conclusions

The results of this study demonstrate that a multidimensional system consisting of a NR column containing immobilized α3β4 nAChR coupled to a C_{18} column can be used for the on-line screening of mixtures for α3β4 nAChR ligands. Due to the poor efficiency of the NR column, the mixture could not be directly resolved on a single column. Instead, the NR column can be used to sort the mixture on the basis of relative affinity for the immobilized receptor. This study used an 8 min yes/no cut-off and was able to isolate seven of the eight α3β4 nAChR ligands contained in the Test Mixture. This approach could be refined by the addition of multiple cut-offs. In this study, fractions taken at 8–10, 10–12 and 12–20 min would place the isolated compounds into low-, medium- and high-affinity groups. The method is reproducible, the NR column is stable (the column used in these studies had been in use for over 1 year) and relatively quick as the total analysis time from the injection onto the NR column to the identification of the compounds was 32 min. In addition, the NR column was also able to identify NCIs of the α3β4 nicotinic receptor subtype. This is a unique capability of the NR column and the studies confirming this feature will be reported elsewhere.

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