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Review

Immobilized nicotinic receptor stationary phases: going with the flow in high-throughput screening and pharmacological studies

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This paper is dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday. It has been a pleasure and an honor to work with him and his students during the past 12 years. He has been a great inspiration and an excellent teacher to everyone who has had the opportunity to work with him. He also has a great singing voice.

Abstract

The nicotinic acetylcholine receptor (nAChR) subtypes α3β4-nAChR and α4β2-nAChR have been immobilized and the resulting stationary phases used to determine binding affinities. The α3β4-nAChR column was coupled to a C_{18} column and a mixture of 18 compounds was sorted into ligands and non-ligands for the α3β4-nAChR. The results demonstrate that the nAChR stationary phases can be used for on-line high-throughput screening (HTS).
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1. Introduction

Genomics and proteomics have produced new therapeutic opportunities through the identification of thousands of potential drug targets. In order to exploit these new targets drug discovery programs have been designed around high-throughput screening (HTS). At the present time the reigning HTS experimental paradigm utilizes targets immobilized in microtiter well plates and affinity binding or enzymatic activity as the discriminating factor. This approach has been extensively reviewed, cf. [1–3].

As currently practiced, HTS produces a great deal of data. However, a key problem is the extraction of information from this data [1]. This is partially due to the yes/no format of the microtiter well plate experiments as well as the need for post-screening separation and structural identification of the 'hits'. In addition, affinity binding probes are designed to identify competi-

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tive binding ligands, and compounds that bind non-competitively to the target are usually missed.

An alternative approach to the microtiter well plate format is affinity chromatography. In this format, chromatographic retention and/or peak shape are the probes of ligand-target interactions. While affinity chromatography cannot match the absolute quantity of data obtained from 1536-, 2080- and 2456-well plates, the information content of the data is greater and direct on-line structural information can be obtained.

The higher information content contained in affinity chromatographic data reflects the fact that the fundamental processes of drug action, absorption, distribution, excretion and receptor activation, are dynamic in nature and, in this manner, similar to the basic mechanisms involved in chromatographic retention. Indeed, the same basic intermolecular interactions (hydrophobic, electrostatic, hydrogen bonding) determine the behavior of compounds in both biological and chromatographic environments [4].

In the past few years, this laboratory has developed an approach to the on-line screening of complex mixtures using quantitative affinity chromatographic techniques and stationary phases containing immobilized trans-membrane receptors and transporters and cytosolic and membrane-bound enzymes [5]. The phases have included immobilized nicotinic acetylcholine receptors (nAChRs) alone [6,7] or co-immobilized with the γ-amino-butyric acid receptor and *N*-methyl-D-aspartate receptor [8]. The drug transporter P-glycoprotein has also been immobilized [9,10] as have the enzymes glutamine synthetase [11], phenyl *N*-methyl transferase [12] and dopamine β-hydroxylase [13].

In this presentation, the utility of the chromatographic approach in HTS and in the determination of pharmacological properties will be demonstrated using stationary phases based upon nAChRs.

2. Neuronal nicotinic acetylcholine receptors (nAChRs)

nAChRs are ligand-gated ion channels formed from five homologous subunits oriented around a center pore. At present, 12 different neuronal subunits have been identified, nine α subunits (α2–α10) and three β subunits (β2–β4). These subunits combine to form multiple nAChR subtypes [14,15].

The nAChR contains two cholinergic agonist binding sites located at the interface between the α and β subunits and on the extracellular N-terminal of the α subunits [16]. These sites are key targets for drug discovery in variety of diseases, including Alzheimer's, Parkinson's, epilepsy and Tourette's Syndrome [14,15].

The nAChR contains two other binding sites at which non-competitive inhibitors (NCIs) bind [15]. The first site is present in the channel lumen within the pore. The NCIs that bind to this site include mecamylamine [15], ketamine [17], bupropion [18] and dextromethorphan [19]. A second site is located at the interface of the lipid membrane and the NCIs acting at this site include estrogens and xenoestrogens [15].

Drug discovery programs aimed at nAChR mediated diseases primarily use a high-throughput screen for competitive agonists and antagonists. These experiments involve competitive binding affinity experiments in microplate format using recombinant receptor systems and radiolabeled marker ligands [14]. NCI activity is experimentally determined by measuring concentration dependent effects on whole cell currents or $^{86}Rb^{+}$ efflux yielding IC_{50} or EC_{50} values [17–19]. The latter studies are time consuming, exacting and the results often differ from laboratory to laboratory and from cell line to cell line.

3. Preparation of the α3β4-nAChR and α4β2-nAChR stationary phases [6,7]

Liquid chromatographic columns containing immobilized α3β4-nAChR or α4β2-nAChR subtypes have been prepared using a cell line expressing α3β4-nAChR [6,7] or from solubilized rat forebrain (α4β2-nAChR) [7]. The receptors were immobilized on an immobilized artificial membrane (IAM) stationary phase {Regis Chemical Co., Mortin Grove, IL, USA} or on Superdex gel

beads. The experimental procedures were as follows.

3.1. Preparation of α4β2 or α3β4-nAChR-detergent solution

Rat whole forebrain or transfected cells were suspended in 50 mM Tris–HCl, pH 7.4 (buffer A), homogenized for 30 s with Brinkmann Polytron, and centrifuged at $35\,000 \times g$ 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 $35\,000 \times g$ for 30 min, and the supernatant containing nAChR-cholate solution was collected.

3.2. Immobilization of nAChR on IAM particles

Dried IAM particles were suspended in 4 ml of the obtained detergent solutions containing proteins. For the immobilization of the various 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 nAChR–IAM support was then washed with buffer A, centrifuged and the solid collected.

3.3. Immobilization of nAChR on Superdex 200 gel beads

Dried lipid mixture of 60 mg L-α-lecitin (20% phosphatidylcholine), 10 mg L-α-phosphatidylserin, and 20 mg cholesterol was solubilized with 4 ml of obtained nAChR–detergent solution. The nAChR–lipid-cholate solution was mixed with 50 mg dry Superdex 200 beads. The suspension was dialyzed against buffer A for 24 h at 4 °C. Non-immobilized liposomes were removed by centrifugal washing with buffer A.

3.4. Results

About 63 mg protein isolated from the membrane of transfected cells and 14 mg of protein prepared from the brain tissues were, respectively, immobilized per gram of IAM particles or Superdex 200 gel beads. The binding activity of the immobilized nAChRs was investigated using a filtration binding assay with [^{3}H]-epibatidine ([^{3}H]-EB) as the marker ligand, (−)-nicotine as the displacer and blank IAM particles and Superdex 200 gel beads as the negative controls [7]. (−)-Nicotine specifically displaced [^{3}H]-EB from the supports containing the immobilized nAChRs, but no specific displacement of [^{3}H]-EB was detected on IAM particles and Superdex 200 gel beads. The results demonstrated that the immobilized nAChRs retained their specific binding activities, Table 1.

4. Frontal chromatographic studies: do the immobilized receptors still work

Frontal affinity chromatography was used to characterized the activity of the immobilized nAChRs. This technique permits the direct determination of binding affinities and the number of active binding sites [20]. Once the properties of the immobilized-nAChR stationary phase have been established, the column can be used in HTS using zonal chromatographic techniques.

4.1. Chromatographic conditions

The nAChR–IAM particles or nAChR–Superdex gel beads were packed in an Amersham

Table 1
The characteristics of the immobilized nAChR stationary phases based upon IAM particles and Superdex 200 gel beads determined using filtration binding assays with [^{3}H]-EB as marker ligand (for experimental details see [7])

Sample	Specific binding (%)	nAChR density (nmol/g protein)
α4/β2 NR-detergent solution[a]	62	0.14
α4/β2 NR–IAM[a]	49	0.81
α3/β4 NR-detergent solution[b]	100	8.57
α3/β4 NR–IAM[b]	97.8	5.09
α3/β4 NR-liposome Superdex 200[b]	29.4	1.45

[a] Prepared from rat forebrain with detergent deoxycholate.
[b] Prepared from transfected cells with detergent cholate.

Pharmacia HR 5/2 glass column (5 mm i.d. × 2 cm) and connected to a HPLC pump. [^{3}H]-EB was used as a marker and an on-line flow scintillation detector monitored the elution profile. All chromatographic experiments were performed at flow rate 0.4 ml/min at room temperature. A 50 ml sample superloop was used to apply a series of radioactive ligands through the nAChR column to obtain elution profiles showing a front and plateau regions. The chromatographic data was summed up in 1 min intervals and smoothed using the MICROSOFT EXCEL program with a ten point moving average.

4.2. Data analysis

The data from the frontal chromatography experiments were used to calculate dissociation constants, K_d, for the marker and displacer ligands using a previously described approach [6]. The experimental approach is based upon the effect of escalating concentrations of a competitive binding ligand on the retention volume of a marker ligand that is specific for the target receptor. For example, if the nAChR is the target, EB can be used as the marker ligand [6,7]. Then the association constants of EB, K_{EB}, and the test drug, K_{drug}, as well as the number of the active binding sites of the immobilized nAChR, P, can be calculated using Eqs. (1) and (2):

$$(V_{max} - V)^{-1} = (1 + [EB]K_{EB})(V_{min}[P]K_{EB})^{-1} + (1 + [EB]K_{EB})^2(V_{min}[P]K_{EB}K_{drug})^{-1} \times [drug]^{-1} \quad (1)$$

$$(V - V_{max})^{-1} = (V_{min}[P]K_{EB})^{-1} + (V_{min}[P])^{-1}[EB] \quad (2)$$

In the above equations, V is the retention volume of EB; V_{max}, the retention volume of EB at low concentration (60 pM) and in the absence of drugs; V_{min}, the retention volume of EB when the specific interaction is completely suppressed. The value of V_{min} can be determined by running [^{3}H]-EB in a series of concentration of drugs and plotting $1/(V_{max} - V)$ versus 1/(drug) extrapolating to infinite (drug). From the above plot and a plot of $1/(V - V_{min})$ versus (EB), dissociation constant values, K_d, for [^{3}H]-EB and the drugs can be obtained.

4.3. Chromatographic results with the α3β4-nAChR column

The retention volume of 60 pM [^{3}H]-EB was 23 ml (Fig. 1, profile A). This retardation was primarily due to the specific binding to saturable sites of the receptors as indicated by a decrease in retention volume to 8 ml when the concentration of [^{3}H]-EB was increased to 450 pM (Fig. 1, profile B).

The binding of [^{3}H]-EB to the α3β4-nAChR–IAM stationary phase could be reduced in competitive displacement experiments using known α3β4-nAChR ligands in the mobile phase. For example, the retention volume of 60 pM [^{3}H]-EB decreased from 23 to 18 ml when a 60 nM concentration of the nAChR–ligand (−)-nicotine was added to the mobile phase (Fig. 1, profile C) and fell to 0.9 ml when the (−)-nicotine concentration was increased to 1000 nM (Fig. 1, profile D). The decreases in retention volumes of [^{3}H]-EB relative to mobile phase concentrations of a

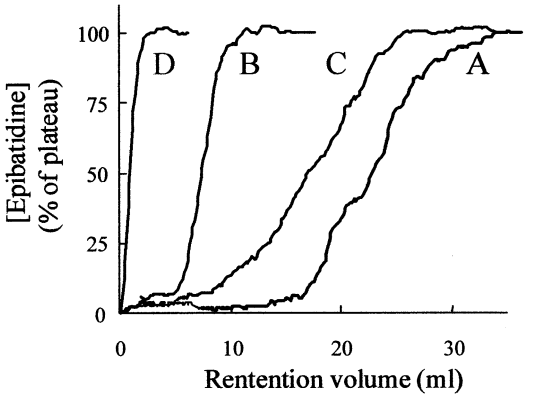

Fig. 1. Example of the elution profiles of [^{3}H]-EB on α3/β4 NR–IAM stationary phase (0.5 × 1.7 cm). 60 pM[^{3}H]-EB (A), 450 pM [^{3}H]-EB (B), and 60 pM [^{3}H]-EB in the presence of 60 nM (−)-nicotine (C) and 1000 nM (−)-nicotine (D) were included, respectively, in buffer A. Flow rate, 0.4 ml/min (reprinted from [6]).

displacer reflect the binding affinity of the displacer for the receptor.

Using this technique, the relative affinities of nicotinic drugs for the α3/β4-nAChR could be readily classified by determining the concentrations required to decrease the retention volumes of [^{3}H]-EB to a predetermined level.

To decrease the retention volumes of 60 pM [^{3}H]-EB from 9.5 to 6 ml on an α3/β4-nAChR column (0.5 × 1.25 cm), required mobile phase concentrations of 0.12 nM of EB, 17 nM of A85380, 45 nM of (−)-nicotine, 1200 nM of carbachol or 21 000 nM of atropine, respectively. The relative affinities of these drugs for the α3/β4-nAChR determined by this method were, therefore, EB > A85380 > (−)-nicotine > carbachol > atropine which is consistent with results from ligand binding assays using membrane homogenates.

The relative affinities can be classified by the dissociation constants (K_d) calculated from the resulting data (Table 2). The K_d values showed the same rank order as those of the values measured with binding assays using membrane homogenates. The low affinity of atropine (K_d: 17 200 nM) is also consistent with literature values. While the calculated affinities for the four ligands determined using both methods differed by up to fivefold, linear regression analysis showed that the two data sets correlated with a $r^2 = 0.9972$ ($P = 0.0014$). This indicated that the observed differences were quantitative, not qualitative.

Table 2
Dissociation constant, K_d, of ligands at α3β4-nAChR calculated using frontal chromatography on an immobilized α3β4-nAChR stationary phase and displacement experiments on membranes from cell expressing the α3β4-nAChR

Ligand	K_d (nM)[a]	K_d (nM)[b]
(±)-Epibatidine	0.27 ± 0.05	0.38 ± 0.07
A85380	17.2 ± 0.5	73.6 ± 6.3
(−)-Nicotine	88 ± 33	475 ± 52
Carbachol	1280 ± 30	3839 ± 276
Atropine	14 570 ± 2600	–

[a] Frontal chromatography with α3/β4-IAM stationary phase (0.5 × 1.3 cm).
[b] Binding assay using cell membrane homogenates.

4.4. Comparison of the α3β4-nAChR and α4β2-nAChR stationary phases

The results of binding to immobilized receptors showed that [^{3}H]-EB and (−)-nicotine had higher binding affinities at the α4/β2-nAChR subtype than at α3/β4-nAChR subtype, Table 3. These results are consistent with the results determined from ligand binding assays using membrane homogenates, Table 3. The K_d-values obtained from α4/β2-nAChR–liposome-Superdex 200 column were similar as those determined using α4/β2-nAChR–IAM column, Table 3.

4.5. Stability and reproducibility of nAChR columns

One α3/β4-nAChR–IAM column was used continuously over a 10-day period and then stored for 40 days at 4 °C. The retention volumes for 60 pM [^{3}H]-EB were 9.50 ± 0.05 ml (from day 1 to 10) and 9.70 ± 0.08 ml (day 50).

The relative affinities of EB and (−)-nicotine obtained on three α3/β4-nAChR–IAM columns prepared from different batches of cell lines were reproducible, Table 4. However, the retention volumes of EB at the same concentration differed from column to column.

Table 3
Comparison of the K_d-values of EB and (−)-nicotine at α3β4-nAChR and α4β2-nAChR calculated using frontal chromatography on an immobilized α3β4-nAChR and α4β2-nAChR stationary phases and displacement experiments on membranes from cell expressing the α3β4-nAChR and α4β2-nAChR

Formats of NRs	K_d of EB (nM)	K_d of (−)-nicotine (nM)
α3/β4-nAChR–IAM	0.27 ± 0.05	88 ± 33
α3/β4-nAChR membrane	0.38 ± 0.07	475 ± 52
α4/β2-nAChR–IAM	0.044 ± 0.005	1.0 ± 2.3
α4/β2-nAChR membrane	0.053 ± 0.002	7.2 ± 1.3
α4/β2-nAChR-Superdex	0.020 ± 0.08	7.4 ± 2

Table 4
Comparison of the dissociation constants and binding sites determined on three α3/β4-nAChR–IAM columns

Column size (cm)	K_d of EB (nM)	K_d of (−)-nicotine (nM)	Binding sites (pmol/ml bed)
0.5 × 1.8	0.34 ± 0.04	52 ± 10	7.5 ± 0.2
0.5 × 1.3	0.27 ± 0.05	88 ± 33	13.5 ± 0.3
0.5 × 1.7	0.21 ± 0.06	130 ± 45	15.0 ± 0.4

4.6. Conclusions drawn from the studies

The results from the frontal chromatographic studies demonstrate that the α3β4-nAChR and α4β2-nAChR can be solubilized and then immobilized on an IAM LC stationary phase with retention of their binding activities. The resulting receptor columns can be used to determined relative binding affinities of a series of compounds for a single nAChR subtype or for the comparison of these affinities between receptor subtypes.

5. Initial zonal chromatographic studies: are the columns efficient [7]

Once the activity of the immobilized nAChR had been established, the chromatographic efficiency of the columns was investigated. In addition, the effect of mobile phase composition on retention was determined in order to optimize the zonal chromatographic conditions.

5.1. Chromatographic conditions

The nAChR–IAM particles were packed in a HR5/2 glass column and connected to a HPLC pump. [^{3}H]-EB was used as a marker and an on-line flow scintillation detector monitored the elution profile. The radioactive signal (CPM) was recorded every 6 s, the CPMs recorded over a 30-s period were added and treated as a single data point. The resulting data were smoothed using the MICROSOFT EXCEL program with a five-point moving average to produce the elution profiles.

All chromatographic experiments were performed at a flow rate of 0.4 ml/min at room temperature. A 100 μl injection loop was used to supply the sample. The mobile phases consisted of ammonium acetate in concentrations from 5 to 200 mM all at pH 7.4 and 50 mM ammonium acetate solutions ranging in pH from 4 to 9.5.

5.2. Columns used in the experiments

The primary column used in these experiments contained immobilized α3β4-nAChR. Additional columns were constructed from α3 subunits only, β4 subunits only and a mixture of the two subunits.

5.3. Chromatographic results

There was no significant retention of [^{3}H]-EB {$t < 2$ min} on the α3-subunit column (peak 1, Fig. 2A), β4-subunit column (peak 2, Fig. 2A) and the α3-plus β4-subunit column (peak 3, Fig. 2A). However, [^{3}H]-EB was retained for 5 min on the

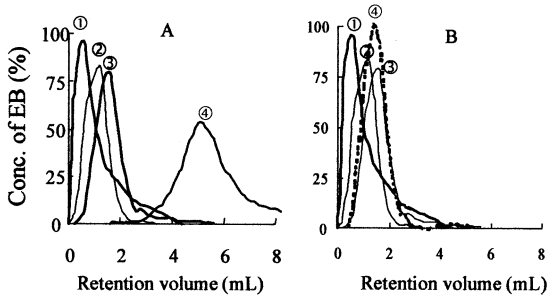

Fig. 2. Elution profiles of [^{3}H]-EB (0.5 nM) in zonal chromatography based on the α3 NR column (0.5 × 1.2 cm) (Peak 1), the β4 NR column (0.5 × 1.1 cm) (Peak 2), the α3 + β4 NR column (0.5 × 1.3 cm) (Peak 3) and the α3β4 NR column (0.5 × 1.5 cm) (Peak 4). Mobile Phase: Tris–HCl buffer (50 mM, pH 7.4) (A) No (−)-nicotine in the mobile phase. (B) 1 μM (−)-nicotine present in the mobile phase (reprinted from [7]).

α3/β4-nAChR column (peak 4, Fig. 2A). When 1 μM (−)-nicotine was added to the mobile phase, the retention of [^{3}H]-EB was reduced to $t < 2$ min, peak 4 (dash line) Fig. 2B, and no significant change in the retention of [^{3}H]-EB was observed in the other three columns, Fig. 2B. The results indicate that the retention of [^{3}H]-EB on the α3β4-nAChR column was specific retention due to the intact receptor and not the result of interactions with the individual subunits.

5.4. Effects of ionic strength and pH on the retention of [^{3}H]-EB

On the α3β4-nAChR column, the retention volumes of [^{3}H]-EB increased from pH 4.0 to 7.0 (1–6 ml) and then remained constant in the pH range 7.0–9.5. When 5 mM ammonium acetate (pH 7.4) was used as the mobile phase, the retention volume of [^{3}H]-EB was 17 ml. The retention volume decreased with increasing ionic strength of the buffer and at an ammonium acetate concentration of 200 mM (pH 7.4) the compound eluted in a volume of 5 ml.

The binding of competitive agonists and antagonists to the nAChR is based upon electrostatic interactions. The effects of pH and ionic strength on the retention volume of [^{3}H]-EB are indicative of an electrostatic-based retention mechanism. This indicates that the same functional binding mechanism operates on both the immobilized and free nAChR. In addition, the data presents a means to optimizes chromatographic performance on the nAChR column through the manipulation of mobile phase pH and ionic strength.

5.5. Conclusions drawn from the studies

The results of this study demonstrate that the immobilized α3β4-nAChR column could be used in standard zonal chromatographic studies. In addition, the chromatographic retention observed on the α3β4-nAChR column was due to the intact receptor and reflected specific binding to that receptor.

6. The application of the α3β4-nAChR column to HTS using multidimensional chromatography [21]

The frontal and zonal chromatographic studies demonstrated that the immobilized nAChR columns could be used to chromatographically separate compounds based upon their affinities for the nAChR. However, the efficiency of the α3β4-nAChR was too low to directly resolve a mixture into its individual components, Fig. 3a. Thus, a multidimensional chromatographic system was constructed by coupling a C_{18} column to the α3β4-nAChR, Fig. 4. In this manner, a mixture could be sorted into binders and non-binders, and the compounds with affinity for the α3β4-nAChR transferred to the C_{18} column for separation and identification.

6.1. Chromatographic system and conditions

The α3β4-nAChR column {50 × 2 mm i.d.} was connected via a switching valve to a C18 column

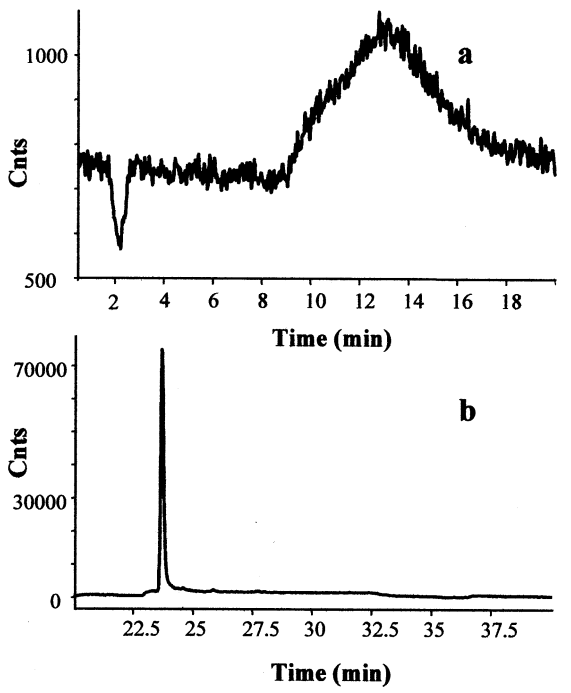

Fig. 3. A representative chromatogram from the chromatography of EB on: (a) the α3β4-nAChR column alone; (b) the multidimensional system (reprinted from [21]).

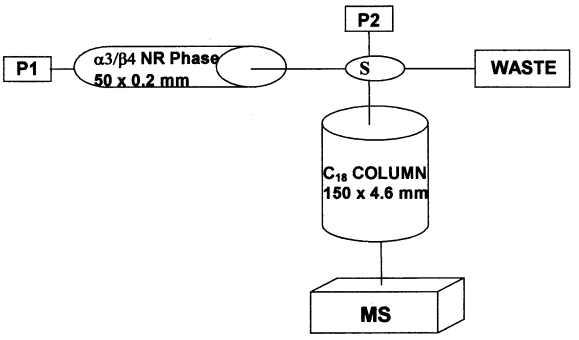

Fig. 4. The multidimensional system used in this study (reprinted from [7]).

{150 × 4.6 mm i.d.}. The system was then connected on-line to a single quadrupole mass spectrometer, Fig. 4.

The mobile phase consisted of ammonium acetate (10 mM, 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 α3β4-nAChR column connected to the C_{18} column. The eluent from the α3β4-nAChR column was directed to the C_{18} column for 12 min. At $t = 20$ min, the switching valve was rotated and the α3β4-nAChR column was disconnected from the C_{18} column. The compounds trapped on the C_{18} column were eluted onto the mass spectrometer using a mobile phase of ammonium acetate (10 mM, pH 7.4): methanol (40:60, v/v) at a flow rate of 1.0 ml/min. The analysis time was 12 min. Detection was accomplished using total-ion monitoring.

6.2. Compounds used in the study

Eighteen compounds were used in the study, seven compounds that bind to the α3β4-nChR and 11 that do not bind, Table 5. Of the seven compounds that displayed affinity for the α3β4-nChR, five were competitive ligands and two, ketamine and norketamine, were NCIs.

6.3. Results of the study

The chromatographic profile of EB on the α3β4-nAChR column is presented in Fig. 3a and the post-column peak compression achieved on the C_{18} column is presented in Fig. 3b. The chromatographic experiment was completed in 25 min and resulted in a 70-fold increase in the detection sensitivity for EB.

The chromatographic profile, presented as total-ion current, from the on-line fractionation of the mixture of 18 compounds is presented in Fig. 5. The chromatogram contains seven compounds, one of which had no known affinity for the α3β4-nAChR and six, which had known affinity for the receptor, Table 5. The one compound that appears to be misplaced, naltrexone, is a weak NCI of the α3β4-nAChR with an IC_{50} of 100 μM (K. Kellar, personal communication). This may be the reason that the compound was retained on the immobilized nAChR column.

The other 11 component of the mixture were not present in the fraction transferred to the C_{18} column from the α3β4-nAChR column at $t = 8$ min. This group included ten compounds with no known affinity for the α3β4-nAChR and one α3β4-nAChR ligand, Table 5. The α3β4-nAChR ligand was acetylcholine. Previous studies with the KXα3β4R2 cell line (the source of the α3β4-nAChR used in this study) have shown that acetylcholine has a lower affinity for the nAChR than nicotine (K_i acetylcholine = 881 nM, K_i nicotine = 475 nM) [22]. Since the screen was set using the affinity of nicotine as the cutoff point, it is not surprising that acetylcholine was not present in the fraction transferred to the C_{18} column.

A Fisher's exact test for sensitivity and specificity was performed to determine if the chromatographic system could differentiate between non-nAChR ligands and nAChR ligands. The two-sided P-value was 0.0025 (extremely significant) and the Likelihood ratio was 6.364 indicating that the system can confidently distinguish between non-nAChR and nAChR ligands.

6.4. Conclusions drawn from the study

The results from the study indicate that the coupled α3β4-nAChR–C_{18} system can be used for the on-line screening of a mixture. The compounds are sorted based upon binding affinity for the immobilized receptor and then directly identified by mass spectrometry.

Table 5
The compounds used in this study, their respective affinities for the α3β4-nAChR and retention times on the column containing immobilized α3β4-nAChR

Numbers	Compound	Affinity for α3β4-nAChR	Retention time (min)
1	Anabasine	No	$t < 8$
2	Acetylcholine	Yes	
3	Benzamidine	No	
4	Butyrlcholine	No	
5	Caffeine	No	
6	Cytisine	No	
7	2,3-Dihyroxybenzoic acid	No	
8	Norepinephrine	No	
9	Glutamic acid	No	
10	Epinephrine	No	
11	3-Hydroxytyramine	No	
12	Epibatidine	Yes	$t \geq 8$
13	Ketamine	Yes	
14	Naltrexone	No	
15	Nicotine	Yes	
16	4-Dimethylaminopyridine	Yes	
17	Norketamine	Yes	
18	Nornicotine	Yes	

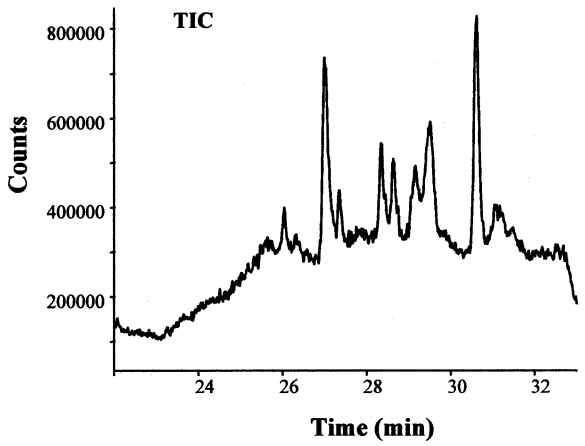

Fig. 5. The chromatogram from the chromatography of a mixture of 18 compounds on the multidimensional system (reprinted from [7]).

7. Summary

The nAChR subtypes α3β4-nAChR and α4β2-nAChR have been immobilized on the IAM liquid chromatographic stationary phase. Frontal affinity chromatography experiments have demonstrated that the resulting stationary phases can be used to determine the binding affinities (K_d-values) of compounds to the receptor. The K_d-values determined chromatographically were comparable to those found using standard membrane affinity binding techniques. Frontal affinity chromatography could also be used to detect differences in the binding affinity of a compound between the receptor subtypes.

In zonal chromatography, the retention time of a compound observed on the α3β4-nAChR stationary phase was also comparable to its affinity for the receptor. However, the chromatographic efficiency of the column was not great enough to adequately resolve a mixture of compounds based on their relative affinities for the α3β4-nAChR. Therefore, the α3β4-nAChR column was coupled to a column containing a C_{18} stationary phase. A mixture of 18 compounds were sorted into two groups, those with an affinity for the α3β4-nAChR < that of nicotine and those with an affinity ≥ nicotine. The compounds with a affinity ≥ nicotine were transferred to the C_{18} column, separated and identified by mass spectrometry using total-ion monitoring.

The results demonstrate that the immobilized nAChR stationary phases can be used for the on-

line screening for ligands to the nAChR. In this study, 18 compounds were sorted and identified in 32 min. The mixture did not saturate the α3β4-nAChR column and the throughput can be increased by adding more compounds to the mixture {to date up to 28 compounds have been analyzed in a single run} or by moving to a micro-column format with parallel throughput. These developments will be reported elsewhere.

The studies summarized in this report as demonstrate that the nAChR columns can be used to identify both competitive and non-competitive ligands to this receptor. This represents a significant advance from the microtiter well plate format.

The results from these studies also demonstrate the possibility of the extensive use of immobilized liquid chromatographic stationary phases containing drug targets in on-line HTS. This is also illustrated by the successful development of stationary phases containing immobilized receptors, transporters and enzymes [5] including the γ-amino-butyric acid receptor and *N*-methyl-D-aspartate receptor [8], the drug transporter P-glycoprotein [9,10], and the enzymes glutamine synthetase [11], phenyl *N*-methyl transferase [12] and dopamine β-hydroxylase [13]. The usefulness of this approach is only limited by the ability to identify potential targets and the construction of the appropriate chromatographic system.

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