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Review

Enantioselective interactions of dextromethorphan and levomethorphan with the α3β4-nicotinic acetylcholine receptor: comparison of chromatographic and functional data

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Abstract

The enantioselectivity of the interaction of dextromethorphan (DM) and levomethorphan (LM) with an immobilized α3β4 subtype of the nicotinic acetylcholine receptor (nAChR) liquid chromatographic stationary phase has been compared to DM- and LM-induced non-competitive blockade of nicotine-stimulated $^{86}Rb^+$ efflux from cells expressing the α3β4-nAChR. The association rate constants (k_{on}) and dissociation rate constants (k_{off}) for the formation of the DM and LM complexes with the nAChR were determined using non-linear chromatographic techniques and the k_{off} value for DM ($1.01 \pm 0.01\,s^{-1}$) was significantly lower than the k_{off} for LM ($1.55 \pm 0.002\,s^{-1}$) while the k_{on} values did not significantly differ (23.66 ± 0.61 and $18.61 \pm 0.36\,\mu M^{-1}\,s^{-1}$, respectively). In thermodynamic studies using the van't Hoff approach, the enthalpy change (ΔH°) of the DM–nAChR complex was $330\,cal\,mol^{-1}$ more stable than the LM–nAChR complex, while there was no significant difference in the entropy change (ΔS°). In the functional in vitro cell-based studies, there was no significant difference in the observed IC_{50} values for DM ($10.1 \pm 1.01\,\mu M$) and LM ($10.9 \pm 1.08\,\mu M$), but the recovery from the DM-induced blockade was slower than the recovery from LM-induced blockade; after 7 min: $38.25 \pm 15.46\%$ recovery from DM blockade, $63.30 \pm 16.08\%$ from LM blockade; after 4 h: $76.20 \pm 4.51\%$ recovery from DM blockade and $93.12 \pm 8.76\%$ from LM blockade. The enantioselective differences in the functional effects are consistent with the chromatographic and thermodynamic data and indicate that this difference is due to increased stability of the DM–nAChR complex. The results suggest that the chromatographic approach can be used to probe the interaction of non-competitive inhibitors (NCIs) with nAChRs and to predict relative duration of functional blockades.
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Keywords: Reviews; Enantiomer separation; Dextromethorphan; Levomethorphan; Nicotinic acetylcholine receptor

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Abbreviations: DM, dextromethorphan; LM, levomethorphan; nAChR, nicotinic acetylcholine receptor; NCI, non-competitive inhibitor

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1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChR) contain at least two binding sites at which non-competitive inhibitors (NCIs) can bind [1,2]. A key site is located in the channel lumen within the central pore of the receptor. At this site, NCIs inhibit nAChR function by a reversible channel blockade or shorten channel opening time in a voltage-sensitive manner [3]. The NCIs that bind to this site include mecamylamine [2,4], ketamine [5], and dextromethorphan (DM) [6].

The interactions between nAChRs and NCIs at the channel lumen have been described primarily in terms of IC_{50} values, which were experimentally determined by measuring concentration-dependent effects on whole cell currents or $^{86}Rb^+$ efflux [5–8]. The binding interactions, i.e. association and dissociation equilibrium constants (K_a/K_d) as well as association and dissociation rate constants (k_{on}/k_{off}), between NCIs and nAChRs have not been extensively described.

The determination of K_a/K_d and k_{on}/k_{off} values for NCI–nAChR interactions has been hampered by the absence of a rapid and direct method for these measurements. We have recently reported one possible approach to these determinations employing a liquid chromatographic stationary phase containing immobilized nAChR and non-linear chromatography techniques [9]. Non-linear theory has been applied to affinity chromatography and assumes that (1) the stationary phase contains a limited number of specific active sites; (2) the chromatographic interactions are described by a non-linear adsorption isotherm; (3) interphase solute transfer is not infinitely fast; (4) kinetic rates of adsorption and desorption are the primary source of band broadening and peak skew [10,11]. Using non-linear chromatography approach, concentration-dependent peak asymmetries can be used to determine K_a, k_{on} and k_{off}.

In the previous study, the NCIs: mecamylamine, ketamine, bupropion and DM were chromatographed on an immobilized α3β4-nAChR liquid chromatographic stationary phase and the K_a, k_{off} and k_{on} values determined for each ligand–receptor interaction [9]. However, these studies could not determine if a relationship existed between chromatographically determined kinetic parameters and functional properties of the NCIs. A key difficulty was the fact that the calculated non-linear chromatography parameters may reflect both non-specific interaction between the NCIs and the chromatographic support and specific interactions between the NCI and immobilized nAChR.

In liquid chromatography, one technique to separate specific from non-specific interaction is to use enantiomeric compounds. Enantiomers have identical physiochemical properties and, therefore, all non-specific interactions between the enantiomers of a chiral NCI and an immobilized nAChR stationary phase should be equivalent. Any differences in the chromatographic retention between the NCI enantiomers will be due to specific binding interactions with the immobilized nAChR.

Supporting this approach, enantioselective interactions between nAChR and NCIs have been previously reported in functional experiments [12]. In a study of the interaction of the mecamylamine enantiomers with the α3β4-nAChR, (*S*)-mecamylamine and (*R*)-mecamylamine had equivalent activity, expressed as IC_{50} values, relative to the inhibition of acetylcholine-induced whole cell currents. However, the (*S*)-mecamylamine-induced blockade lasted longer than the (*R*)-mecamylamine-induced blockade. In addition, Friederich et al. [13] have reported that the IC_{50} value for (*S*)-ketamine is four-fold lower than the IC_{50} value for (*R*)-ketamine in a SH-SY5Y cell line (human peripheral neuroblastoma cells), however, Sasaki et al. [14] found no enantioselectivity in the ketamine-induced inhibition of nAChR-mediated current in PC12 cells (a rat pheochromocytoma cell line).

In the present study, we hypothesized that chromatographic studies of interactions between DM and its enantiomer levomethorphan (LM) with an immobilized α3β4 subtype of nAChR employing non-linear chromatography analysis and van't Hoff approach in thermodynamic analysis would reflect results from functional studies of DM- and LM-induced blockade of nicotine-stimulated $^{86}Rb^+$ efflux experiments on KXα3β4R2 cells (human kidney embryonic 293 cells stably transfected with the rat nAChR α3 and β4 subunit genes [4]).

2. Materials and methods

2.1. Materials

(−)-Nicotine hydrogen tartate, dextromethorphan hydrobromide, and poly-D-lysine were purchased from Sigma (St. Louis, MO, USA). Levomethorphan was purchased from Cerilliant Co. (Round Rock, TX). HPLC grade methanol, ammonium acetate, 0.1 M ammonium hydroxide solution and other chemicals were purchased from Fisher scientific (Pittsburgh, PA, USA).

Tissue culture medium and penicillin/streptomycin were purchased from Gibco Invitrogen Corp. (Carlsbad, CA, USA). Geneticin (G-418) was purchased from Cellgro by Mediatech Inc. (Herndon, VA, USA). Fetal bovine serum was obtained from Biosource International (Camarillo, CA, USA). ^{86}RbCl was purchased from Perkin-Elmer (Boston, MA, USA).

2.2. Preparation of the α3β4-nicotinic acetylcholine receptor (α3β4-nAChR) stationary phase

The preparation and characterization of the α3β4-nAChR stationary phase has been previously reported [15,16]. In brief, the KXα3β4R2 cell line with expressed α3β4-nAChR [4] was used to create the column. The 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 × 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 nAChR-cholate solution was collected.

The receptors were immobilized on an immobilized artificial membrane (IAM) stationary phase (IAM-PC (12 μm, 300 A), Regis Chemical Co., Morton Grove, IL, USA). To do this, dried IAM particles were suspended in 4 ml of the detergent solution containing the nAChR proteins and the mixture was stirred for 1 h at room temperature. The suspension was then dialyzed against 2 × 1 l buffer A for 24 h at 4 °C. The nAChR-IAM support was then washed with buffer A, centrifuged, the solid collected and packed into a 0.5 cm (i.d.) × 0.8 cm HR5/2 glass column purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

2.3. Chromatographic system

The LC-MS system composed of a SIL-HTA autosampler and LC10AD pump (Shimadzu, Columbia, MD, USA), and a Micromass Q-TOF mass-spectrometer (Micromass, Beverly, MA, USA). The column temperature was controlled by placing the column containing the immobilized α3β4-nAChR stationary phase inside a Plexiglas column jacket (Aura Industries, New York, NY, USA) that was connected to a Haake D1 circulating thermostated water bath (Thermo Electron Corporation, Waltham, MA, USA).

DM and LM were monitored in the positive ion mode (ESI+). The compounds were detected using single ion monitoring at $m/z = 272$ $[\mathrm{MW} + \mathrm{H}]^+$ ion. The chromatograms were recorded and processed using MassLynx v. 3.5 (Micromass).

2.4. Non-linear chromatographic studies

Aqueous solutions (10 μM) of each compound were prepared and 20 μl aliquots were injected into column. The mobile phase was composed of ammonium acetate (10 mM, pH 7.4) modified with methanol in the ratio 85:15 (v/v). The flow rate was $0.2\,\mathrm{ml\,min^{-1}}$ and the experiments were carried out at ambient temperature.

The data from the non-linear studies was analyzed using PeakFit v4.11 for Windows Software (SPSS Inc., Chicago, IL). The mathematical approach was the non-linear chromatography model derived from Impulse Input Solution [10] and described by Eq. (1) (PeakFit User's Manual, pp. 8–25)

$$y = \frac{a_0}{a_3}\left[1 - \exp\left(-\frac{a_3}{a_2}\right)\right] \times \left[\frac{\sqrt{(a_1/x)}I_1(2\sqrt{a_1 x}/a_2)\exp((-x-a_1)/a_2)}{1 - T(a_1/a_2),(x/a_2)[1-\exp(-a_3/a_2)]}\right] \quad (1)$$

where y is intensity of signal, x is reduced retention time, $T(u, v) = \exp(-v)\int_0^u \exp(-t) I_0(2\sqrt{vt})\,\mathrm{d}t$; $I_0(\cdot)$ and $I_1(\cdot)$ are modified Bessel functions, a_0 is area parameter, a_1 is center parameter, reveal to true thermodynamic capacity factor, a_2 is width parameter, and a_3 is distortion parameter.

Obtained chromatograms were smoothed with MassLynx v. 3.5 procedure and next extracted to Excel worksheet as a set of two columns: retention time and signal intensity. The input data were further processed with PeakFit v4.11 software: after standard linear baseline subtraction, each peak profile was fitted to the Eq. (1). The minimization procedure was repeated at least three times in order to find the global minimum. The set of parameters (a_0, a_1, a_2 and a_3) was collected for each profile and used for the calculation of descriptors of the kinetic interactions with the immobilized nAChR, dissociation rate constant (k_{off}); equilibrium constant (K_a); association rate constant (k_{on}) real thermodynamic capacity factor (k'_{NLC}), according to the following equations:

$$k'_{\mathrm{NLC}} = a_1 \quad (2)$$

$$k_{\mathrm{off}} = \frac{1}{a_2 t_0} \quad (3)$$

$$K_\mathrm{a} = \frac{a_3}{C_0} \quad (4)$$

$$k_{\mathrm{on}} = k_{\mathrm{off}} K_\mathrm{a} \quad (5)$$

where t_0 is the dead time of a column; C_0 is a concentration of solute injected multiplied by a width of the injection pulse (as a fraction of column dead volume).

2.5. Thermodynamic chromatographic studies

The mobile phase was composed of ammonium acetate (10 mM, pH 7.4) modified with methanol in the ratio 85:15 (v/v). The flow rate was maintained at $0.2\,\mathrm{ml\,min^{-1}}$. Chromatograms were obtained at 5 °C intervals from 5 to 30 °C. At each temperature, DM and LM were independently injected (20 μl of a 10 μM aqueous solution) onto the column and the chromatograms recorded.

The chromatograms were smoothed with MassLynx v. 3.5. The retention times corresponding to the peak maxima

were determined and the capacity factors, k's, calculated according to the equation:

$$k' = \frac{t_R - t_0}{t_0} \quad (6)$$

where t_R is retention time at peak maximum and t_0 is dead time of the system. The t_0 value was determined by injecting the pure water.

The enantioselectivity of the chromatographic process was expressed as the enantioselectivity factor, α, calculated according to the equation:

$$\alpha = \frac{k'_2}{k'_1} \quad (7)$$

where k'_1 is the capacity factor of the first eluted compound and k'_2 is the capacity factor of the second eluted compound.

The thermodynamic characteristics of chromatographic process were calculated from the retention data at the experimental temperatures using the relationship:

$$\ln k' = \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{R}\frac{1}{T} \quad (8)$$

where ΔS° and ΔH° are standard entropy change and standard enthalpy change, respectively; R is gas constant ($1.9872\,\mathrm{cal\,mol^{-1}\,K^{-1}}$); and T is temperature in Kelvin.

2.6. Nicotine-stimulated $^{86}Rb^+$ efflux experiments on KXα3β4R2 cells

The KXα3β4R2 cells were established and maintained as described previously [4]. The function of nAChR expressed in KXα3β4R2 cells was measured using a $^{86}Rb^+$ efflux assay [6]. Briefly, cells in growth medium were plated onto 24-well plates. On the day of experiment, cells were loaded in medium containing $2\,\mu Ci\,ml^{-1}$ $^{86}Rb^+$ for 4 h at 37 °C. After loading, cells were washed three times, and 1 ml of buffer with or without drugs was added to each well for 2 min. The efflux buffer was collected, and the cells were lysed in 1 ml of 0.1 M NaOH. The radioactivity in the efflux samples and cell lysates was measured by liquid scintillation counting. The amount of $^{86}Rb^+$ efflux was expressed as the percentage of the total $^{86}Rb^+$ loaded (fractional release). Stimulated $^{86}Rb^+$ efflux was defined as the difference between efflux in the presence and absence of nicotine (total efflux − basal efflux). For obtaining an IC_{50} value, inhibition curves were constructed in which a range (0.03–500 μM) of concentrations for each antagonist was included in the assay to inhibit efflux stimulated by 100 μM nicotine. In the recovery from antagonists block studies, cells were preincubated with DM or LM (10 μM) for a 24 h time period before loading with $^{86}Rb^+$ (in presence or absence of drug). Cells were washed four times over a 7 min and 4 h period and nicotine-stimulated efflux was measured as described above. The IC_{50} values and curve fittings were determined by non-linear regression analyses using Prism software (GraphPad Software, San Diego, CA).

3. Results

3.1. Non-linear chromatographic studies

The peak profiles for DM and LM obtained under the chromatographic conditions utilized in this study are presented in Fig. 1. The chromatographic capacity factor (k') for LM was 25 and the k' for DM was 40.5. The calculated

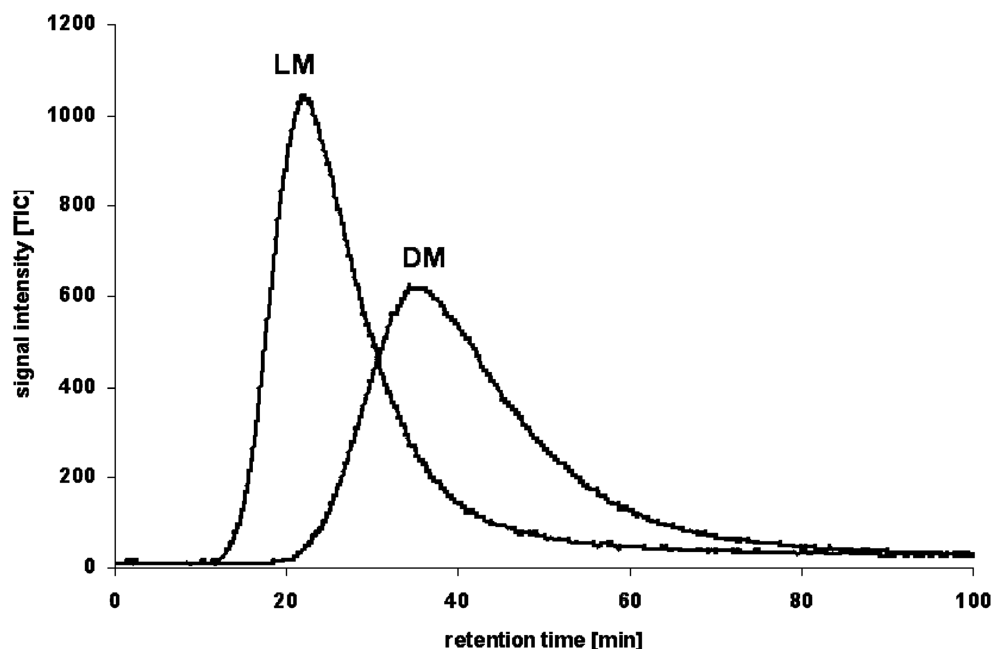

Fig. 1. The comparison of peak profiles of DM and LM obtained in independent experiments consequent injections.

Table 1
DM and LM parameters determined in non-linear chromatography study

Compound	k'_{NLC}	k_{on} ($\mu M^{-1}\,s^{-1}$)	k_{off} (s^{-1})	K_a (μM^{-1})
DM	61.30 (±0.27)	23.66 (±0.61)	1.01 (±0.01)	23.40 (±0.36)
LM	35.81 (±0.15)	18.61 (±0.38)	1.549 (±0.002)	12.01 (±0.23)

See Section 2 for details.

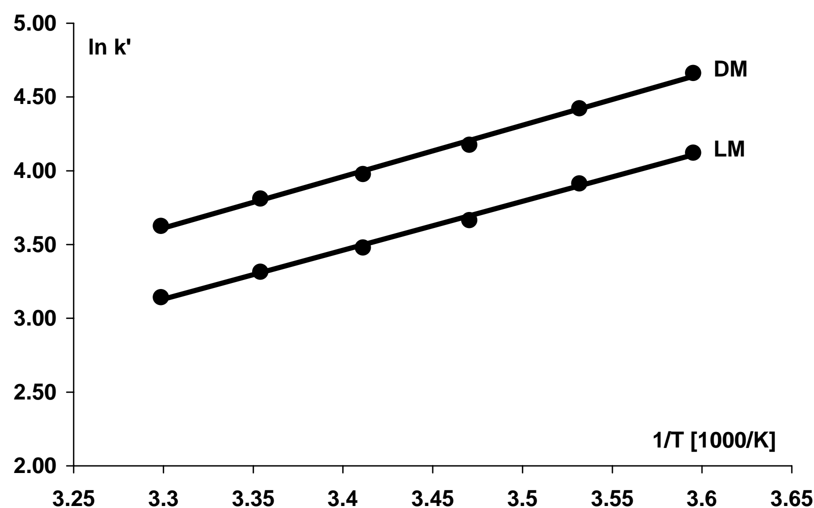

Fig. 2. The relationship between chromatographic retention expressed as $\ln k'$ of DM and LM and temperature expressed as $1/T$.

enantioselectivity factor (α) was 1.62. Based on this value, $\Delta\Delta G$ of separation can be calculated by the following equation:

$$\Delta\Delta G = -RT\ln\alpha \tag{9}$$

and (at 20 °C) equals to $-0.28\,\text{kcal mol}^{-1}$.

Both compounds produced broad and asymmetric peaks that were consistent with previously reported profiles obtained in studies on the α3β4-nAChR column [9]. The resulting non-linear chromatography derived kinetic parameters, the true thermodynamic capacity factors (denoted as k'_{NLC}), association and dissociation rate constants (k_{on} and k_{off}, respectively) and chromatographically determined equilibrium constants (K_a), are shown in Table 1.

3.2. Thermodynamic studies

The effect of temperature on the chromatographic retention of DM and LM on the nAChR column was determined using a sequence of temperatures ranging from 5 to 30 °C. The compounds were independently injected at each temperature and their respective k's established (Table 2). van't Hoff plots were constructed for each enantiomer by plotting $\ln k'$ versus $1/T$ (Fig. 2). The correlations for DM and LM were linear with R^2 values of 0.9984 and 0.9985, respectively.

The standard changes in enthalpy (ΔH°), entropy (ΔS°) and free energy (at 20 °C) (ΔG°) of the binding interactions between DM and the immobilized nAChR and LM and the immobilized nAChR were calculated from the slopes and intercepts of the van't Hoff plots (Table 2).

Table 2
Temperature dependence of the retention of DM and LM and determination of the thermodynamic parameters of the interaction of DM and LM with the immobilized nicotinic acetylcholine receptor using Eq. (8)

T (K)	$\ln k'_{DM}$	$\ln k'_{LM}$
278	4.66	4.12
283	4.42	3.91
288	4.18	3.66
293	3.98	3.48
298	3.81	3.31
303	3.63	3.14
	DM	LM
ΔH° (kcal mol^{-1})	−6.92 (±0.19)	−6.59 (±0.18)
ΔS° (cal mol^{-1} K^{-1})	−15.7 (±0.7)	−15.2 (0.6)
ΔG° (kcal mol^{-1})	−2.33 (±0.4)	−2.04 (±0.4)

Table 3
The results of nicotine-stimulated $^{86}Rb^+$ efflux experiments of DM and LM

Enantiomer	IC_{50} (μM)	Percent recovery after 7 min	Percent recovery after 4 h
DM	10.1 (±1.10)	38.25 (±15.46)	76.20 (±4.51)
LM	10.9 (±1.08)	63.30 (±16.08)	93.12 (±8.76)

IC_{50} values (determined as explained in Section 2) and percent recoveries from blockade (data from 300 μM nicotine experiments).

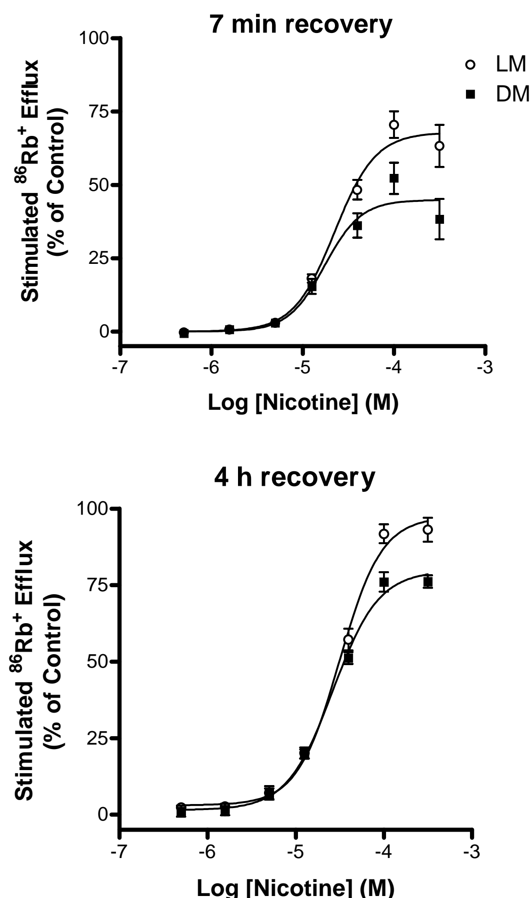

Fig. 3. Recovery from blockade of nicotine-stimulated function by DM and LM in KXα3β4R2 cells. Cells were preincubated for 24 h with 10 μM of DM and LM and then allowed to recover in media for 7 min or 4 h wash period.

3.3. Nicotine-stimulated $^{86}Rb^{+}$ efflux

The IC_{50} values determined for DM and LM using nicotine-stimulated $^{86}Rb^{+}$ efflux experiments are presented in Table 3. No significant difference was observed between the two enantiomers.

The percent recovery from the blockade of nicotine-stimulated efflux by DM and LM was studied at a series of nicotine concentrations and assessed after recovery in media for 7 min or 4 h (Fig. 3). At the higher nicotine concentrations, the percent recovery following DM block was significantly less than the percent recovery seen after treatment of the cells with LM at both time points (Fig. 3). The representative values from experiments utilizing 300 μM nicotine stimulation (average of five experiments) are presented in Table 3. At both time points there was a statistically significant difference in percent recovery after DM and LM blockade, in 7 min, $p = 0.0363$; in 4 h, $p = 0.0049$ (data analyzed by t-test for independent samples).

4. Discussion

In liquid chromatography, chromatographic retention is the summation of non-specific and specific interactions between the solutes and the stationary phase. When the stationary phase contains an immobilized chiral selector and the solutes are enantiomeric compounds, the non-specific interactions between each enantiomeric solute and the stationary phase will be equivalent and will not produce a chromatographic separation of the two enantiomers. Any observed enantioselective separation will reflect specific and unequal interactions between the solutes and the chiral selector.

In this study, the immobilized chiral selector was the α3β4-nAChR and the enantiomeric solutes were DM and LM. Previous studies with this system employing mecamylamine as a displacer, established that DM binds to high affinity site(s) located in the channel lumen within the pore of the receptor [9].

The regression lines constructed from the temperature-retention studies (Fig. 2) support this assumption. Linearity within van't Hoff plots indicates an invariant retention mechanism over the temperature range studied [17]. Thus, DM and LM interacted with the immobilized α3β4-nAChR stationary phase via a single mechanism.

The use of van't Hoff plots to examine the binding thermodynamics of agonists and competitive antagonists to nAChRs and other receptors has been previously reported [18,19]. In the present study, the calculated ΔS° and ΔH° values were both negative and the ligand–receptor interactions were enthalpy driven (Table 2). In addition, for DM and LM there was no significant difference in the ΔS° values, while the ΔH° value for the DM–nAChR interaction was significantly ($p < 0.05$) lower than that of the LM–nAChR interaction. Thus, the enantioselective difference between the two complexes reflected in the chromatographic experiments was enthalpy-based with an $\Delta\Delta H^{\circ}$ value of -0.33 kcal mol^{-1} and $\Delta\Delta G^{\circ}$ (at 20 °C) value of -0.29 kcal mol^{-1}. The latter value is in agreement with the $\Delta\Delta G$ value derived from the chromatographic separation factor α -0.28 kcal mol^{-1}.

The higher stability of the DM complex was reflected by the data from the non-linear chromatography experiments (Table 1). The LM capacity factor k'_{NLC} was reduced by 42% of value determined for DM, and the difference between the enantiomers was also reflected by the chromatographic equilibrium constants (K_a), where the value for LM was almost half of the value for DM. This data reflects significant enantio-discriminative binding interaction between LM/DM and α3β4-nAChR.

In this study, the calculated k_{off} for the dissociation of the DM–nAChR complex was 53% lower than that calculated for LM–nAChR complex (Table 1) and these results are consistent with the thermodynamic studies (Table 2). Thus, the data suggests that k_{off} values can be used as relative measures of differential stability and, in this case, in-

dicators of the principal source of the observed enantioselectivity.

Unlike the k_{off} values, the calculated k_{on} for the formation of the DM–nAChR complex was 20% higher than that calculated for the LM–nAChR complex (Table 1). However, the difference may not be significant due to the fact that while k_{off} and K_a are determined directly from the experimental parameters (Eqs. (1), (3) and (4)), k_{on} is derived from the k_{off} and K_a values (Eq. (5)).

The data from the studies of the blockade of nicotine-stimulated $^{86}Rb^+$ efflux demonstrate that the recovery of α3β4-nAChR activity was slower after exposure to DM than after exposure to LM (Fig. 3 and Table 3). These results suggest a slower dissociation of the DM–nAChR complex relative to the LM–nAChR complex. This conclusion is consistent with the results from the thermodynamic and non-linear chromatography studies. Thus, the data suggest that the chromatographically determined k_{off} values can be used to predict the relative rate of the reversibility of NCI blockade of nAChR activity.

Since the functional studies determined IC_{50} values as well as the rate of functional recoveries, it is reasonable to explore the relationships between IC_{50} values and chromatographic parameters. One starting point is the relationships between K_d/K_a and IC_{50} established by Cheng and Prussoff [20]. This relationship was determined for competitive antagonists, not for non-competitive inhibitors. But, if one assumes that a similar relationship exists between K_d and IC_{50} values for the binding of NCIs to the nAChR, then the chromatographically determined K_a values ($K_d = 1/K_a$) and functional data can be compared. Using this assumption, the data from the non-linear approach would predict a lower IC_{50} for DM relative to LM (Table 1). However, the chromatographic and functional data do not correlate since the functional data demonstrate no significant difference between the IC_{50} values for DM and LM (Table 3).

The discordance between the chromatographic and functional data reflects the differences between the K_a values for the two enantiomers, which is primarily a function of the difference in k_{off} values (Table 1). Thus, the source of the enantioselective difference in the recovery from DM/LM-induced blockade, the relative stabilities of the complexes, does not appear to play a significant role in the determination of the IC_{50} values. Similar behavior has been described for mecamylamine enantiomers [12].

One can speculate that an alternative approach to the comparison of chromatographic data and IC_{50} values would be the use of the k_{on} values. In the case of DM and LM, these rates did not significantly differ (Table 1). Since association rate constants reflect the rate of formation of the bimolecular non-competitive inhibitor–receptor complexes, this observation suggests that for the compounds and receptors used in this study, the effectiveness of a functional blockade is a reflection of the initial formation of the complex not its equilibrium distribution.

5. Conclusion

Chromatographically derived affinity (k' or K_a) of enantiomers cannot be directly related to in vitro response values (IC_{50}). Both techniques use completely different protocols and experimental conditions. In contrast to studies using functioning cells, chromatography uses “bare” proteins or membranes immobilized on a chromatographic support and measures relative affinity of ligands in simplified conditions (e.g. no nicotine, no transmembrane potential). However, significant correlation between the dissociation of the NCI–nAChR complex on the chromatographic column and the duration of an in vitro effect has been established. Moreover, the hypothesis that association rates k_{on} from non-linear chromatography investigation can be successfully related to the strength of NCI functional effect (IC_{50}) is currently under investigation. This hypothesis is being validated using an extended set of enantiomeric and achiral NCIs as well as different subtypes of nAChRs, and the results will be presented elsewhere. Thus, the whole chromatographic approach of investigation of immobilized nAChRs can be useful in predicting and characterizing non-competitive inhibitory properties of drugs and drug candidates.

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