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Determination of Acid Dissociation Constants of Compounds Active at Neuronal Nicotinic Acetylcholine Receptors by Means of Electrophoretic and Potentiometric Techniques

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The dissociation constants of epiboxidine **2** and a series of bases active at neuronal nicotinic acetylcholine receptors were determined by means of potentiometric and electrophoretic methods, which gave values in good agreement. Although showing different features, the two techniques are complementary for dissociation constant determinations. The choice of the most suitable method is guided by the available amount of sample, its purity, and the time needed for the analysis. The experimental values were compared with the predictions obtained with ACD/pK_a DB software.

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Introduction

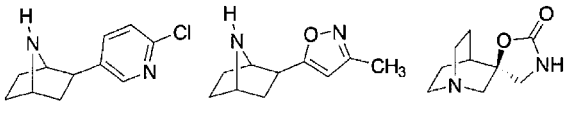
Neuronal nicotinic acetylcholine receptors (nAChRs) are widely distributed in the brain, where they primarily modulate neurotransmitter release and, to a lesser extent, participate to the synaptic transmission.[1] The nAChRs are involved in physiological processes such as cognition, learning and memory, cerebral blood flow and metabolism, as well as a number of pathologies, including Alzheimer's and Parkinson's diseases, schizophrenia, epilepsy, anxiety, depression and nicotine addition.[2-5]

Efforts in this research area have mainly focused on the discovery and development of agonists selectively interacting with $\alpha4\beta2$ and $\alpha7$ subtypes,[6] which are the two main populations of nicotinic receptors in the human brain and thus the most relevant biological targets for potential therapeutic applications. In this frame, epibatidine **1**, a highly toxic alkaloid identified in the skin of the Ecuadorian frog *Epipedobates tricolor*,[7] epiboxidine **2**,[8] an epibatidine analogue in which the chloropyridinyl ring has been replaced by the 3-methylisoxazolyl moiety, and ARR1779 **3**[9] are endowed with high affinity for both the $\alpha4\beta2$ and $\alpha7$ nAChR subtype (Fig. 1). In our research group, a series of racemic compounds structurally related to epibatidine, epiboxidine and ARR1779, among them derivatives **4** – **16** (Fig. 2), were synthesized and tested at rat $\alpha4\beta2$ and $\alpha7$ nAChR subtypes.[8,10]

In addition to the assessment of pharmacodynamic features, compounds of potential therapeutic significance should be evaluated for properties which will contribute to their pharmacokinetic profile *in vivo*, such as brain penetration, solubility, lipophilicity, hydrogen bonding capacity and charge. The latter parameter can be easily estimated once the acidic dissociation constant is known. Moreover, the knowledge of pK_a values is crucial for better understanding the chemical interactions between the compound of interest and its pharmacological target. Many biologically active molecules are fully or partially ionized at physiological pH, and the presence of ionizable groups is often essential in the process of molecular recognition by receptors or enzymes or is needed to prepare an aqueous solution for administration. For these reasons, the acidity constant is a key parameter which characterizes a new biologically active compound.[11,12]

Historically, potentiometric titration is the standard method used to determine pK_a values:[12] the sample is titrated with acid or base using a pH electrode to monitor the course of the titration. From the change of shape of the sample titration curve, in comparison with the blank, it is possible to determine the pK_a value.[13] Since 1990, capillary electrophoresis has been proposed as an alternative technique for the determination of ionization constants,[12] it is based on the change of the electrophoretic mobility of ionizable samples as a function of pH. In its uncharged form the sample has no effective mobility, while in its fully ionized state it reaches its maximum mobility. An intermediate mobility is a function of its dissociation equilibrium; pK_a values can be determined by regression analysis.[14]

In this study we aimed at measuring the pK_a values of epiboxidine **2** and those of its structurally related compounds **4** – **8**,[8] as well as of epibatidine analogues **9** – **11** and derivatives **12** – **16**, the latter belonging to a group of recently patented



1 Epibatidine **2** Epiboxidine **3** ARR1779

Fig. 1 Chemical structures of model compounds **1** – **3**.

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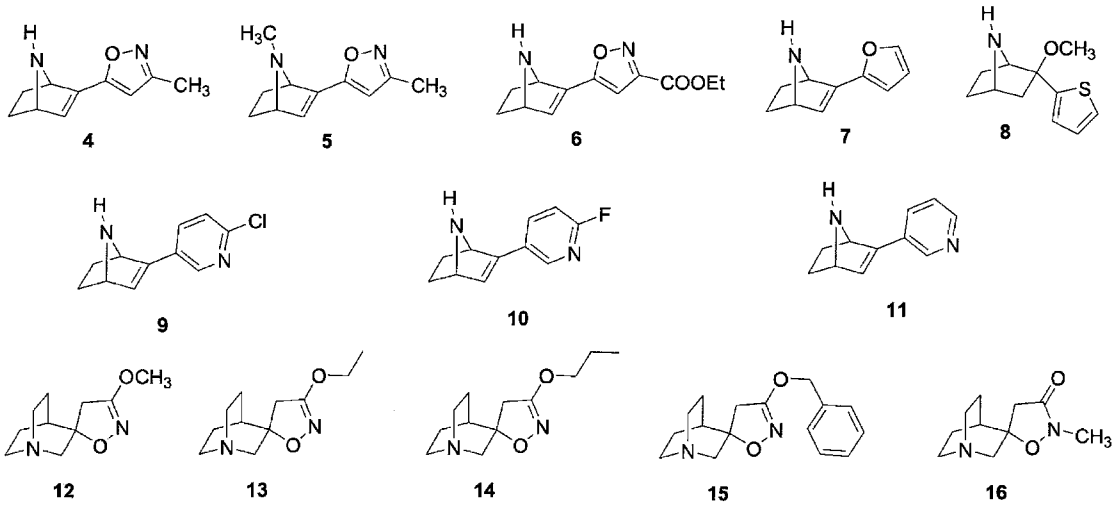

Fig. 2 Chemical structures of investigated compounds **4** – **16**.

analogues of ARR1779[10] (Fig. 2). The pK_a values were determined with both capillary electrophoresis and potentiometric titrations to compare the applicability ranges and the limits of the two techniques.[15,16] Furthermore, calculated values were collected by using the predictor software ACD/pK_a DB from Advanced Chemistry Development, Inc., which has been adopted by the majority of pharmaceutical companies and is considered one of the most reliable commercial prediction programs.[17]

Experimental

Chemicals

Compounds **2** and **4** – **16** were synthesized in our laboratory following published procedures.[8,10] Phosphoric acid, formic acid, acetic acid, boric acid, 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), sodium hydroxide, CO_2 free potassium hydroxide, potassium chloride, potassium hydrogen phthalate (KHP), hydrochloric acid (0.5 M), acetone and methanol were purchased from Sigma-Aldrich. All reagents used were of analytical grade and all reagents and buffers were prepared with water obtained from a Milli-Q water purification system (Millipore).

Apparatus

Capillary electrophoretic (CE) experiments were carried out using a Beckman Coulter ProteomeLab PA 800 system equipped with a diode-array detector scanning from 190 to 600 nm. A 32 Karat software was employed for instrument control, data acquisition and data analysis. Electrophoretic separations were performed under the conventional operating conditions (anodic injection) in an uncoated fused-silica capillary of 32 cm total length, 21 cm effective length and 50 µm i.d. (Composite Metal Service Ltd.). New capillaries were preliminarily conditioned as follows: 30 min with 0.1 M NaOH, 30 min with water and 30 min with the running buffer. Between runs at different pH values, the capillary was activated with 0.1 M NaOH for 3 min, rinsed with water for 3 min and with background electrolyte (BGE) for 5 min. Between runs at the same pH value, the capillary was only washed with water for 3 min and equilibrated with BGE for 5 min. Activation, rinse and equilibration steps were all carried out with a pressure of 20 psi. All injections were performed in the hydrodynamic mode (10 s, 0.5 psi). The capillary was operated at 8 kV, while maintaining its temperature at 25°C; detection was carried out at 280 nm, since at this wavelength the samples showed their peak absorbance. Three replicate injections were carried out for each compound at each pH value and all the data were used to calculate the pK_a values. The pH values of the running buffers were measured with a MP 220 pH meter (Mettler Toledo) equipped with an electrode InLab 418 (Mettler Toledo), calibrated daily. GraphPad Prism Ver. 5.0 software (trial version, GraphPad Software) was used to perform non-linear regression analyses of the electrophoretic data.

Potentiometric titrations were carried out with the Sirius GLpKa instrument coupled with a computer-aided system for the evaluation of pK_a values (Sirius RefinementPro software Ver. 1.0). For electrode standardization, see Supporting Information.[18,19]

Base titrant (0.5 M KOH) was prepared from CO_2-free ampoules of KOH in order to minimize the concentration of CO_2 in the solution. This reagent was standardized by titration with a weighed amount (0.15 to 0.19 g) of KHP dissolved in 20 ml of ISA water. For a better result, three KHP titrations were performed and combined in a MultiSet by means of the RefinementPro software to determine the mean value of the KOH concentration factor. A volumetric standard solution (0.5 M) of hydrochloric acid was employed as the acid titrant, which was standardized by means of a blank titration.

Electrophoretic measurements

To perform electrophoretic experiments, we prepared samples containing one substance dissolved in water/methanol/acetone 94/5/1 v/v/v mixtures. Acetone was used to determine electroosmotic flow (EOF) and methanol was added to improve the solubility of the tested compounds. Samples were set at different concentrations from 0.2 to 0.5 mg/ml depending on their UV response.

Twenty-one buffers spanning the range pH 2.0 – 12.0, with an increment of 0.5 pH units, were prepared according to Geiser *et al.*[20] and their ionic strength was set at 20 mM (Table S1, Supporting Information). The buffers were degassed in an ultrasonic bath prior to use and were replaced every ten runs, thus avoiding electrolytic phenomena. For the calculation of the analyte apparent mobility, see Supporting Information.

The calculated values of μ_{eff} were reported as a function of pH, giving rise to a sigmoidal curve in accordance with the model equations for pK_a determinations.[12] A non-linear regression was performed to determine pK_a values, which were dependent on BGE concentration. These values were corrected by introducing an activity coefficient in order to obtain the "true" pK_a value independent of the experimental procedures.[20]

Potentiometric measurements

For potentiometric titrations, a weighed amount of analyte (5 mg for all the compounds except for **9** and **10**, in this case a larger amount of sample, *i.e.* 10 mg, was necessary to determine the lower pK_a value very close to the limit of the titration interval) was dissolved in 20 ml of ISA water and the pH was lowered to 1.8 with 0.5 M HCl solution before titration with a 0.5 M KOH solution up to pH 12.2. For each sample, three determinations were carried out in the same vial and the results were combined in a MultiSet by means of the RefinementPro software to calculate more accurate values. The potentiometric pK_a values, obtained at a 0.15 M ionic strength, were corrected to a 0.02 M ionic strength in order to compare them with the electrophoretic values using an empirical expression.[21]

Prediction of pK_a values

Theoretical pK_a values were determined by means of the ACD/pK_a DB software (trial version, Advanced Chemistry Development. Inc.), based on Hammett equations derived from a library of more than 31000 experimental pK_a values for approximately 16000 compounds in aqueous solutions and more than 2000 molecules in non-aqueous solvents.

Results and Discussion

All the tested compounds were monoprotic bases except **9**, **10** and **11** which contain two basic centers. CE measurements yielded electrophoretic mobilities which were plotted *versus* the pH of the running buffer. The μ_{eff} values were positive according to the cationic nature of the ionized compounds. In Fig. S1 (Supporting Information), some representative plots of μ_{eff} against pH are reported, while in Fig. S2 (Supporting Information), the titration curves of compounds **9** and **10** are shown. The results obtained with the potentiometric and the electrophoretic methods are listed in Table 1 together with the theoretical values predicted by the ACD/pK_a DB program.

Confidence intervals associated with electrophoretic and potentiometric determinations fall within 0.2 units, while 0.4 units are the confidence interval of the software predictions. Electrophoretic and potentiometric measurements gave pK_a values in good agreement. With the electrophoretic method, we were unable to determine the lowest pK_a value for **9** and **10** since it was outside the investigated pH range or very close to its lowest limit. Because a BGE ionic strength higher than 20 mM is necessary for pH values lower than 2.0 to prevent electric current elevation and Joule effect, we should apply a lower voltage, which causes an increase in the time of analysis. Moreover, at a pH value lower than 2.0, the proton activity coefficient should be taken into account. Alternatively, very low pK_a values may be determined by applying the potentiometric method, even though a relatively high amount of sample (*i.e.* >10 mg) is needed to single out the shape of the titration curve from the background.

Inspection of Table 1 reveals that a good general correspondence is observed when the predicted pK_a values and the related experimental data are taken into account. However, a meaningful discrepancy exists in the case of epiboxidine **2** and, notably, in all the spirocyclic compounds **12** – **16**. However, we have no explanations accounting for this trend within the set of compounds under study.

Table 1 Measured and predicted pK_a values for the compounds under study

Compound	Potentiometry	CE	ACD/pK_a DB
2	8.91	8.87	10.14
4	8.59	8.72	9.12
5	8.35	8.25	7.68
6	8.47	8.31	8.92
7	9.06	9.08	9.50
8	8.81	8.71	9.13
9	9.26	8.84	9.05
	1.79	—	0.26
10	9.28	9.25	9.03
	2.45	—	–0.79
11	9.15	9.60	9.28
	4.04	3.99	4.87
12	8.67	8.63	9.89
13	8.56	8.94	9.92
14	8.59	8.75	9.91
15	8.55	8.82	9.90
16	8.39	8.56	9.79

In summary, both electrophoretic and potentiometric measurements gave reliable results and the two techniques showed complementary features. The electrophoretic method needs a very small amount of sample, not necessarily characterized by a high degree of purity, whereas the potentiometric method requires high-purity samples of known concentration. The electrophoretic method does not need an analytical solution because only mobilities are used to determine pK_a values. Moreover, since CZE is a separation method with a high resolving power, various components could be in principle studied in the same run. The weaknesses of the electrophoretic technique are both the difficulty to determine very low or very high pK_a values and the time needed for data collection. Conversely, the potentiometric method is faster and it can be applied to the determination of extreme pK_a values, but it requires a sizable amount of high-purity samples and analytical solutions of known concentration.

Conclusions

In this study we were interested in measuring, by means of potentiometric and electrophoretic methods, the acid-base dissociation constants of a group of novel weak bases that bind at the neuronal nicotinic acetylcholine receptors. On the whole, the two protocols gave comparable results. Due to their different features, electrophoresis and potentiometry can be regarded as complementary techniques for dissociation constant determinations, the choice of the most suitable approach being guided by the amount of sample, its purity, and the time required for the analysis.

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Supporting Information

Electrode standardization; calculation of the analyte apparent mobility; Table S1 (buffer composition); Fig. S1 (relationship between μ_{eff} and pH of BGE for compounds **2**, **9**, **11** and **12**) and Fig. S2 (titration curves for compounds **9** and **10**).

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