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Determination of the dissociation constants (pK_a) of basic acetylcholinesterase inhibitors by reversed-phase liquid chromatography

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Abstract

An RP-HPLC study for the pK_a determination of a series of basic compounds related to caproctamine, a dibenzylamine-diamide reversible inhibitor of acetylcholinesterase, is reported. The 2-substituted analogues, bearing substituents with different electronegativity, were analysed by RP-HPLC by using C_{18}, C_4 stationary phases with a mobile phase consisting of mixture of acetonitrile and triethylamine phosphate buffer (pH range comprised between 4 and 10). Typical sigmoidal curves were obtained, showing the dependence of the capacity factors upon pH. In general, the retention of the investigated basic analytes increased with increasing of the pH. The inflection point of the pH sigmoidal dependence was used for the dissociation constant determination at a fixed acetonitrile percentage. When plotting pK_a vs. percent of acetonitrile in the mobile phase for two representative compounds, linear regression were obtained: the *y* intercept gave the aqueous $pK_{a(w)}$. The pK_a estimation by HPLC method was found to be useful to underline the difference of benzylamine basicity produced by the ortho aromatic substituents. The variation of pK_a values (6.15–7.80) within the series of compounds was correlated with the electronic properties of the ortho-substituents through the Hammett σ parameter, whereas the ability of substituents to accept H-bond was found to play a role in determining the conformational behavior of the molecules.

Keywords: Dissociation constants; Quantitative structure–property relationships; Acetylcholinesterase inhibitor; Caproctamine; Dibenzylamine-diamides, 2-substituted

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive decline of cognitive functions. The significant deficits in pre-synaptic cholinergic markers observed in brains of AD patients have led to the formulation of the cholinergic hypothesis of AD [1]. To date, only few inhibitors of acetylcholinesterase (AChE), such as tacrine, donepezil and rivastigmine, are used in medicine for the treatment of AD symptoms by increasing acetylcholine (ACh) content in the synapse [2–4]. Moreover, some evidence suggests that the AChE peripheral binding site may play a key role in the development of the senile plaques, accelerating β-amyloid peptide deposition [5].

In order to make available novel ligands based on a polyamine backbone having inhibitory activity for

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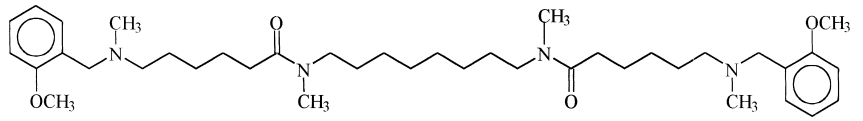
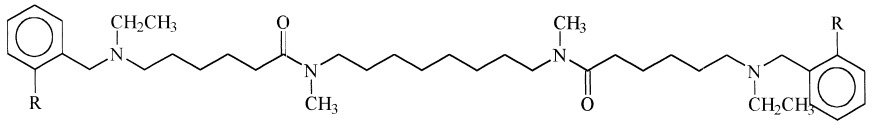


Caproctamine



Compound	R
1	CF_3
2	NO_2
3	Cl
4	H
5	CH_3
6	$O(CH_2)_2CH_3$
7	OCH_3

Fig. 1. Structures of the analysed compounds.

both active and peripheral binding sites of AChE, caproctamine (Fig. 1) was found to be the prototype of a new class of diamine diamides as AChE non-covalent inhibitors, displaying also a significant competitive antagonism toward muscarinic M_2 receptors [6].

A previous study, dealing with the assessment of the effect on affinity of the substituents on both the aromatic rings and on the basic nitrogen atoms, showed that the insertion of a 2-OCH_3 group and an ethyl function, resulted in the best activity profile [7]. A preliminary molecular modeling study showed that diprotonated diamine diamides are required to establish favourable interactions possibly with both Trp 84 in the active site and Trp 279 in the peripheral site of AChE [6,8].

It was therefore postulated that the nature of the 2-benzyl substituent could affect the basicity of the benzylamine tertiary amine group. In order to establish the role of the aromatic ring substituents on the basicity of the benzylamine function, which was considered a key physical chemical property [9] to improve the affinity on the AChE, the determination of the dissociation constants (pK_a) of a series of caproctamine derivatives (Fig. 1) was performed following an HPLC method. The synthesis, SAR study and biological activity of these novel compounds are being published elsewhere.

The determination of dissociation constants (pK_a) of basic drugs or active compounds is important to predict the ionisation state at physiological pH and this physical chemical parameter can be important for SAR studies [9]. The determination of this physical chemical parameter is therefore essential in understanding the biological activity of drugs, allowing a more defined picture of the macromolecule–ligand binding interaction. Moreover, the biological test system may have several compartments of different pH, hence, ionisation equilibria may influence the total concentration of drug available for the interaction with the enzyme active site.

A methodological approach of choice is pK_a estimation by isocratic reversed-phase HPLC [10–14]. In fact, as most of the organic compounds tend to be poorly soluble in water, the classical potentiometric techniques for studying acido–basic (protolytic) equilibria are not practical. Another advantage of the HPLC method is that a small

amount of sample, which need not to be of high purity, is required.

The protonation of the weak base in HPLC is regarded as a secondary chemical equilibrium, the first being the distribution equilibrium of the solute between the mobile and stationary phase. The secondary equilibrium is controlled by both the pH of the mobile phase and the ratio of the organic modifier.

The basic theory of these equilibria has been worked out in the 1970s and 1980s [15,16]. The analyte retention in reversed-phase chromatography represents the molecular fraction weighted average of the retention of the dissociated and undissociated forms.

The expression of the observed retention factors ($k = t_r - t_o/t_o$, where t_r is the analyte retention time and t_o is the void volume) can attain the form:

$$k_{obs} = k \cdot x^{+}_{HB} + (1 - x^{+}_{HB}) \cdot k_B$$

x^{+}_{HB} is the pH dependent molar fraction of the protonated basic compound HB^{+}, $(1 - x^{+}_{HB})$ is the pH dependent molar fraction of the unprotonated basic compound B.

Introducing the equilibrium constant defined as:

$$K^{+}_{HB} = [H^{+}][B]/[HB^{+}]$$

the expression of the observed retention factor attains the form:

$$k_{obs} = k^{+}_{HB}[H^{+}] + k_B K^{+}_{HB}/[H^{+}] + K^{+}_{HB}$$

Therefore the pH dependence of the capacity factors follows the dissociation curve.

Here it is reported a HPLC study for the pK_a determination of a series of basic compounds related to caproctamine [6], a dibenzylamine-diamide reversible inhibitor of AChE. The 2-substituted analogues, bearing substituents with different properties, were analysed by RP-HPLC by using C_4 and C_{18} stationary phases with a mobile phase consisting of a mixture of acetonitrile and triethylamine phosphate buffer (pH range comprised between 4 and 10). An interpretation of the variation of pK_a values was attempted, by determining quantitative structure–property relationships (QSPR) for the series, and by analysing the conformational characteristics of each compound as well.

2. Experimental

2.1. Materials and methods

2.1.1. Materials

2-Methoxy benzylamine (MBA) was purchased by Aldrich (Milan, Italy). The studied compounds (Fig. 1) were synthesised as previously reported [6,7]. Their solutions (1 m*M*) were prepared in acetonitrile and diluted with mobile phases before injection.

HPLC grade acetonitrile (Romil Ltd, Cambridge, UK) and water obtained from Milli-RX apparatus (Millipore, USA) were used to prepare solutions and mobile phases. Phosphoric acid (85%) and triethylamine of analysis quality (Carlo Erba Reagenti, Milano Italy) were used to prepare the triethylamine-phosphate (50 m*M*) buffer solutions in the pH range between 4.0 and 10.0.

The buffer solutions were filtered through a 0.45 μm membrane filter and degassed before their use for HPLC.

2.1.2. Apparatus

The solvent delivery system was a Jasco PU-980 Intelligent HPLC pump equipped with a Reodyne Model 7125 injector with a 20 μl sample loop. The eluents were monitored by a Jasco MD 910 Multiwavelength Detector (DAD) connected to a computer station. For routine analyses the detector wavelength was set at 220 and 260 nm. The flow-rate was 1 ml/min.

2.1.3. Determination of the apparent dissociation constant (pK_a) (monocratic method)

The chromatographic separations were performed on a 5 μm Phenomenex Prodigy C_{18} 100 μm (150×4.6 mm I.D.) and a 5 μm Phenomenex Jupiter C_4 300 μm (150×4.6 mm I.D.) columns at 25 °C. The flow-rate was 1 ml/min.

The mobile phases consisted of acetonitrile–(50 m*M*) triethylammonium phosphate buffer 50:50 (v/v), at different pH values between 4.0 and 10.0.

The stock solutions of tested compounds (1 m*M* in acetonitrile) were diluted with appropriate buffer to the final injected concentration (300 μ*M*). A 20 μl injection of every compound was made in triplicate into the chromatographic system equilibrated with the mobile phases at the defined pH value.

The dead volume of the system was measured as the first distortion of the baseline after injection of pure acetonitrile. At each mobile phase composition, the retention factor k was calculated according to $k = (t_r - t_0)/t_0$ where t_r and t_0 are the retention times of analyte and the non-retained compound, respectively.

Observed retention factors vs. pH of the mobile phases were fitted to the following equation (Boltzmann sigmoid $Y = \text{bottom} + (\text{top} - \text{bottom})/[1 + \exp((V_{50} - X)/\text{slope})]$ using a non-linear least square program (Prism 3.0). Computer generated plots of k vs. pH were obtained and the pH value at the inflection point (V_{50}) was taken as a valuable index of pK_a (Table 1).

2.1.4. Determination of p$K_{a(w)}$ (polycratic method)

According to their chromatographic behaviour, the retention times of the solutes (**2**, **7** and MBA) as a function of pH of the mobile phase were determined at four different acetonitrile–phosphate buffer mixtures ranging from 35 to 55% (v/v) acetonitrile.

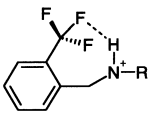
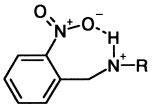
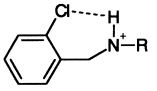
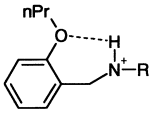
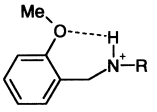
The p$K_{a(w)}$ values (pK_a at 100% aqueous mobile phase) were obtained from the y intercepts of plots of pK_a vs. percent of acetonitrile in the mobile phase (Table 2). Correlation studies were performed using a statistical program (GraphPad Prism).

2.1.5. QSPR and molecular modeling

Electronic and steric parameters for the substituents were retrieved from the database of the QSAR program [17], and one-variable regressions were calculated by means of the same program.

The 3D models of the molecules were built by means of the SYBYL program [18], and conformational searches were carried out on each molecule in order to estimate the tendency to establish an intramolecular H-bond interaction. The protonated form

Table 1
Chromatographic (monocratic method) dissociation constants

Compound	R	pK_a (obs.)	σ	%[a]	H-bond
1	CF_3	6.15±0.04	0.54	30	
2	NO_2	6.26±0.03	0.78	28	
3	Cl	6.92±0.04	0.23	22	
4	H	7.42±0.04	0.00	0	–
5	CH_3	7.46±0.02	−0.17	0	–
6	$O(CH_2)_2CH_3$	7.75±0.05	−0.25	33	
7	OCH_3	7.80±0.06	−0.27	25	

[a] Percentage of conformers showing intra-molecular H-bond calculated on the total conformer population.

Table 2
Dissociation constant values obtained from regression analysis of pK_a values at different acetonitrile percentage in reversed-phase chromatography

Compound	*y* Intercept = $pK_{a(w)}$	Slope	Corr. coeff.
2	8.52±0.22	−0.0462±0.0054	0.9727
7	9.61±0.34	−0.0584±0.0063	0.9881
MBA	8.22±0.21	−0.0224±0.0081	0.8909

of the compounds was studied by means of Monte Carlo conformational analysis [19], as implemented in the MacroModel package (Ver. 5.5) [20]. To account for the solvent effects on the compounds during the conformational analyses, the GB/SA solvation model [21] was exploited. In order to classify the conformations obtained for each molecule a geometrical cluster analysis was carried out on the output of Monte Carlo conformational searches, and the conformer population was determined.

2.2. Results and discussion

2.2.1. Determination of the apparent dissociation constant (pK_a) (monocratic method)

In order to determine the pK_a of the caproctamine derivatives of Fig. 1, and to underline the basicity differences produced by the ortho benzyl substituent, the compounds were analysed by RP-HPLC by using a C_4 and C_{18} stationary phases with a mobile phase consisting of a mixture of fixed acetonitrile percentage (50%) and triethylamine phosphate buffer (pH range between 4 and 10). This buffer was selected because it can cover a wide pH range (3–11) and because TEA can mask free silanols, avoiding peak tailing and secondary interactions with the stationary phase. This experiment was performed to obtain relative index of basicity, i.e. relative acidic dissociation constants. The two basic nitrogens in the analytes were assumed to have the same dissociation constant, bearing the same groups and being far apart from each other.

Typical sigmoidal curves were obtained (Fig. 2), showing the dependence of the analyte capacity factors upon the pH of the mobile phase. In general, the retention of the investigated basic analytes increased with increasing of the pH (Fig. 3). Experimental *k* values were determined from three different injections at every mobile phase composition and at each pH considered. Relative standard deviations were lower than 2%.

The inflection point (V_{50}) of the pH sigmoidal dependence was used for the dissociation constant determination at a fixed 50% acetonitrile percentage (Table 1).

The difference between the top *k* value and the bottom one of the sigmoidal trend was found to be related to the lipophilicity of the molecule: for example, at basic pHs, compound **1**, substituted with $-CF_3$ was more retained than **3** (–Cl), which showed a higher *k* value than **7** ($-OCH_3$).

It was found that the lipophilicity of the stationary phase could not affect the relative pK_a determination, but influenced only the analyte retention times. Therefore, in order to analyse the compounds at the same percentage of acetonitrile in the mobile phase and with a reasonable retention (*k*), the analytes which resulted in more retained, i.e. the ones which had higher lipophilicity (**1**, **2**, **3** and **5**) were analysed on a C_4 stationary phase and the others (**4**, **6**, **7**) also on a C_{18} stationary phase.

2.2.2. QSPR and molecular modeling

As shown in Table 1, CF_3-, NO_2-, and Cl-substituted derivatives showed lower pK_a values than the unsubstituted parent compound **4**, whereas the methoxy- and *n*-propyloxy-derivatives resulted in more basic. The ortho-substituted analogues of caproctamine bear substituents on the phenyl rings characterized by different electronic properties, and, in principle, such atoms or groups can exert electron-withdrawing or -donating, inductive or mesomeric effects. Therefore, it was hypothesised that the basicity of the tertiary benzylamine was influenced by the vicinal ortho substituent. However, this influence might also be of steric nature, considering the possibility that the ortho function can hinder or

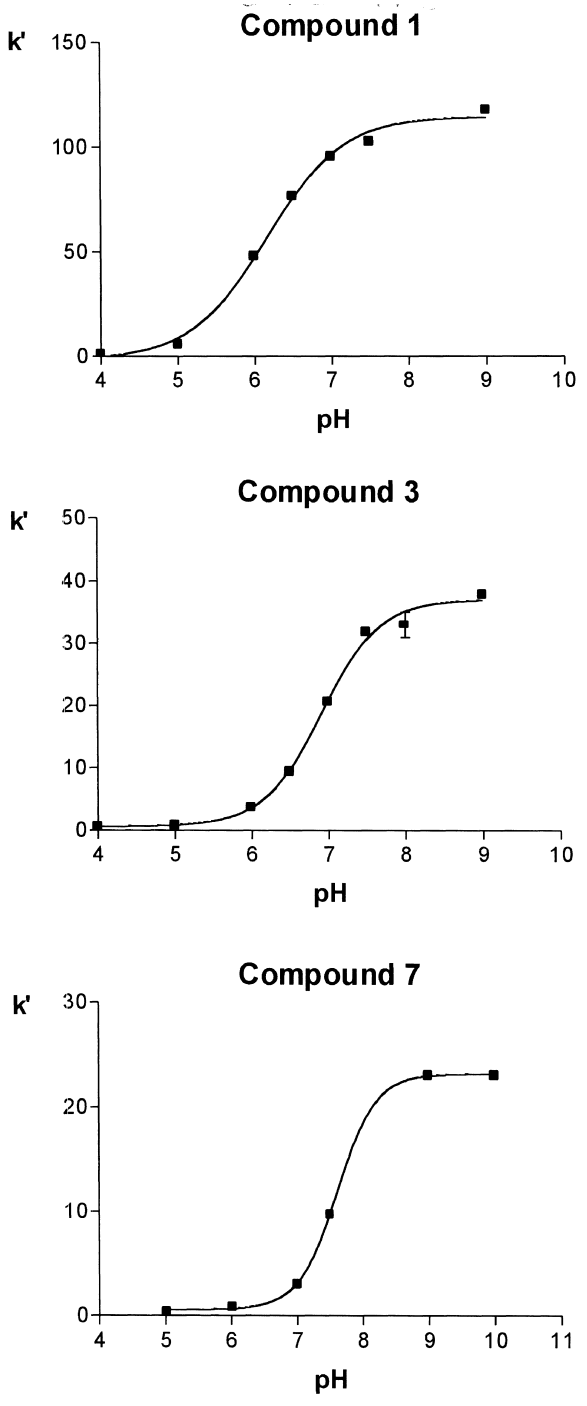


Fig. 2. Variation of the retention factor (k') of some tested compounds as function of pH in 50% (v/v) organic modifier.

facilitate (through H-bond formation) the protonation of the amine.

To interpret the relationship between the observed chromatographic basicity and the properties of ortho substituents, we looked for correlations (QSPR) between pK_a values and electronic and steric constants of the caproctamine derivatives.

All the available electronic and steric substituent constants [17] for compounds **1–7** were taken into consideration in the search for a statistically significant relationship, and the best result was obtained with the classical Hammett constants (Table 1). The correlation is described by the following equation

$$\begin{aligned} \mathrm{p}K_a &= -1.61\,(\pm 0.46)\sigma + 7.31\,(\pm 0.18) \\ n &= 7 \quad s = 0.178 \quad r^2 = 0.943 \quad q^2 = 0.833 \end{aligned} \tag{1}$$

where σ is the electronic parameter, n is the number of compounds, s is the standard deviation, and r^2 and q^2 are the simple and crossvalidated square correlation coefficients of the regression, respectively. It is remarkable that just σ, but not σ_{ortho}, σ^0, F, or R, explain almost 95% of the variance of the pK_a values. The mentioned alternative constants are supposed to account for purely electronic ortho effects, effects on insulated reaction centers, purely inductive or resonance effects, respectively [22]. An interpretation of the correlation expressed by Eq. (1) can be that the substituents in compounds **1–7** affect N-protonation through mixed inductive and mesomeric effects, the same way as they affect ionization in the model reaction, i.e. the ionization of benzoic acid. This is rather surprising if one thinks to the different relative positions of substituents and reaction centers in benzoic acids and benzyl amines, but it might be explained as a consequence both of the small variance of the electronic properties, and of the similarity and collinearity between the constants (e.g. $r^2(\sigma/\sigma^0) = 0.986$) in the set under study. Moreover, it must be remembered that σ_{ortho} values are not reliable for many substituents, as it is witnessed by the rather wide range of parameter values available for some of them [23].

Consideration of the steric properties of substituents parameterized by the classical E_s values or by the Sterimol parameters did not give equations better than Eq. (1). Thus, it seems that there is no direct steric influence of the substituents on the

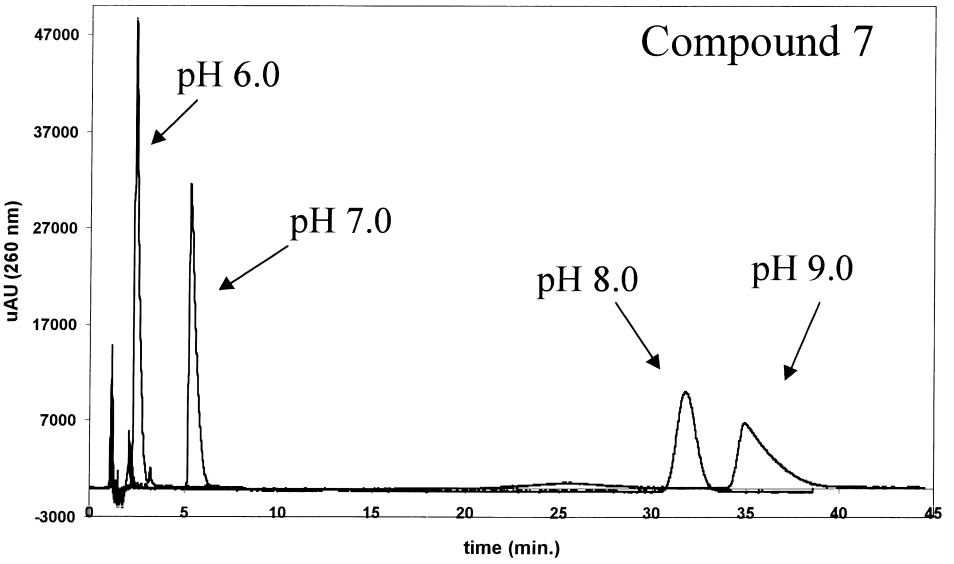


Fig. 3. Overlaid chromatograms showing the retention behaviour of compound **7** as a function of pH in 50% (v/v) organic modifier on C_4 stationary phase, UV detection at 260 nm, flow-rate 1 ml/min.

protonation reaction. To clarify this aspect, we carried out a conformational analysis on the protonated forms of the compounds, and on each population of conformers we calculated the percentage of those displaying intra-molecular H-bonds. These percent values are shown in Table 1. It appears that the ability to make an intra-molecular H-bond in the protonated form is dependent on the nature of the substituent, but, anyway, this property does not overcome the electronic effect in determining the pK_a value. To exemplify, compounds **2** and **7** (2-nitro- and 2-methoxy-substituted derivatives, respectively) show similar percentages of H-bonded conformers, but the pK_a values are still different, being higher than that for the derivative bearing the electron-donating substituent, i.e. OCH_3 (negative σ value). Summarizing, it might be hypothesized that, in this series of compounds, the protonation of the tertiary amine is affected by the electronic properties of the ortho-substituents, while the possibility to establish H-bonds affects the conformational behavior of the protonated molecules, by favoring the intra-molecularly bonded conformers.

2.2.3. Determination of p$K_{a(w)}$ (polycratic method)

After the determination of the relative analyte basicity, it was considered interesting to determine acidic dissociation constants p$K_{a(w)}$, i.e. extrapolated to 100% water. The two compounds showing the highest and lowest pK_a values were selected with the purpose of establishing the upper and lower p$K_{a(w)}$ scale values. **MBA**, whose pK_a was calculated by the potentiometric method (pK_a = 9.77) [24] was chosen as a reference compound.

Apparent pK_a values were therefore obtained at

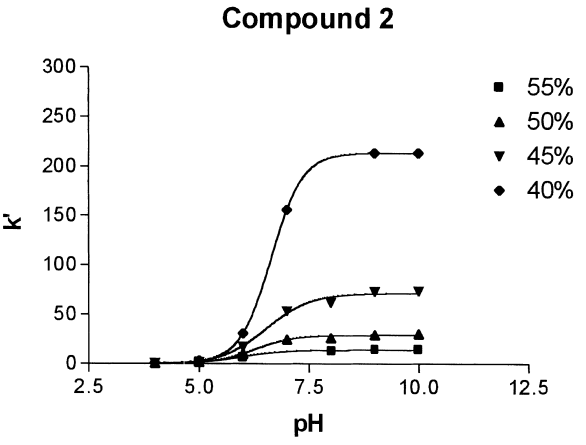


Fig. 4. Observed capacity factors (k') vs. pH for compound **2** in acetonitrile–buffer mixtures.

four acetonitrile percentages in the range 55–35% for **7** and **2** (Fig. 4) and 30–5% for **MBA**. The pK_a values were found to increase by decreasing the acetonitrile content of the mobile phase. Therefore, when plotting pK_a vs. percent of acetonitrile in the mobile phase, linear regression with negative slopes were obtained: the *y* intercept gave the aqueous p$K_{a(w)}$. In Table 2 the p$K_{a(w)}$ values are reported. The tested compounds showed basicity values higher than **MBA** and correlated to the relative pK_a values. These results are compatible with their tertiary amine group, a stronger base than the primary amine function of **MBA**.

However, **MBA**, the reference compound, was found to have a p$K_{a(w)}$ value = 8.2, lower than the value found by the potentiometric method [24]. This might be partially due to the fact that the p$K_{a(w)}$ was obtained by extrapolating to 0% acetonitrile, i.e. to 100% of an aqueous solution with ionic strength different from the potentiometric method (0.5 *M* KNO_3) [24], even if only the ionic strength difference might not account for the pK_a difference of 1.5. Moreover, in previous papers [13,14], pK_a values determined by HPLC were not found in perfect agreement with the potentiometric ones. So one may deduce that also the values obtained for our tested compounds are underestimated.

One problem encountered for **7**, a stronger base, was that as the pK_a increases by decreasing the percentage of acetonitrile, the needed pH value to reach the retention plateau (i.e. prevalence of the unprotonated form) was out of scale, possibly compatible with the HPLC system used. Besides, the relatively high lipophilicity of the tested compounds did not allow to work with percentages of acetonitrile lower than 35%. In these conditions therefore, the extrapolation was obtained using experimental pK_a in a narrow range of values.

3. Conclusions

The reported HPLC method for the determination of acidic constants pK_a of basic AChE inhibitors was found useful to obtain relative indices of basicity. The pK_a estimation by the HPLC method was found useful to underline the difference of benzylamine basicity produced by the ortho aromatic substituents.

The main advantage for the HPLC system, using fixed acetonitrile percentage, is that preferential solvation in acetonitrile–water mixtures produces lower pK_a values than expected, reaching therefore the capacity factor plateau of the sigmoidal curve at pHs lower than 12. This pH value is the limit value to be used with stationary phase in HPLC.

The variation of pK_a within the series of compounds was correlated with the electronic properties of the ortho-substituents through the Hammett σ parameter, whereas the ability of substituents to accept H-bond was found to play a role in determining the conformational behavior of the molecules.

The estimation of absolute p$K_{a(w)}$ constants, obtained by extrapolation of the relative pK_a values determined at fixed acetonitrile percentages was attempted for two representative compounds (**2**, **7**). However, we found a lower p$K_{a(w)}$ value than the one obtained by the potentiometric method for **MBA**, the selected reference.

The fact that the HPLC method may produce different p$K_{a(w)}$ values from the classical potentiometric method has been already experienced by other researchers in previous papers [13,14]. Moreover, this determination is quite laborious and it does not add any further information for activity and chemical physical correlation studies, already given by the relative pK_a values, which satisfactorily described the basicity variation within the series.

The extrapolated p$K_{a(w)}$ method, in our opinion, seems to have a better application for basic compounds with pK_a lower than 10 and characterised by medium lipophilicity values.

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