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Liquid Chromatographic Determination of pK_a Value of 1-(2-methylbenzonitrile)-3-benzylbenzimidazolium bromide as a Drug Candidate in Acetonitrile-Water Binary Mixtures

İlkay KONÇE [1], Senem AKKOÇ [2], Zehra ÜSTÜN [3], Ebru ÇUBUK DEMİRALAY [2]*

1 Department of Chemistry, Faculty of Science and Literature, Suleyman Demirel University, Isparta, Turkey.
2 Department of Basic Pharmaceutical Sciences, Faculty of Pharmacy, Suleyman Demirel University, Isparta, Turkey.
3 Department of Medical Services and Techniques, Atayalvaç Vocational School of Health Services, Suleyman Demirel University, Isparta, Turkey.
* Corresponding Author. e-mail: ebrucubuk@sdu.edu.tr; ebrucubukdemiralay@gmail.com

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ABSTRACT: The ionization/protonation (pK_a) constant, which is a physicochemical parameter that directly affects the pharmacokinetic properties of a drug such as absorption, distribution, metabolism, and excretion (ADME), is currently determined by analytical methods. The effect of pH of the mobile phase on the chromatographic behavior of 1-(2-methylbenzonitrile)-3-benzylbenzimidazolium bromide (**2**) which its synthesis, fully characterization, and antiproliferative activity properties were studied previously, and the protonation constant (pK_a) value were determined in this study. The pK_a value of compound **2**, benzimidazolium salt, was determined by the reverse-phase liquid chromatographic (RPLC) method at 25 °C. ${}^{s}_{s}pK_a$ values were evaluated using retention time (t_R) in acetonitrile-water binary mixtures with acetonitrile (ACN) percentages of 40%, 45%, 50% and 55% (v/v). The aqueous pK_a (${}^{w}_{w}pK_a$) value of the synthesized compound was calculated from the ${}^{s}_{s}pK_a$ value using the macroscopic parameters (dielectric constant and mole fraction) which play an important role in the solvent properties. Obtained ${}^{w}_{w}pK_a$ values were found to be 11.290 and 11.241, respectively. In addition, the degree of ionization of the related compound was calculated using the ${}^{w}_{w}pK_a$ values.

KEYWORDS: Benzimidazolium salt; hydro-organic mixture; pK_a value; macroscopic parameter.

1. INTRODUCTION

Busulfan (1,4-butanediol dimethanesulfonate) is a chemotherapy drug used in the treatment of chronic myeloid leukemia (CML) and other leukemias, lymphomas, and myeloproliferative disorders. In a conducted study by Akkoç *et al.*, a benzimidazolium salt demonstrated more toxic effect than busulfan against human epithelial colon colorectal adenocarcinoma cell line (ATTC® CCL-221™) (DLD-1) and human epithelial breast adenocarcinoma cell line (ATTC® HTB-26™) (MDA-MB-231) for 72 h [1]. Therefore, in this study, the physicochemical properties of this compound were investigated. The protonation constant (pK_a) value, which can predict the behavior of a drug under *in vivo* conditions and considered as the main item in its biophysical characterization, was determined for the compound **2** that was synthesized and characterized as a new cancer drug candidate (Figure 1). The pK_a value is the basic physicochemical parameter used to determine the degree of ionization of the molecules in the solution at different pH values. This parameter is crucial to predict absorption, distribution, metabolism, excretion, and formulation evolution. In addition, this value determined in the organic solvent-water binary mixture is extremely useful for the estimation of the chromatographic optimization of the compounds [2,3].

Konçe İ, Akkoç S, Üstün Z, Çubuk Demiralay E. Liquid chromatographic determination of pka value of 1-(2-methylbenzonitrile)-3-benzylbenzimidazolium bromide as a drug candidate in acetonitrile-water binary mixtures. J Res Pharm. 2022; 26(3): 655-662.

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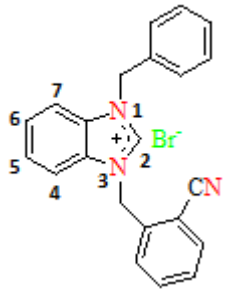

Figure 1. Chemical structure of compound 2

Analytical methods with high precision and accuracy such as reverse-phase liquid chromatography (RPLC), spectrophotometry is used to determine the pK_a values of such heterocyclic compounds [3-7]. In the analyzes performed in these methods, the compound must be dissolved with a solvent. To eliminate the harmful effects of organic solvents, water is the first solvent preferred in the analyzes. However, in cases the solubility of the compounds in water is insufficient, it is necessary to use an organic solvent-water binary mixture to dissolve the compound. On the other hand, in most of the analyses performed in 100% water environment with the RPLC method, problems such as non-repeating retention times (t_R), deterioration of peak shapes, and phase precipitation are observed in the RPLC column [8]. For these reasons, it is necessary to add a small amount of organic modifiers to the mobile phase to eliminate these problems [9, 10]. Acetonitrile (ACN) is the most preferred solvent among organic solvents. The determinations made using binary mixtures formed with water have excellent chromatographic properties [5,11].

Since a great majority of drugs include basic functional groups, RPLC behavior of basic compounds has attracted significant interest. Therefore, RPLC separation of organic bases of different pK_a values is of particular importance in the pharmaceutical industry. In general, the analysis of such compounds with conventional columns used in the RPLC method is difficult due to the peak symmetry, not choosing the appropriate mobile phase pH. For this reason, it is necessary to use columns suitable for the analysis of basic compounds. Conventional C18 silica columns are not preferred due to limited operating pH ranges, peak symmetry and poor reproducibility. To extend the pH range up to pH 12, silica backbone soluble organic-silica hybrid phases have been developed. Especially in the determination of basic compounds, this type of modified columns should be used instead of conventional columns [12].

In RPLC, there is a difference between the retention of a neutral compound and the retention of an ionizable compound. The retention of a neutral compound remains constant in a fixed mobile phase concentration at different mobile phase pH values. On the other hand, there is an equilibrium between molecular (B) and ionized forms (BH^+) of an ionizable compound, depending on its pK_a value at different pH values in a fixed mobile phase concentration. Since these forms of the compound have different affinities to the stationary phase, they have different retention values. Moreover, BH^+ forms of ionizable compounds have shorter retention times than B forms [9,10,13].

Temperature is an effective parameter in chromatographic determination. In studies with ionizable compounds, besides the pH value of the mobile phase, temperature levels significantly affect the t_R value of the compound. The mobile phase pH and temperature are also important factors in determining the pK_a value. Liquid chromatographic studies are common to determine pK_a values, mostly at 25 °C and 37 °C [14-16]. Determination of the pK_a value at these temperatures is necessary to understand the biological mechanism of the transfer of ionizable compounds into the cell [16].

A limited number of studies have been conducted on the pK_a values of azolium salts [17-20]. When these literatures are examined, there is no systematic study to determination the pK_a values of aromatic benzimidazolium salts in ACN-water binary mixtures. In the present study, $^{s}_{s}pK_a$ values in water-ACN binary mixtures of compound **2** were determined by the RPLC method. Using these data, the aqueous pK_a value of this compound, which has low water solubility, in the aqueous medium was calculated using some macroscopic data (X_{ACN} and ε).

2. RESULTS

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In this study, the RPLC method was developed to determine the pK_a value of the physicochemical property of this benzimidazolium salt, which may be a drug candidate. 1-(2-methylbenzonitrile)-3-benzylbenzimidazolium bromide (**2**) is a very strong basic compound. This compound, which has a benzimidazole ring, has two related ionizable functional groups, acidic and basic groups. The dissociation equilibria of compound 2 was shown in Figure 2.

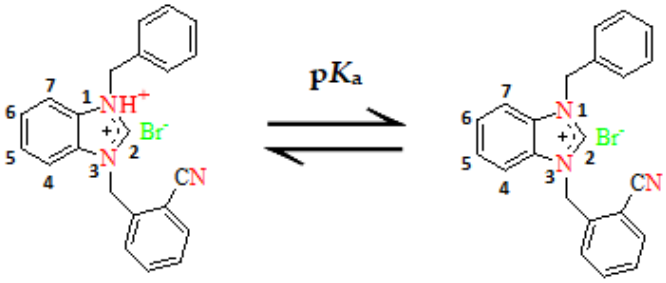

Figure 2. Dissociation equilibrium of compound 2

The protonation constant value ($^{s}_{s}pK_a$) of the N(1)-substituted benzimidazole compound with the benzene ring was calculated in hydro-organic mixtures. To determine the $^{s}_{s}pK_a$ value of this synthesized benzimidazolium salt, the t_R values of the compound **2** were analyzed in ACN-water binary mixtures containing 40%, 45%, 50%, and 55% (v/v) ACN. Chromatograms showing the change in t_R at pH 7.0 and pH 11.75 depending on the mobile phase pH in these media are given in Figure 3.

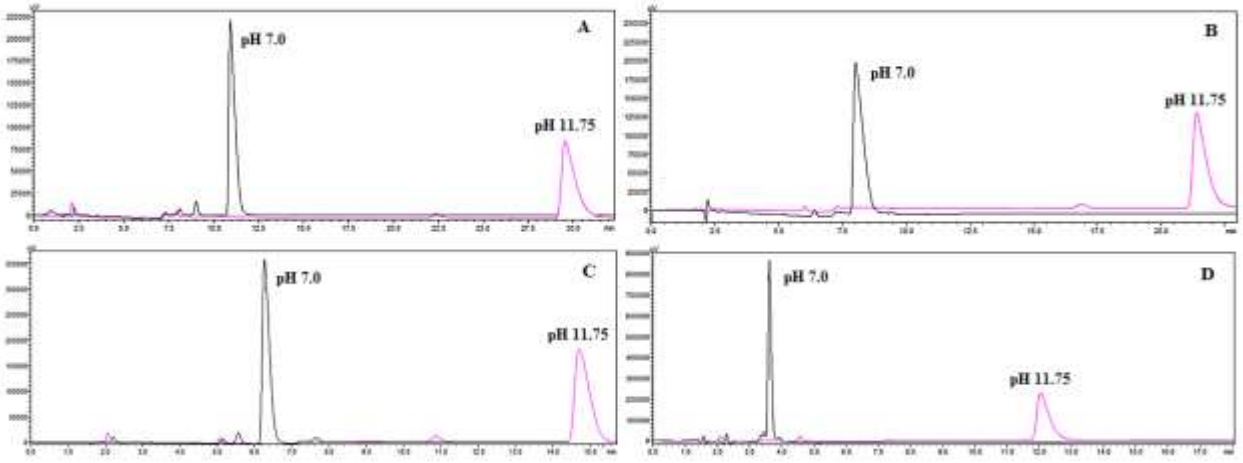

Figure 3. Chromatograms of synthesized compound. A) 40% B)45% C)50% D)55% (v/v) ACN

t_R values were determined in the selected pH ranges (7.0-11.75) for each mobile phase composition, and $^{s}_{s}pK_a$ and intrinsic t_R values (t_{RBH^+}, t_{RB}) were calculated using the obtained data with the NLREG program. Data calculated with standard deviation values are given in Table 1. The working pH range was started from 7. No significant change was observed in the t_R value of the compound in the study performed between pH 2 and pH 7. The ionization constant of the N(3)-substituted benzimidazole compound could not be calculated because of its small value. For this, the study was started from pH 7.

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Table 1. Retention time and the pK_a value of compound **2** in various ACN ratios calculated by NLREG program

	ACN concentration (v/v)			
	40%	**45%**	**50%**	**55%**
$^{s}_{s}pK_a$	10.777±0.114*	10.695±0.060	10.606±0.064	10.504±0.202
t_{RBH^+}	10.827±0.490	8.462±0.187	6.468±0.134	4.868±0.329
t_{RB}	31.291±1.345	22.481±0.465	15.502±0.302	11.509±0.670

*Standard deviation

Due to the lower polarity of ACN (α 0.19) compared to water (α 1.17), the solute-solvent polar interaction decreases with increasing ACN content of the mobile phase. Therefore, as the pH value of the mobile phase increases, the t_R value increases, while the retention time decreases as the ACN composition of the mobile phase increases. On the ACN rich side (0.15≤ X_{ACN}≤0.75), preferential solvation by water exists, which could explain the low decrease of $^{s}_{s}pK_a$ values of the studied compound when the percentage of ACN in the mobil phase increases [24].

When the t_R-mobile phase pH relationship was examined in all the binary mixtures studied, it was seen that the experimental data were in agreement with the theoretical data (Figure 4). The inflection points of these curves give the $^{s}_{s}pK_a$ value of the compound.

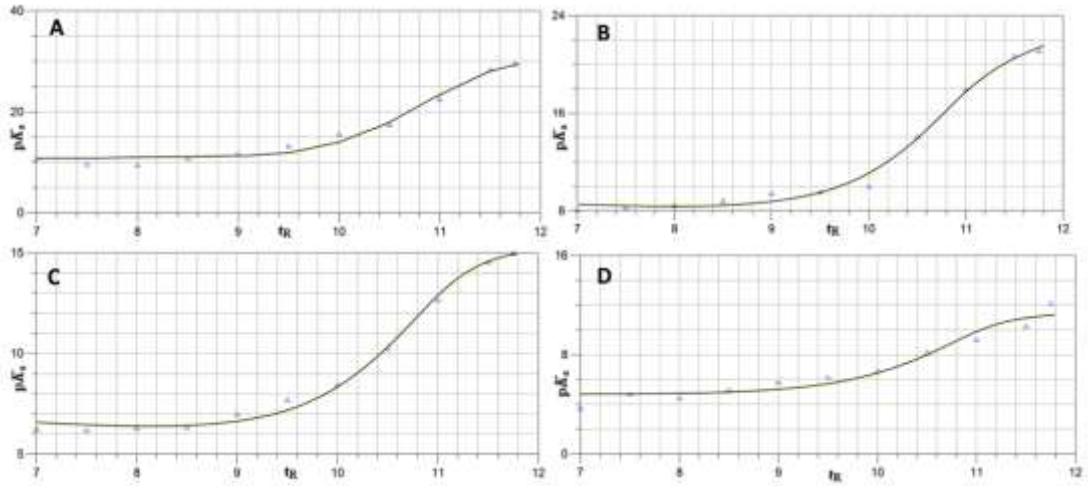

Figure 4. Plots of the retention times versus the pH of the mobile phase for A) 40% B)45% C)50% D)55% (v/v) ACN. The solid lines indicate the predicted retention times

3. DISCUSSION

Water-ACN binary mixtures in which compound **2** with low water solubility dissolves were analyzed by the RPLC method. ACN is a polar aprotic solvent with a high dielectric constant (ε 38.8). It plays an active role in improving the separation and selectivity of analytes in HPLC studies. In addition, the Gemini-NX C18 column selected in the present study consisted of a silica matrix grafted with ethylene groups to give a hybrid silica surface- functionalized with octadecyl methyl silane. This new generation column with a wide pH range (1-12) has high reproducibility, efficiency, and stability in the analysis of acidic and basic compounds.

Since this compound has low solubility in the aqueous medium, $^{s}_{s}pK_a$ value cannot be determined in the water medium. For this purpose, some macroscopic parameters of ACN were used to determine the aqueous pK_a ($^{s}_{s}pK_a$) value of the related compound. In the conducted study by Barbosa et al. [25] the macroscopic parameters of ACN, mole fraction (X_{ACN}), and dielectric constant (ε), were used to calculate the $^{w}_{w}pK_a$ value. The calculated $^{s}_{s}pK_a$ values in this study were plotted against the X_{ACN} and 1/ε values. The intercept value of the linear graph plotted using the X_{ACN} -$^{s}_{s}pK_a$ relationship gives the $^{s}_{s}pK_a$ value of the compound (Figure 5). The $^{w}_{w}pK_a$ value was calculated using the linear equation obtained using the 1/ε-$^{s}_{s}pK_a$ relationship and ε=78.34 in the aqueous medium (Figure 5). The $^{w}_{w}pK_a$ values calculated by these two approaches are given in Table 2.

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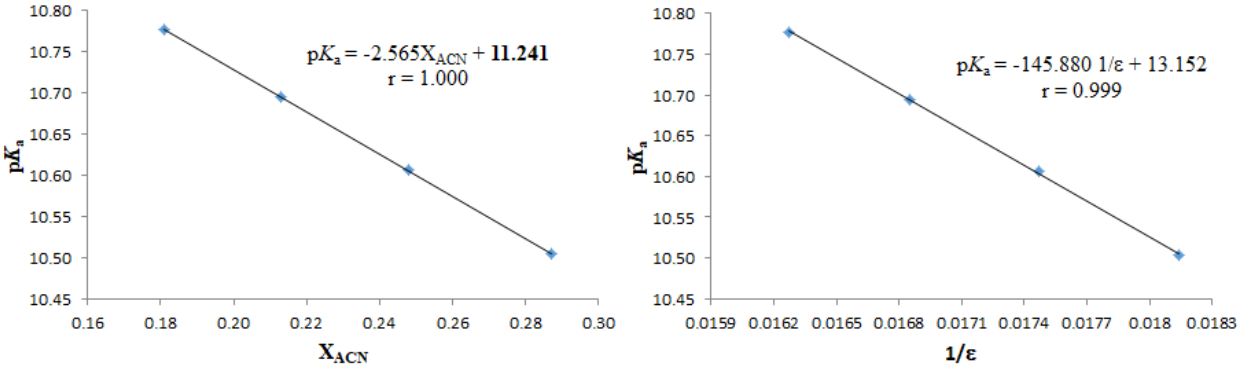

Figure 5. Graphs showing the linear relationship of X_{ACN} -$^{s}_{s}pK_a$and $1/\varepsilon$-$^{s}_{s}pK_a$

Table 2. Calculated $^{w}_{w}pK_a$ values

Analyte	$^{w}_{w}pK_a$ values	
	X_{ACN} -$^{s}_{s}pK_a$	1/ε-$^{s}_{s}pK_a$
Compound 2	11.241	11.290

When Figure 5 is examined, it is seen that the slopes of the graphs are negative. The negative slope is due to the basic functional group of the compound. It is seen that the $^{w}_{w}pK_a$ values calculated using these two macroscopic constants are compatible with each other.

There are several studies in the literature regarding the pK_a values of azolium salts [17-20]. There is only one study with ^{1}H-NMR to determine the pK_a value of benzimidazolium salts [17]. In addition, pK_a values of benzimidazolium salts in the study were determined by the potentiometric method. The pK_a value at 1 position of benzimidazolium salts containing 2-(4-methoxyphenyl)ethyl group was determined as 11.30±0.07 by ^{1}H-NMR method and 10.36±0.18 by potentiometric method.

An active drug substance can cross the cell membrane in a non-ionized form. Therefore, the degree of ionization must be known. The degree of ionization is necessary for the determination of ADME properties and can be calculated using the Henderson-Hasselbach equation [26]. In this study, the drug candidate with the basic functional group, whose $^{w}_{w}pK_a$ value was determined, becomes 50% completely ionized at the $^{w}_{w}pK_a$ value of compound **2**. Ionized and non-ionized forms of the compound were determined by using the % ionization values calculated in the pH 1.5-14 ranges (Figure 6). In basic compounds, two units above the pK_a value represent the non-ionized state of the compound.

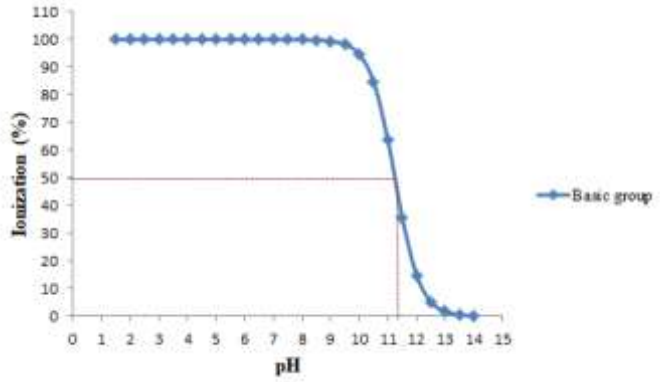

Figure 6. Percentage ionization of studied compound as a function of pH

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4. CONCLUSION

The protonation constant value of the drug candidate benzimidazolium salt 1-(2-methylbenzonitrile)-3-benzylbenzimidazolium bromide (**2**) was determined in the water-ACN binary mixture. The pK_a value of this synthesized benzimidazolium salt was determined for the first time in ACN-water binary mixtures with the RPLC method with high reproducibility and accuracy. The value of this basic compound, which has low solubility in the aqueous medium was determined using the macroscopic constants of ACN at 25 °C. The obtained data provide useful information for physicochemical studies as well as for pharmacological characterization.

5. MATERIALS AND METHODS

5.1. Preparation of compound 2

Compound **2** (404.3 g/mol) was synthesized, in our previous study [1], from 1 mmol of 1-benzylbenzimidazole and 1 mmol of 2-(bromomethyl)benzonitrile in ethyl alcohol at 80 °C for 24 h [1]. This known compound **2** was purified with crystallization method in ethyl alcohol and obtained as a white solid in very high yield 95% (Figure 7).

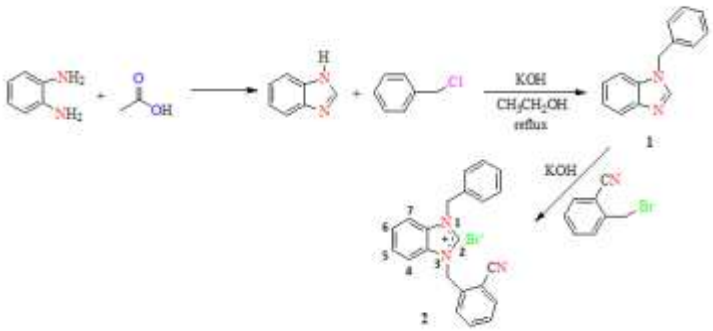

Figure 7. Synthesis of a benzimidazolium salt 2

The structural characterization data of the related compound are given in following [1]. IR: 1554.5, 2152.4, 2933.5, 2964.4, 3022.2 cm^{-1}. ^{1}H NMR (400.13 MHz, DMSO-d$_6$), δ: 5.72, 6.08, 7.20-8.08, 10.08. ^{13}C NMR (100.13 MHz, DMSO-d$_6$), δ: 49.07, 50.56, 111.36, 114.29, 114.68, 117.44, 127.41, 127.73, 128.81, 129.22, 129.43, 130.06, 131.43, 131.83, 134.24, 134.31, 134.44, 134.50, 137.32, 137.41, 143.99, 144.76. HRMS [L-Br]$^+$ calcd. for $C_{22}H_{18}N_3$: 324.4, found m/z: 324.14.

5.2. Chemicals and reagents

1-(2-methylbenzonitrile)-3-benzylbenzimidazolium bromide (2) was prepared according to literature procedure [1]. Acetonitrile (as organic modifier), orthophosphoric acid, sodium hydroxide, ammonium chloride, potassium hydrogen phthalate purchased from Merck (Darmstad, Germany). Ammonia was obtained from Sigma-Aldrich (St. Louis, MO). The compounds are of analytical purity and no purification was done prior to use.

5.3. Apparatus

Mettler Toledo InLab 413 Ag/AgCl glass electrode combined with a Mettler Toledo MA 235 pH/ion analyzer and was used for pH measurements of RPLC. Potassium hydrogen phthalate ($C_8H_5KO_4$) (0.05 molkg^{-1}) was chosen for electrode calibration in binary mixtures of ACN-water [21,22]. In adjusting the mobile phase pH, the temperature was kept constant at 25 °C.

In the determination of the chromatographic behavior of investigated compound, an HPLC system (Shimadzu, Kyoto, Japan) was used. This system includes a UV-visible detector (SPD-20A), column oven (CTO-20A), pump (LC-20AD), and degassing unit (DGU-20A$_3$).

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The Direct-Q®3 UV water purification system (Millipore, Bedford, MA, USA) was used to produce ultrapure water.

5.4. Chromatographic study

The pK_a value of benzimidazolium salt **2** was determined in water-ACN binary mixtures containing 40%, 45%, 50%, and 55% (v/v) ACN with the RPLC method. The pH of the mobile phase containing 25 mM *o*-H_3PO_4 was adjusted between pH 7.0 and 8.0 by adding 1 M NaOH and the pH of the mobile phase containing 25 mM NH_4Cl was adjusted between pH 8.5 and 11.75 by adding concentrated NH_3 solution.

The stock solution of the synthesized pure compound was prepared in 100 µgmL^{-1} water-ACN binary mixture. 20 µL of the stock solution was injected into the HPLC system. The retention times (t_R) of the compound were analyzed in five replicates. The relative standard deviation value (RSD%) of the t_R value was calculated below 1%. The separation was carried out in the Gemini NX C18 analytical column (250 x3.0 mm I.D., 5 µm) at a constant temperature of 25 °C. In the HPLC system, the flow rate of the mobile phase was fixed at 1 mLmin^{-1}. Analysis of the compound with UV-visible detector gave maximum absorbance value at 230 nm.

5.5. Data treatment

pK_a and t_R values of the ionized and neutral forms (t_{RBH^+}, t_{RB}) of compound **2** in studied ACN-water hydro-organic mixtures were calculated with nonlinear regression (NLREG) program [23].

Supplementary Material: ^{1}H NMR, ^{13}C NMR and IR spectra of compound **2** are given in the supporting information file.

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