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Thermodynamic protonation constant values of meclizine and buclizine in acetonitrile-water binary mixtures

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ABSTRACT: In the study, the retention times (t_R) values for meclizine (MCZ) and buclizine (BCZ) structurally related benzhydrylpiperazine were determined to modeling the dependence of retention on the acetonitrile concentration in the mobile phase. Reversed-phase liquid chromatography (RPLC) analysis of the MCZ and BCZ was realized with polar embedded amide (15x0.46 cm I.D, 5μm) column. The mobile phase pH range is selected from 5.0-9.0 to determine the structural changes due to protonation that result from a change in pH. The different retention time were attributed to their different hydrophobicities and ionization degree. The thermodynamic protonation constant (${}_{s}^{s}pK_a$) value for investigated compounds was calculated using the t_R relationship to pH of the mobile phase and effect of the activity coefficients. Linear correlation between ${}_{s}^{s}pK_a$ values and macroscopic parameters (X_{ACN}, $1/\varepsilon$) was also determined.

KEYWORDS: Benzhydrylpiperazine compounds; thermodynamic value; extrapolation methods; reversed-phase liquid chromatography.

1. INTRODUCTION

H_1 antihistamines are used widely in the treatment of allergic disorders. These medications are most effective in relieving the symptoms of seasonal rhinitis and conjunctivitis. Some first-generation H_1 antagonists (e.g., dimenhydrinate, cyclizine, meclizine, buclizine, and promethazine) have been widely used to prevent motion sickness. Antiemetic effects of these H_1 antihistamines can be beneficial in treating vertigo or postoperative emesis [1].

The structure and biological behavior of meclizine (MCZ) are similar to buclizine (BCZ). These compounds are a piperazine-derivative antihistamine. Members of the piperazine class of agents are structurally related to both the ethylenediamine and the benzhydryl ethers of ethanolamines. Their structures include the 2-carbon separation between nitrogen atoms, which is incorporated into the piperazine ring. Diarylmethylene groups (benzhydryl substituents) are attached to one of the nitrogen atoms, and an alkyl substituent is attached to the other nitrogen [2].

The chromatographic retention behavior of compounds in the reversed-phase liquid chromatography (RPLC) method depends on the interaction between the mobile phase and the stationary phase [3]. RPLC column selection is an important part of developing an analytical method. Traditionally, C18 columns are preferred for analysis of apolar compounds in method optimization studies. Where these compounds are difficult or impossible to elute from the column, different packing materials are preferred. Columns play an active role in retention. Besides, in the chromatographic analysis of the compounds, the correct selection of the concentration of the organic modifier in the mobile phase and pH value is very important. Different approaches are used to estimate the retention time of the compound with the correct choice of mobile phase [3-6].

If the protonation constant (pK_a) value of the basic analyte to be determined at different organic modifier concentrations is known, the retention time (t_R) in RPLC can be easily estimated using thelinear solvation relationships (LSERs) method [7-9].

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According to this approach, the t_R value of basic compounds can be calculated using the Eq. (1) [8,9]

$$t_R = \frac{t_{RHA}+t_{RA}10^{\pm(pH-pK_a)}}{1+10^{\pm(pH-pK_a)}} \qquad \text{[Eq. 1]}$$

The t_{RHA} and the t_{RA} are expressed as the retention time of ionized and non-ionized forms, respectively. A negative sign is used to calculate basic compounds [8,9]. Eq. (2) can be derived from Eq. (1) as follows:

$$t_R = t_{RHA}\left[\frac{1+\left(\frac{t_{RA}}{t_{RHA}}\right)10^{\pm(pH-pK_a)}}{1+10^{\pm(pH-pK_a)}}\right] \qquad \text{[Eq. 2]}$$

If in Eq. (2) the logarithm of the equation is taken:

$$log(t_R) = log\left\{t_{RHA}\left[\frac{1+\left(\frac{t_{RA}}{t_{RHA}}\right)10^{\pm(pH-pK_a)}}{1+10^{\pm(pH-pK_a)}}\right]\right\} \qquad \text{[Eq. 3]}$$

$$log(t_R) = log(t_{RHA}) + log\left(\frac{1+f10^{\pm(pH-pK_a)}}{1+10^{\pm(pH-pK_a)}}\right) \qquad \text{[Eq. 4]}$$

The f in the equation is the ratio of t_R values (t_{RA}/t_{RHA}). The equation in parenthesis is a hyperbolic function. The logarithm of this function is the sigmoidal function and this equation can be expressed as [8,9]:

$$log\left(\frac{1+f10^{\pm(pH-pK_a)}}{1+10^{\pm(pH-pK_a)}}\right) = \frac{U}{1+V10^{\pm(pH-pK_a)}} \qquad \text{[Eq. 5]}$$

U and V are two fitting coefficients. The initial estimates for U and V were set -1 and 4, respectively [8,9].Using Eq. (5)generated by this LSER approach, the log(t_R) value of the analyte can be calculated. Residual values for compounds were calculated as the difference between the estimated and measured log(t_R). Then these values were squared and summed. The best fits were obtained by minimizing the sum of squares of residuals (SSR). Microsoft Excel SOLVER was then started with the default regression [8,9].

Many drug compounds include at least one acidic and/or basic functionality, and the dissociation state of these compounds play a large role in determining the physicochemical properties (lipophilicity, solubility, protonation constant) of a compound. Protonation constant value (pK_a) is a physicochemical parameter that allows the estimation of ionization of basic compounds at different pH conditions. Information on pK_a value plays the main role in the expansion of drug delivery formulations [9]. This physicochemical parameter affects the ADME (absorption, distribution, metabolism, and excretion) properties of the active pharmaceutical ingredients (APIs) [10-12]. On the other hand, intestinal drug absorption is a key step for oral availability and systemic exposure of drug candidates.

For ionizable compounds insoluble in water, the determination of pK_a is difficult. Because of this, organic or hydro-organic solvent mixtures are needed for ${}_{s}^{s}pK_a$ determination [2,9]. In recent years, studies showing solvent-solvent and solute-solvent interaction in organic modifier-water mixtures in the liquid chromatographic analysis have attracted considerable attention [13,14].

Acetonitrile (ACN)-water mixtures are widely used in RPLC and other analytical methods to affect the solubility, absorption property, and chromatographic behavior of the compound [15-17]. One of the well-defined separation methods to estimate pK_a values is the RPLC method. It is a method based on differences in the retention of protonated and unprotonated compounds [18].

By this method, the t_R values are determined at pH values of the mobile phase in pK_a determination [19]. Furthermore, the organic solvent added to the mobile phase has a great effect on retention. t_R values were measured at each pH of the mobile phase, depending on the percentage of aprotic ACN selected in this study. ${}_{s}^{s}pK_a$ values of MCZ and BCZ in hydro-organic mixtures were also calculated by using this nonlinear relationship [20].

Although pK_a values of MCZ and BCZ with software packages based on the molecular structure can be found in the literature [21,22], the dissociation behavior of compounds in ACN-water hydro-organic mixture was still unknown. There is only one study in the literature regarding the pK_a value of MCZ [23]. There is no study done with the RPLC method. Therefore the RPLC method was chosen to determine the ${}^{s}_{s}pK_a$ values of the benzhydrylpiperazine antihistamine compounds with high precision and accuracy [18, 24- 26]. The study aimed was to determine the influence of ACN content on retention times in the microheterogeneity region. The t_R values of BCZ and MCZ in varying ACN content and the pH range of 5.0- 9.0 were measured. The ${}^{s}_{s}pK_a$ values for these benzhydrylpiperazine antihistamines were determined using the pH- t_R relationship. Aqueous pK_a (${}^{w}_{w}pK_a$) values for MCZ and BCZ were calculated using the mole fraction and dielectric constant of ACN [27].

2. RESULTS

2.1. Determination of t_R values for antihistamines

Octadecyl stationary phases (C18) are most commonly used in the analysis of these compounds by the RPLC method, but an investigation of chromatographic behavior of MCZ and BCZ by an amide-based column has not been reported so far. C18 columns were preferred for liquid chromatographic analyzes of MCZ and BCZ. Peak shapes and very good repeatability results were obtained in the analysis performed with this chosen Ascentis RP-Amide (15×0.46 cm I.D, 5µm) column. Acetonitrile, which is a polar aprotic solvent, readily soluble in water and preferred for the analysis of nonpolar compounds in RPLC analysis, was used as an organic modifier.

In this work, the t_R values of MCZ and BCZ were determined using the simultaneous action of ACN and pH in the mobile phase. The logt_R values of the compounds were estimated using Eq. (5). The differences between the experimental and predicted logt_R values were taken and the differences were squared. The results were obtained by minimizing the sum of squares of residuals (SSR) value. For ACN- water binary mixture containing 65% (v/v) ACN, the logt_R values calculated with the LSER approach are given in Table 1. SOLVER program was then started with the default regression. This procedure was repeated for the t_R values at the pH of the mobile phase and studied ACN concentration.

The differences between the experimental and predicted t_R values calculated according to this approach are quite small. When t_R values calculated by the LSER approach are plotted against experimental t_R values, a linear function is obtained (the correlation coefficient is at least 0.99). A regression between predicted and experimental t_R values should be a slope of 1.00 and an intercept of 0.0. In this study, the regression slope and intercept values were approximately close to 1 and 0, respectively (Figure 1).

2.2. Determination of pK_a values

Experimental t_R values of the MCZ and BCZ were determined at the pH of the mobile phase and composition. The change of t_R value in mobile phase pH in ACN-water binary mixture containing 62% ACN (v/v) is given in Figure 2 [28,29]. It is given in the blank sample at each pH value in the chromatogram. Low aqueous solubility is a major problem in drug development. MCZ and BCZ are sparingly soluble in water (logS_{MCZ}:-5.18; logS_{BCZ}:-6.18) [22]. Hence the determination of ${}^{s}_{s}pK_a$ values for these compounds by the RPLC method was performed in the ACN-water binary mixtures.

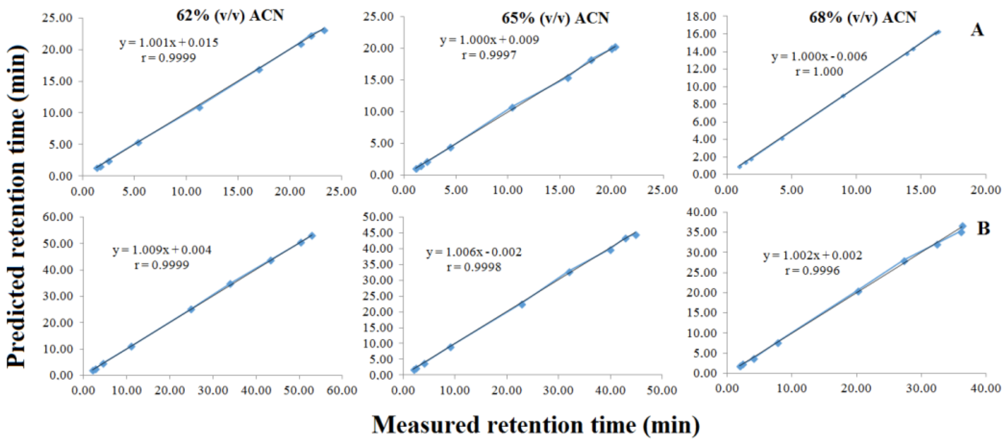

Figure 1. Predicted *vs.* measured retention times for A)MCZ and B)BCZ.

Table 1. Predicted t_R values by LSER approach for MCZ and BCZ

	pH	t_R (experimental)	t_R (predicted)		$logt_R$ (exp.)	$logt_R$ (pre.)	$(logt_{Rexp}-logt_{Rpre})^2$
MCZ	5.0	1.088	1.100		0.037	0.041	$2.27.10^{-05}$
	5.5	1.553	1.522		0.191	0.182	$7.55.10^{-05}$
	6.0	2.212	2.147	***U* = 0.143**	0.345	0.332	$1.69.10^{-04}$
	6.5	4.454	4.422	***V* = 2.876**	0.649	0.646	$9.60.10^{-06}$
	7.0	10.406	10.780	***f* = 29.263**	1.017	1.033	$2.36.10^{-04}$
	7.5	15.791	15.473		1.198	1.190	$7.82.10^{-05}$
	8.0	18.005	18.280		1.255	1.262	$4.33.10^{-05}$
	8.5	20.033	20.007		1.302	1.301	$3.18.10^{-07}$
	9.0	20.355	20.267		1.309	1.307	$3.54.10^{-06}$
						SSR*	$6.38.10^{-04}$
BCZ	5.0	1.909	1.932		0.281	0.286	$2.78.10^{-05}$
	5.5	2.418	2.400		0.383	0.380	$1.07.10^{-05}$
	6.0	4.015	3.900		0.604	0.591	$1.60.10^{-04}$
	6.5	9.032	9.133	***U* = 0.554**	0.956	0.961	$2.35.10^{-05}$
	7.0	22.723	22.650	***V* = 3.011**	1.356	1.355	$1.93.10^{-06}$
	7.5	31.879	32.803	***f* = 46.102**	1.504	1.516	$1.54.10^{-04}$
	8.0	39.880	39.732		1.601	1.599	$2.61.10^{-06}$
	8.5	42.867	43.571		1.632	1.639	$5.00.10^{-05}$
	9.0	44.810	44.521		1.651	1.649	$7.90.10^{-06}$
						SSR	$4.39.10^{-04}$

*SSR: Sum of squares of residuals

The ${}^{s}_{s}pK_a$values at studied ACN percentages were obtained by fitting the t_R-pH data, usually bynonlinear regression. ${}^{s}_{s}pK_a$ values depend on the ACN composition [30] and the ${}^{s}_{s}pK_a$ values of MCZ and BCZ were determined from the combined effect of pH of the mobile phase and experimental t_R values. The sigmoidal behavior was observed when the t_R values of MCZ and BCZ measured in the pH 5.0-9.0 range were plotted (Figure 3). ${}^{s}_{s}pK_a$ values of the compounds were calculated by nonlinear regression (NLREG) program using pH- t_R data. The thermodynamic ${}^{s}_{s}pK_a$ values and intrinsic t_R values of MCZ and BCZ in different ACN concentrations were calculated by using nonlinear squares data. Table 2 list the calculated data by the NLREG program [31], together with the standard deviations. The ${}^{s}_{s}pK_a$ values were also calculated by the SOLVER program (Table 2). The results obtained by these two approaches are compared in Table 2.

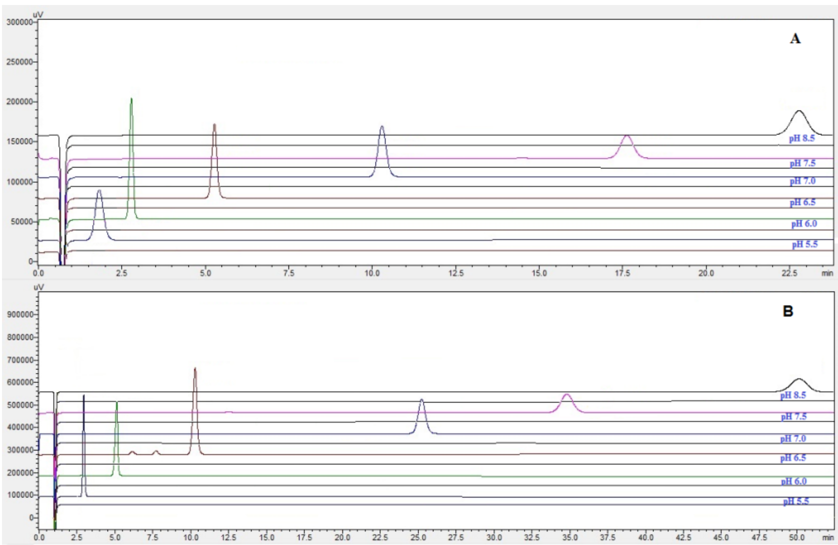

Figure 2. Overlaid chromatograms obtained from studied compounds for a mobile phase composed of ACN-water 462 binary mixture (62:38, v/v%) at different mobile phase pH. A)MCZ and B)BCZ.

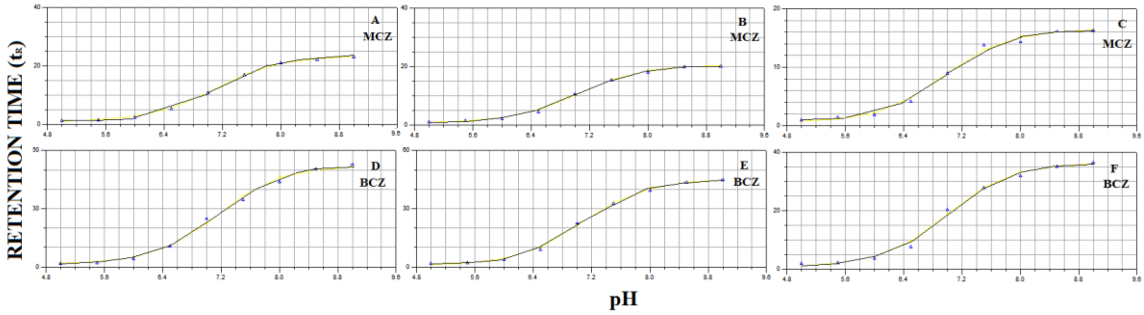

Figure 3. The retention time of the studied compounds is plotted against as a function of mobile phase pH using the NLREG program. A-D) ACN-water mixture containing 62% (v/v) ACN; B-E) ACN-water mixture containing 65% (v/v) 479 ACN; C-F) ACN-water mixture containing 68% (v/v) ACN.

Table 2. NLREG and SOLVER program data for studied compounds.

Compounds	ACN (v/v)%	NLREG			SOLVER
		t_{RBH^+}	t_{RB}	${}_s^s pK_a$	${}_s^s pK_a$
BCZ	60	2.371 (0.147)*	60.626 (1.586)	7.200 (0.066)	7.185
	62	1.661 (0.038)	52.159 (1.172)	7.149 (0.057)	7.127
	65	0.977 (0.057)	45.042 (0.609)	7.069 (0.035)	7.036
	68	0.869 (0.038)	36.407 (0.787)	6.992 (0.058)	6.931
MCZ	60	1.289 (0.098)	28.119 (0.224)	7.152 (0.021)	7.131
	62	1.067 (0.092)	23.410 (0.100)	7.102 (0.011)	7.086
	65	0.701 (0.028)	20.513 (0.296)	7.037 (0.039)	7.005
	68	0.622 (0.015)	16.483 (0.365)	6.957 (0.061)	6.914

*Standard deviation value

These two programs gave concordant results. The ${}_s^s pK_a$ values were found to decrease slightly with the increasing percentage of ACN in the mobile phase. The data given in Table 2 are consistent with the pK_a values of the piperazine ring [32-34].

As can be seen, the chromatographic retention of studied compounds with a benzhydrylpiperazine substituent increases strongly in the pH range 5.0-9.0. The sigmoidal curves were shown as continuous lines and the plotted points (Figure 3). Both compounds were an ionized form (BH^+) at pH 5.0. Protonation of the base causes peak tailing in C18, C8 columns [28,29]. In the study with different C18 columns (XBridge, Kinetex C18, Gemini NX, *etc.*) at pH 5.0, MCZ and BCZ were shown very long interaction with these columns and shown high retention times. The retention time of the amide column selected for this study is very low compared to the retention times of other C18 columns. The results also clearly indicate that benzhydrylpiperazine antihistamines with piperazine substituents behave quite differently from those with a substituent on the side chain.

As shown in Table 2, these two compounds have one ${}_s^S pK_a$ value corresponding to the basic functionalgroup. ${}_s^S pK_a$ value of the piperazine nitrogen linked to the methyl chain was influenced by the nature and the position of the substituent on the phenylmethyl attached to the 1-(4-chlorobenzylhydryl)piperazine. The dissociation equilibriums are given in Figure 4.

System suitability tests are an integral part of HPLC method development. The US Pharmacopoeia (24th) suggests that system suitability tests can be performed prior to analysis. For this test, combined effect of organic modifier content and ${}_s^S pH$of the mobile phase on the retention behavior of MCZ and BCZ was determined. The optimum separation condition of MCZ and BCZ was determined as ACN-water mixture containing 62% ACN (v/v) and pH 5.5. The parameters include capacity factor, theoretical plate number, retention time, tailing factor, selectivity, and RSD% of peak height or area for repetitive injections. The tailing factor and capacity factors were obtained as 1.17 and 1.22 for MCZ, 1.20 and 2.96 for BCZ, respectively. The theoretical plate numbers (N) were 3928 for MCZ, 4227 for BCZ. The selectivity factor was 2.43. The variation in retention time and peak area for five replicate injections of all compounds reference solutions gave RSDs of 0.06% and 0.32% for MCZ, 0.01% and 0.18% for BCZ, respectively. The results obtained from system suitability tests are inagreement with the USP requirements.

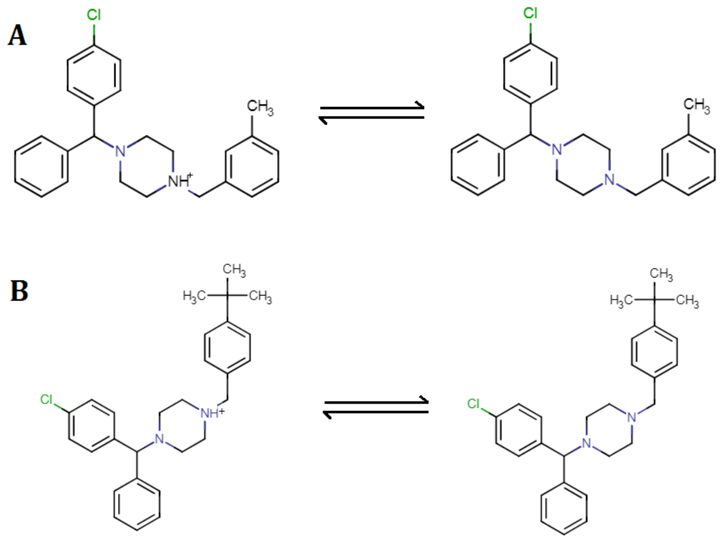

Figure 4. Protonation equilibriums of A) MCZ and B) BCZ.

3.DISCUSSION

MCZ and BCZ show one dissociation equilibrium corresponding to the deprotonation of N-4 the piperazine nitrogen linked to the methyl chain. On the ACN-rich side ($0.15 \leq X_{ACN} \leq 0.75$), preferential solvation by water exists, which could explain the low decrease of ${}_{s}^{s}pK_a$ values of MCZ and BCZ when the percentage of acetonitrile increases [35]. pK_a value is generally determined under standard conditions (25 °C, constant ionic strength). MCZ and BCZ were determined at 37 °C with this study. The pK_a value should be determined at bio relevant temperature (37 °C) to determine the absorption and distribution of the properties of the drug.

Since these hydrophobic antihistamines are not soluble in a water, ${}_{s}^{s}pK_a$ values in the water medium cannot be determined by the RPLC method. For this, aqueous ${}_{w}^{w}pK_a$ values were calculated by using the mole fraction (X_{ACN}) and dielectric constant (ε) values of acetonitrile determined by Barbosa et al.[27] in the ACN concentrations studied. In the present study, macroscopic parameters (dielectric constant and mole fraction) were used to calculate the ${}_{w}^{w}pK_a$ values of hydrophobic compounds in water. Considering the NLREG data depicted in Table 2, the ${}_{s}^{s}pK_a$ value of MCZ and BCZ were plotted against the reciprocal of the dielectric constant (1/ε), and the X_{ACN} (Figure 5). The data are shown as average value of three repetitive analyses with error bars as the standard deviations. The correlations between ${}_{s}^{s}pK_a$ values with the 1/ε and X_{ACN} of the studied binary mixtures are linear with correlation coefficients greater than 0.99 (Figure 5). The intercept value of the linear equation in the X_{ACN} -${}_{s}^{s}pK_a$ relationship gave the ${}_{w}^{w}pK_a$ value. In the linear equation in the 1/ε-${}_{s}^{s}pK_a$ relationship, ε = 78.51 is taken. Using this value, ${}_{w}^{w}pK_a$ value was calculated. ${}_{w}^{w}pK_a$ values calculated using these linear equations were given in Table 3. The basic protonation site has a negative slope as expected for the basic group. ${}_{w}^{w}pK_a$ values calculated from these two approaches were compatible with each other.

Data on ${}_{s}^{s}pK_a$values in ACN-water binary mixtures are scarce and only the pK_a value of MCZ was found in the literature [23]. This value was determined in chloroform and the method used was different [23]. In this work, the calculated ${}_{w}^{w}pK_a$ value for compounds is consistent with the values calculated from the Chemicalize program (pK_a: 7.71 for BCZ; pK_a: 7.60 for MCZ) [22].Since MCZ and BCZ are structurally analogous, there are limited studies in the literature regarding the simultaneous chromatographic separation of these compounds. Arayne and co-workers [36] were reported that separation of MCZ and BCZ simultaneously was carried out in C18 column. Separation was carried out in ACN-water (80:20, v/v) and pH 2.6 at room temperature. In this study, experiments have been made by changing the mobile phase composition, pH and flow rate, and the optimum separation condition has been found. As a further example, Sher and co-workers [37] were reported that simultaneous separation of MCZ and BCZ was carried out in C18 column at 25 °C. In the mobile phase, ACN was used as an organic modifier and heptane sulfonic acid was used as an ion pair former. No optimization study has been found in this study. There is no method optimization study for these trial and error method. In this study, both the pK_a values of the compounds and the optimum separation conditions of the compounds were determined by using the pH-t_R and pH-k relationship. In addition, in this study, chromatographic separation was carried out on an amide column. Determinations made with this column is the first study in the literature.

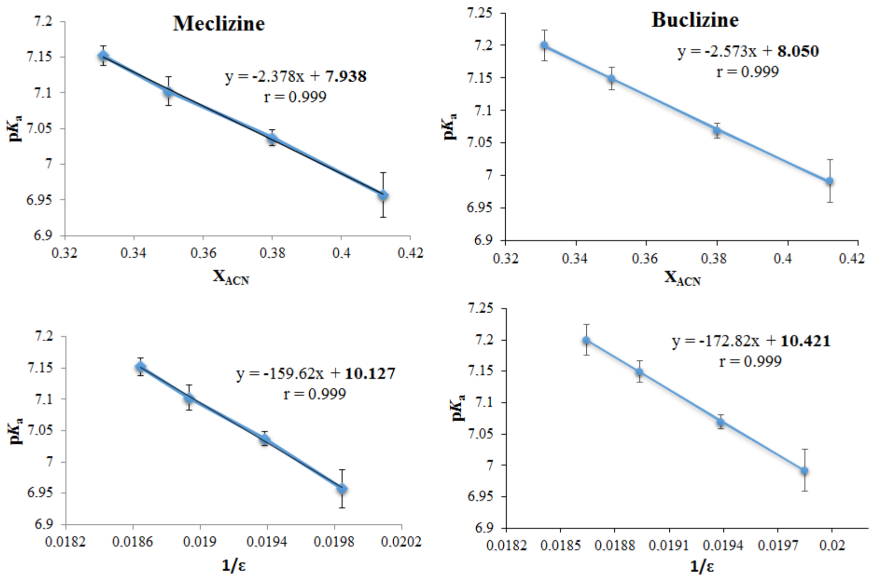

Figure 5. Linear relationships between ${}^{s}_{s}pK_a$ values and macroscopic parameters.

Table 3. Calculated aqueous pK_a values for MCZ and BCZ.

Compounds	${}^{w}_{w}pK_a$	
	1/ε-${}^{s}_{s}pK_a$	X_{ACN}- ${}^{s}_{s}pK_a$
MCZ	8.090	7.938
BCZ	8.216	8.050

The extent of ionization of a drug has an important effect on its ADME properties. Understanding how changes in pH alter the ionization of drugs is very important since unionized drugs cross membranes. Percentages of ionization for the studied drugs in ionized and non-ionized forms at certain pH values were calculated by Henderson-Hasselbach equation [38]. These values were calculated using ${}^{w}_{w}pK_a$ values calculated for basic compounds. Graphs showing the % ionization values in pH range 1.0-11.0 are given in Figure 6. Compounds containing basic functional groups are exactly 50% ionized at their pK_a values. It is known that the basic compounds pK_a value is above 2 units completely in non-ionized form.

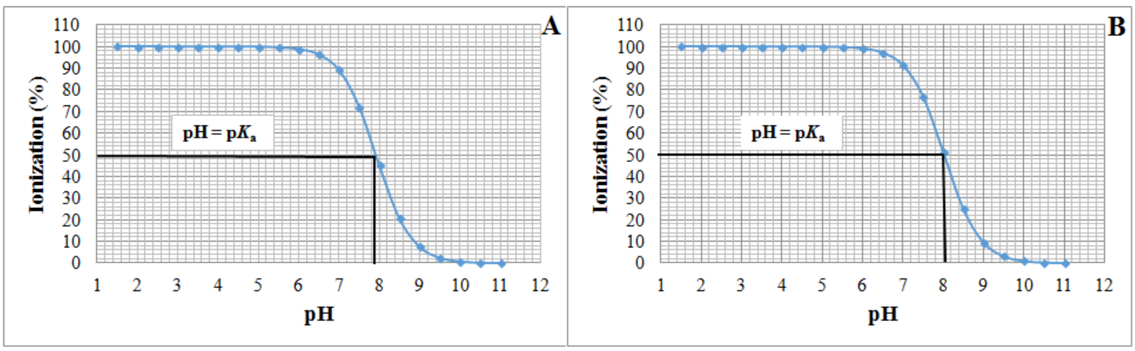

Figure 6. Percentage ionization of A)MCZ and B)BCZ as a function of pH.

4. CONCLUSION

In this study, the first data for which retention times are estimated by using the LSER approach for MCZ and BCZ and ${}^{s}_{s}pK_a$ values are calculated with SOLVER and NLREG programs are presented. The pH value (${}^{s}_{s}pH$) in the ACN-water binary mixture was used as the mobile phase was used instead of the pH (${}^{w}_{w}pH$) value in the water. For this, an RPLC assay was performed at four different concentrations of ACN and the ${}^{s}_{s}pK_a$ values of the compounds were calculated by using the relationships between retention times and eluent pH. Experimental studies were carried out using a special amide column to obtain appropriate interaction between analyte and column. This is the first study to describe the chromatographic behavior of MCZ and BCZ in ACN-water mixtures at 37°C. The retention values of the compounds depending on the mobile phase pH can be used for method optimization in chromatographic analysis of MCZ and BCZ. Percent ionization of the studied compounds in body fluids and their regions was estimated by this study.

5. EXPERIMENTAL

5.1. Chemicals

None of the chemicals used in this study were purified. MCZ and BCZ were sourced from Sigma-Aldrich (St. Louis, USA). Acetonitrile (ACN), sodium hydroxide (NaOH), o-phosphoric acid (o-H_3PO_4), potassium hydrogen phthalate (KHP) were purchased from Merck (Darmstadt, Germany).

5.2. Apparatus

A chromatographic determination was carried out by an HPLC system (Shimadzu Technologies, Kyoto, JAPAN). HPLC system consists of a UV-Vis detector (SPD-20A), a column oven (CTO-20A), a pump (LC-20AD), a degasser (DGU-20A3), and an injection system. Absorbance measurements were made with a UV detector at 230 nm. The polar embedded Ascentis RP-Amide (15×0.46 cm I.D, 5µm, Sigma-Aldrich®) column was chosen for the chromatographic determination of the compounds. pH measurements were performed with a pH meter (Mettler Toledo, Schwerzenbach, Switzerland). In electrode calibration, KHP solution (0.05 mol/kg) was prepared in ACN-water binary mixture [40]. Ultra pure water was supplied from a Direct-Q®3 UV water purification system (Millipore, Bedford, MA, USA).

5.3. Chromatographic study

Determination of t_R values of MCZ and BCZ was carried out in ACN-water hydro organic mixtures containing 60%, 62%, 65%, and 68% (v/v) ACN at pH 5.0-9.0. *o*-H_3PO_4 was added as the buffer component in the mobile phase. 30 mM was kept constant in all mobile phases. In the mobile phases prepared at these high ACN concentrations, the buffer in the medium did not precipitate. The studied mobile phase pH value was adjusted with 1 M NaOH.

Solutions of standard compounds were injected into the HPLC system at a constant flow rate (2 mL/min). The samples were injected into the HPLC column in a volume of 20 µL. The relative standard deviation (RSD%) of measured t_R values is less than 1.0% (n = 3). The chromatographic study was performed at 37 °C.

5.4. Preparation of standard solutions

When MCZ and BCZ have less solubility (log*S*: -5.18 for MCZ and log*S*:-6.18 for BCZ) [22] in water, they are prepared by dissolving in ACN-water hydro organic mixtures. The solutions were stored at a constant concentration (100 µg/mL) and at 4 °C, away from sunlight. Capacity factors (k) for each compound and mobile phase were calculated using the expression $k=(t_R-t_0)/t_0$. The dead time (t_0) was measured by injecting uracil solution (Sigma, USA, 0.1%, in water), which was established for each mobile phase composition and pH studied.

5.5. Data evaluation of pK_a

The SOLVER program was used to estimate the $t_R$values of the compounds at each ACN concentration studied and to calculate ${}_s^s pK_a$ values. For this, the differences between the experimental and theoretically calculated $t_R$values were taken and the sum of the differences was minimized [40]. Besides, the non-linear regression (NLREG) analysis program [31] was used to calculate the ${}_s^s pK_a$ values of the compounds, taking into account the activity coefficients (γ). γ values were calculated with the Debye-Hückel equation based on the ionic strength in the studied mobile phases [16].

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