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Determination of pK_a Values of Some Benzoxazoline Derivatives and the Structure–Activity Relationship

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ABSTRACT: The acid ionization constant (pK_a) values of 2-(3*H*)-benzoxazolinone and its 17 derivatives were determined in buffered solutions by UV–vis spectrophotometry, potentiometry, and capillary zone electrophoresis techniques. The pK_a values of the studied compounds are found to be in the range of 9.01 to 7.15. The advantages and limitations of each technique are discussed. The results suggest that the removal of a proton from the molecule occurred on the nitrogen atom of the 2-(3*H*)-benzoxazolinone ring and the analgesic/anti-inflammatory activities of the benzoxazolinone derivatives decrease when the pK_a values of the compounds increase.

1. INTRODUCTION

Between the two main groups of analgesics for the treatment of inflammation and pain, the non-narcotic ones (nonsteroidal anti-inflammatory drugs (NSAIDs)) are generally used since they do not cause drug dependence. The first used NSAID with therapeutic benefits was acetylsalicylic acid (ASA, known as aspirin), which has now been used for more than a century. NSAIDs, by inhibiting cyclooxygenase enzymes (COX-1 and COX-2), prevent the biosynthesis of prostaglandins (PGs) thus showing antipyretic and anti-inflammatory properties.[1] The fact that no dependence develops against their therapeutic effects is another advantage of such drugs. However, administration of NSAIDs, in some cases, causes a lack of PGs that are necessary for physiological functions.[2−4] Therefore, long-term use of such drugs results in gastrointestinal irritation, gastrointestinal ulcers and bleeding, or renal disorders.[2,3] As a consequence, development of effective NSAIDs with minimal side effects has became the main goal of drug research.[4] Since today's widely used NSAID drugs are announced to have hypnotic effects,[5,6] studies have intensified, and derivatives of such anti-inflammatory and analgesic drugs possessing the benzoxazolinone main body have become important.[5−7]

The first studies on 2-(3*H*)-benzoxazolinone derivatives started with the synthesis of 2-(3*H*)-benzoxazolinone from *o*-hydroxyphenylurethane.[8] Then, the new benzoxazolinone derivatives synthesized by structural modifications mostly made on the third, fifth, and sixth positions of the molecule (Figure 1) were shown to have analgesic and anti-inflammatory,[9−18] antifungal,[19−21] antirheumatic,[17] muscle relaxant,[22,23] and antibacterial properties.[24−26]

Most pharmaceutical compounds are either protonated or deprotonated in aqueous solutions.[27,28] The ionization ability is measured by a parameter, the acid ionization constant (K_a),

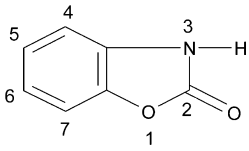

Figure 1. Structure and numbering of 2-(3*H*)-benzoxazolinone.

which is also called the protonation constant, equilibrium constant, or (acid) dissociation constant. Along with the partition coefficient, solubility, and reaction rate, the pK_a (negative logarithm of the K_a) is also an important physicochemical property of a given compound to be formulated into a useful medicine.[29] Depending on a function of its pK_a value(s) and the acidity of the solution, the extent of ionization of a drug controls its solubility and dissolution rate and, as a result, has great impact on gastrointestinal uptake into the bloodstream, distribution, cell permeability, drug-receptor binding, reaction kinetics, metabolism, and elimination.[30,31] Also, knowledge of the pK_a is useful when forming a salt in order to obtain biopharmaceutical properties and solid-state characteristics that may be lacking in the free form of the compound in the preformulation stage. Hence, knowledge of the possible ionization states of a pharmaceutical substance by determining their pK_a values is vital for drug development.[27]

The pH at which 50 % of a compound (with only one ionizable group) is ionized defines its acid dissociation constant value. The ionization of the studied compound can be calculated at any chosen acidity after the pK_a value is determined. The pK_a value is an important parameter for a

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compound bearing an acidic group, a basic group, or both; hence, knowing it leads to understanding the differences in physicochemical properties of the ionized and neutral forms of a species.[29] The most important impact of calculating the pK_a value of a compound on the development of a new drug is in understanding its solubility. While the ionized form of a compound can dissolve in water, the neutral form cannot and, hence, has a higher membrane permeability.

pK_a values of water-soluble compounds that are pure or have impurities that do not interfere with the main compound can be determined by spectrophotometry. The main advantage of spectrophotometric methods is due to their high sensitivity (> 10^{-6} M). On the other hand, the sample has to have a chromophore close to the ionizable groups in order to observe the differences in the spectral properties of the two forms. Spectral data are recorded by a UV−vis spectrometer, and the absorption spectrum of the compound changes when the pH of the aqueous buffered solution is altered. The changes in the absorbance values are usually followed by overlaid plots of recorded spectra, and the largest change occurs when the acidity of the aqueous solution is equal to the pK_a of the studied compound.

On the other hand, potentiometric titration is the most commonly accepted method for determination of pK_a values of compounds, which can dissolve in an aqueous solution. In potentiometric titration, titration of the sample with an acid or base is carried out with the aid of a glass electrode. At the end of the procedure, the acidity constant can be found from the midpoint of the titration curve. A high concentration of the sample (approximately $5{\cdot}10^{-4}$ M) and a 20 min to 40 min analysis time per compound are required for accurate determination of the pK_a value by this technique. At high pH values, carbonate-free solutions must be used to avoid errors in pH measurements. Aqueous and organic solvent mixtures may be used to overcome solubility problems. However, this method is subject to some limitations such as the sample amounts and solution volumes used.[33]

Based on the measurement of the effective mobility[34] of a compound with an ionizable group in electrolytic solutions with various pH values (usually buffered solution) and a constant ionic strength by capillary zone electrophoresis, the pK_a constant can be determined. The pK_a values are achieved by the plot of effective mobility as a function of the varying acidity of the solutions to that of a marker compound. The main advantages of using capillary electrophoresis for pK_a measurements are easy handling of impurities, the requirement of a low amount of sample, and use of automated instrumentation. However, the time of analysis is much longer when it is compared to the above-mentioned methods.[35]

The first purpose of this study is to determine the pK_a values of 2-(3*H*)-benzoxazolinone and its 17 derivatives[7,13,15,36−40] by using a spectrophotometric method. Acidity constants obtained by this technique are aimed to be confirmed by other analytical methods, namely potentiometry and capillary zone electrophoresis. This research is of great importance since knowing the acidity constant of the concerning compound is essential for understanding the chemical interactions and its pharmacological effects.[30] Furthermore, the relationship between the acidity constant and analgesic/anti-inflammatory activities of the drug candidates will be discussed in the manuscript.

2. MATERIALS AND METHODS

2.1. Apparatus and Procedure. *2.1.1. Ultraviolet−visible (UV−vis) Spectrophotometric Method.* The determination of acidity constants by a UV−vis spectrophotometric method included the use of a Shimadzu UV mini-1240 model spectrophotometer. In the spectrophotometric measurements, the concentration of the stock solutions of all studied benzoxazolinone derivatives was 0.01 M in acetonitrile. The stock solution was diluted to give a final concentration of $5{\cdot}10^{-5}$ M in the studied solution with the selected buffer. Buffer solutions were preserved in capped bottles to prevent the entrance of carbondioxide, and double distilled water was used in the preparation of the buffer solutions. The absorbance values at chosen wavelengths were recorded, and these values were transferred to a computer and put into charts. The best wavelength ranges were chosen for all pH values, and the pK_a values of each compound were determined by three independent experiments at each pH value.

2.1.2. Potentiometric Method. pH 4.00, 7.00, and 9.01 buffers in the presence of 10 % acetonitrile were used. In the titrimetric analysis, an Isolab basin and a Heidolph MR Hei-Standard Teflon stirrer were used. A mixture of 0.1 mL of 1 M hydrochloric acid (HCl), 8.9 mL of double distilled water (preserved in capped bottles to prevent the entrance of carbon dioxide) containing 0.154 M potassium chloride to achieve constant ionic strength and the necessary amount from the stock solution of the studied compound, and 1 mL of acetonitrile were prepared in a beaker, and the solution was stirred at room temperature using a magnetic stirrer. The amount of acetonitrile was increased for some 2-(3*H*)-benzoxazolinone derivatives that do not completely dissolve in water. As a result of the titrations performed with 0.5 M potassium hydroxide, the pH values measured were plotted versus the volume of KOH and pK_a values were calculated for each compound from the midpoint of the titration curve and by extrapolating the observed pK_a values to zero organic solvent concentration.

2.1.3. Capillary Zone Electrophoresis Method. The determination of acidity constants by a capillary zone electrophoresis method included the usage of an Agilent 3D device in combination with a diode array detector. A fused silica capillary was obtained from Agilent Technologies and had the following dimensions: 50.0 μm I.D., 40.0 cm effective length, 48 cm total length. The selected wavelength for analysis was 210 nm. The temperature of the capillary column was kept constant at 25 °C, and the separation potential was 20 kV to attain the best separation peak.

A 10 mg sample of each of the compounds was diluted to 10 mL with methanol to prepare stock solutions. For the sample solution to be analyzed, 100 μL of stock solution, 100 μL of buffer solution, and 800 μL of water were mixed. (*N*-Methyl)-2-(3*H*)-benzoxazolinone (compound **3**) was used as a neutral marker instead of using acetone or DMSO. It was injected as 50 μg mL^{-1} for all pH values before the injections of samples in order to determine the electroosmotic flow. Phosphate and borate buffer solutions were prepared by mixing appropriate volumes of stock buffer solutions (0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 for pH range from 5.65 to 8.18; 0.1 M NaOH and 0.2 M H_3BO_3 for pH range from 8.21 to 10.30) and by dilution with water to obtain identical ionic strengths. After filtration of the prepared buffer solutions with a 0.45 μm filter, the ultrasonic bath was used to degas the solutions prior to use.

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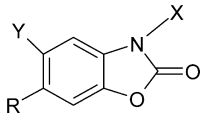
Table 1. List of Compounds Studied



no.	X	Y	R	no.	X	Y	R
1	H	H	H	**10**	H	H	*p*-FPhC(O)−
2	H	Cl	H	**11**	H	H	2,6-$(F)_2$PhC(O)−
3	CH_3	H	H	**12**	H	H	2,5-$(F)_2$PhC(O)−
4	CH_3	Cl	H	**13**	H	H	3,4-$(F)_2$PhC(O)−
5	H	CH_3	H	**14**	H	H	3,5-$(F)_2$PhC(O)−
6	H	H	H_3CC(O)−	**15**	H	H	*o*-ClPhC(O)−
7	H	H	PHC(O)−	**16**	H	Cl	*o*-FPhC(O)−
8	H	H	*o*-FPhC(O)−	**17**	CH_3	H	HC(O)−
9	H	H	*m*-FPhC(O)−	**18**	H	H	3,4-$(Cl)_2$PhC(O)−

Before initial usage of the capillary column, it was activated by flushing for 20 min with 1.0 M NaOH, 0.1 M NaOH, and water, respectively.

Before usage of the capillary column each working day, the column was flushed with 0.1 M NaOH for 20 min. Next, the sample solution added into the vial was injected into the device. Before each measurement, the capillary column was reactivated with 0.01 NaOH for 2 min, rinsed with water for 2 min, and flushed with buffer solution for 4 min. Following these procedures, 50 mbar pressure was applied for 5 s using hydrodynamic injection, and 20 kV voltages were applied after the injection. The compound injection (50 μg mL^{-1}) was repeated 3 times for each sample, and the completion stages of the process were monitored through the signal window. The separation process was performed based on the electroosmotic flow of the solutes, and different speeds of migration for ionic types resolved in electrophoretic buffer inside the capillary. Peaks attained as a result of this process were recorded. Electrophoretic mobilities observed at different pHs were plotted, and the pK_a values were calculated.

2.2. Chemicals and Reagents. Compounds **1** and **2** were purchased from Sigma Aldrich. The rest of the studied compounds (see Table 1) were synthesized according to literature.[7,13,15,36−40] They were of analytical purity. All chemical materials were used without being exposed to any extra purification process. Acetonitrile, methanol, acetic acid, boric acid, sodium chloride, potassium hydroxide, sodium hydroxide, and potassium chloride were supplied by Sigma-Aldrich. Sodium acetate, ammonium chloride, ammonium hydroxide, disodium hydrogen phosphate, sodium dihydrogen phosphate, phosphoric acid, and hydrochloric acid were supplied by Riedel.

Buffer solutions within the pH 1.50 to 3.50 range were prepared from phosphoric acid and sodium dihydrogen phosphate, those within the pH 3.70 to 5.70 range were from acetic acid and sodium acetate, those within the pH 5.80 to 7.80 range were from disodium hydrogen phosphate and sodium dihydrogen phosphate, those within the pH 8.30 to 10.30 range were from ammonia and ammonium chloride, and finally those within pH 10.50 to 12.50 range were from disodium hydrogen phosphate and sodium hydroxide by adjusting the molarities of the buffer components in suitable amounts to achieve the desired pH values. The acidity of each buffer solution has been checked with a Mettler Toledo Seven Easy Model pH-meter that was calibrated with standard buffer solutions.

3. RESULTS AND DISCUSSION

In this study, acidity constant (pK_a) values of 2-(3*H*)-benzoxazolinone and its 17 derivatives were determined by UV−vis spectrophotometry, potentiometry, and capillary zone electrophoresis (CZE) methods. The pK_a value for compound **1** was found to be 9.01 by UV−vis spectrophotometry, 8.98 by potentiometry, and 8.97 by capillary zone electrophoresis. In the literature,[27] pK_a values attained for compound **1** were given as 8.96 by UV−vis spectrophotometry, 8.95 by potentiometry, and 9.18 by capillary zone electrophoresis. The absorption spectrum obtained for compound **1** is given in Figure 2.

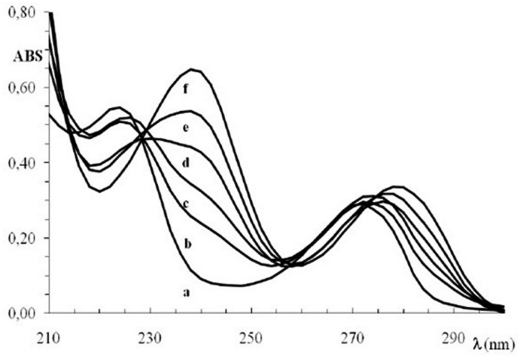


Figure 2. Absorbance vs wavelength plot for $5{\cdot}10^{-5}$ M compound **1** in various buffered solutions. pH values: (a)7.69, (b) 8.62, (c) 8.83, (d) 9.19, (e) 9.41, and (f) 9.87.

Whereas two absorption bands with absorption maxima at 224 and 270 nm were observed in acidic media (pH < 3), these values were observed to shift to 240 and 279 nm in alkaline media, respectively. The presence of isosbestic points on the spectrum indicates that compound **1** undergoes only one acid−base equilibrium.

The plot of absorbance values as a function of pH for compound **1** at 238 nm is given in Figure 3. The plot has the shape of part of a dissociation curve, and the pK_a value is determined from the midpoint of this curve as 9.01. The experiments were repeated four times, and the average value was reported. The same and/or similar pK_a values were obtained from similar plots at other wavelengths as well. In order to verify that the pK_a value obtained by UV−vis spectrophotometry is correct, the potentiometry and capillary zone electrophoresis experiments were also carried out.

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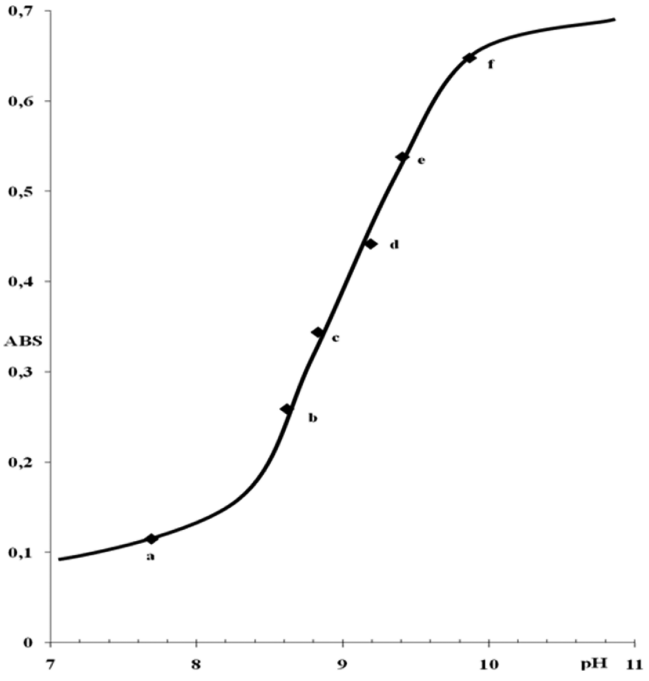

Figure 3. Plot of absorbance values as a function of pH for compound **1** at 238 nm. pH values: (a) 7.69, (b) 8.62, (c) 8.83, (d) 9.19, (e) 9.41, and (f) 9.87.

In potentiometry experiments, 0.154 M KCl was used to keep the ionic strength of the solution constant.[27] However, the solubility of the compound decreased as the concentration increased; hence only pK_a values of some benzoxazolinone derivatives could be obtained by this method. The pK_a value of compound **1** from the midpoint of the curved line was determined as 8.98. The experiments were repeated four times, and the average pK_a values are given. There is no doubt that the pK_a values obtained by potentiometry and UV−vis spectrophotometry are similar ($p < 0.05$) and also the value given in the literature agrees well with both results.[27]

By means of capillary zone electrophoresis (CZE) that was used as a third technique for comparison, the pK_a value of compound **1** was determined. The measured electrophoretic movement was plotted against pH from which the pK_a value is assigned to be 8.97. In order to achieve this experiment compound **1** together with the buffer solution and neutral marker (compound **3**) was passed from the capillary column. Depending on the electrophoretic movements and the migration of the ionic types that were dissolved in the buffer, the peaks were recorded and this procedure was repeated three times. Two different forces would change the migration of an analyte in the capillary electrophoresis. These are electro-osmotic flow (EOF) and electrophoretic mobility. The negatively charged analytes migrate longer in the capillary in comparison to EOF according to their electrophoretic mobilities. It is possible to describe a relationship between the electrophoretic mobility and pK_a of a compound: The field strength (E) in the capillary column related to the total capillary length (L_c) and the applied voltage (V), the effective distance (L_d), and the electrophoretic velocity (υ) affect the electrophoretic mobility (μ) directly. The apparent mobility (μ_{app}) could be described[34] as given below where the migration time of the ion is t_{app}.

$$\mu_{app} = (\upsilon_{app}/E) = (L_c \times L_d)/(t_{app} \times V) \quad (1)$$

Since, the EOF is also related to pH of the background electrolyte, the effective mobility of an ion (μ_e) is not equal to the apparent mobility. Therefore, μ_e could be expressed as follows:

$$\mu_e = \mu_{app} - \mu_{EOF} \quad (2)$$

A marker remaining neutral in the pH of the background electrolyte could be used to determine the EOF under the experimental conditions. Thus, the effective mobility of an ion could be defined as follows:

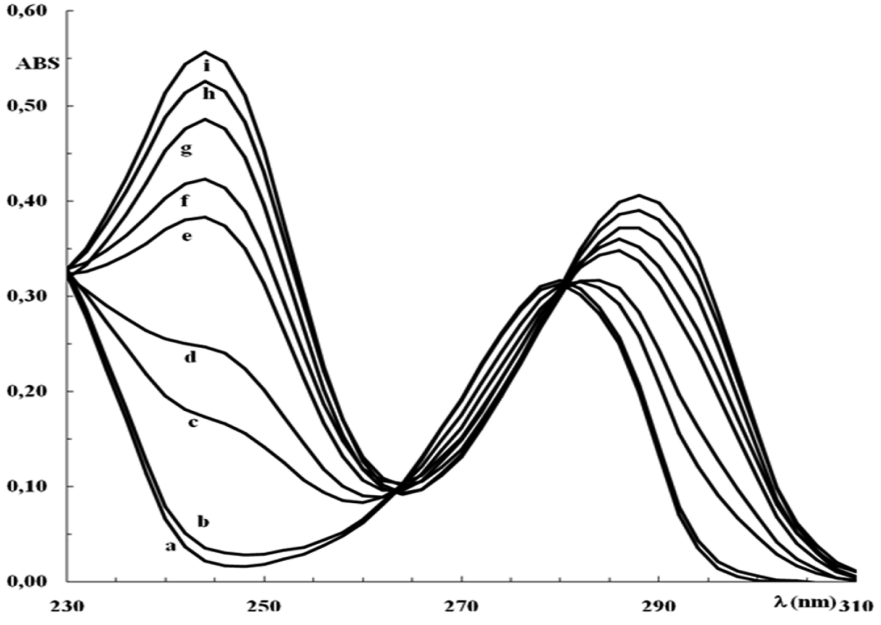

Figure 4. UV spectra obtained at various pH values for compound **2**. pH values: (a) 6.18, (b) 6.74, (c) 7.69, (d) 8.23, (e) 8.62, (f) 8.83, (g) 9.19, (h) 9.41, and (i) 9.75.

$$\mu_e = [(L_c \times L_d)/V] \times [((1/t_{app}) - (1/t_{EOF}))] \quad (3)$$

Compound **2** (5-chloro-2-(3*H*)-benzoxazolinone) is being used as a drug that acts as a neuromuscular blocker.[7] In order to determine the acidity constant (pK_a) of it, UV−vis spectrophotometric and capillary zone electrophoresis methods were used accordingly. Potentiometry was proven to be a useless technique in this case due to the low solubility of compound **2** in aqueous solutions. The absorption spectrum for compound **2** is given in Figure 4. The isosbestic points at 230, 262, and 280 nm indicate the presence of only one acid−base equilibrium. The decrease in absorbance values with increasing acidity at each wavelength shows the equilibrium between the protonated and neutral forms of the compound. The absorbance values measured at 246 nm for compound **2** was plotted as a function of pH from which the pK_a value was determined. The plot had the shape of a dissociation curve and pK_a value of the compound **2** was assigned from the midpoint as 8.26. The similarity between the experimental data and the theoretical curve shows how well the acidity constant values agree. Also, the acidity constants were calculated at other wavelengths and similar results were obtained. With capillary electrophoresis, the calculated effective mobility (μ_e) was plotted against pH. The plot, which was used for determining the pK_a value of compound **2**, is given in Figure 5.

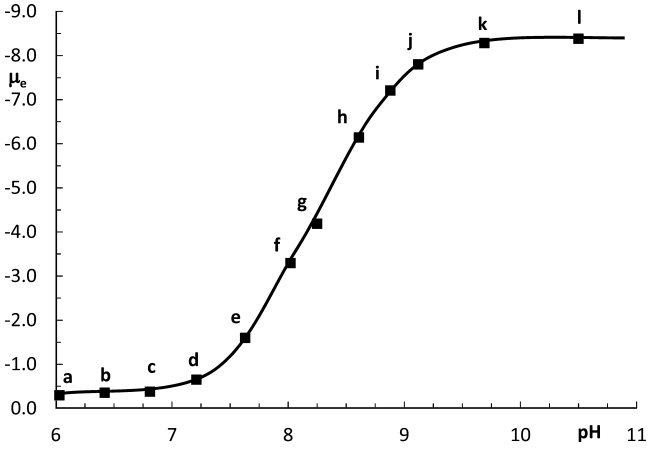

Figure 5. Effective mobility values of compound **2** as a function of pH. pH values: (a) 6.03, (b) 6.42, (c) 6.81, (d) 7.21, (e) 7.63, (f) 8.02, (g) 8.18, (h) 8.61, (i) 8.96, (j) 9.12, (k) 9.69, and (l) 10.50.

In the studies that have been carried out for compounds **3** and **4**, which possess a methyl group on the nitrogen, the acidity constants at the pH range studied (1.83 to 13.0) could not be determined because no change was observed in spectrophotometric measurements. However, it is foreseen that the acidity constants of these compounds should be lower than the lowest pH value of the range. Therefore, only the upper limit of the acidity constants for them could be given (see Table 2).

In the spectrophotometric measurements, the absorbance values did not change over the studied pH range for compounds **3** and **4**. This observation could be due to the aging of these compounds. For that reason, the effect of time on these compounds was studied by running the spectra on different days and the absorbance values were measured. No change in the spectra was observed, and hence it was stated that no aging of the solutions of these compounds occurred. Particularly, since the acidity constant of compound **3** is small, this compound can be used as a neutral marker in the capillary zone electrophoresis experiments.

The results from the investigations on compounds **3** and **4** lead to the conclusion that the protonation should take place at the nitrogen atom on the benzoxazolinone ring. The proposed equilibrium is given in Figure 6.

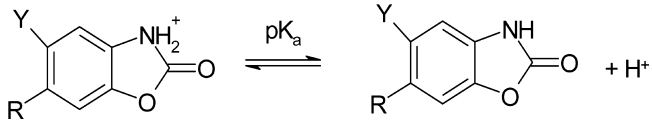

Figure 6. Equilibrium for the protonation of the 2-(3*H*)-benzoxazolinone ring.

By means of the same techniques that were used for the investigation of the first four compounds, the acidity constant of 5-methyl-2-(3*H*)-benzoxazolinone (compound **5**) was studied as well. Since the solubility of compound **5** is much higher than that of compound **2**, its acidity constant could be determined by potentiometry as well. The acidity constants determined by all three techniques for compound **5** are reported in Table 2. Analysis of the pK_a values of these five compounds indicates that when there is an electron-withdrawing group (chlorine for compound **2**) on the fifth position of the ring, the electronic dispersion is affected, and when compared to a compound that bears an electron-donating group (methyl as in compound **5**) the acidity of the compound is increased. The acidity constant values attained for first five 2-(3*H*)-benzoxazolinone derivatives and others using the three methods mentioned above are given in Table 2.

Our systematic studies were continued by the investigation of compounds which have benzoyl substituents on the sixth

Table 2. Experimental pK_a Values Determined by UV−vis Spectrophotometry, Potentiometry, and Capillary Zone Electrophoresis (CZE) for 2-(3*H*)-Benzoxazolinone Derivatives

no.	UV−vis	potentiometry	CZE	no.	UV−vis	potentiometry	CZE
1	9.01 ± 0.06	8.98 ± 0.05	8.97 ± 0.04	**10**	7.70 ± 0.05	–	7.60 ± 0.07
2	8.26 ± 0.05	–	8.25 ± 0.06	**11**	7.35 ± 0.08	–	7.23 ± 0.04
3	<2.10	–	*marker*	**12**	7.45 ± 0.06	7.36 ± 0.04	–
4	<1.83	–	–	**13**	7.61 ± 0.05	7.54 ± 0.06	–
5	8.92 ± 0.05	8.97 ± 0.08	8.82 ± 0.04	**14**	–	–	–
6	7.86 ± 0.04	7.78 ± 0.09	7.85 ± 0.05	**15**	7.35 ± 0.03	7.29 ± 0.07	7.23 ± 0.03
7	7.70 ± 0.07	7.81 ± 0.07	7.75 ± 0.05	**16**	7.15 ± 0.06	–	–
8	7.40 ± 0.06	7.32 ± 0.07	–	**17**	<2.10	–	<3.30
9	7.65 ± 0.06	7.61 ± 0.08	–	**18**	8.30 ± 0.05	–	–

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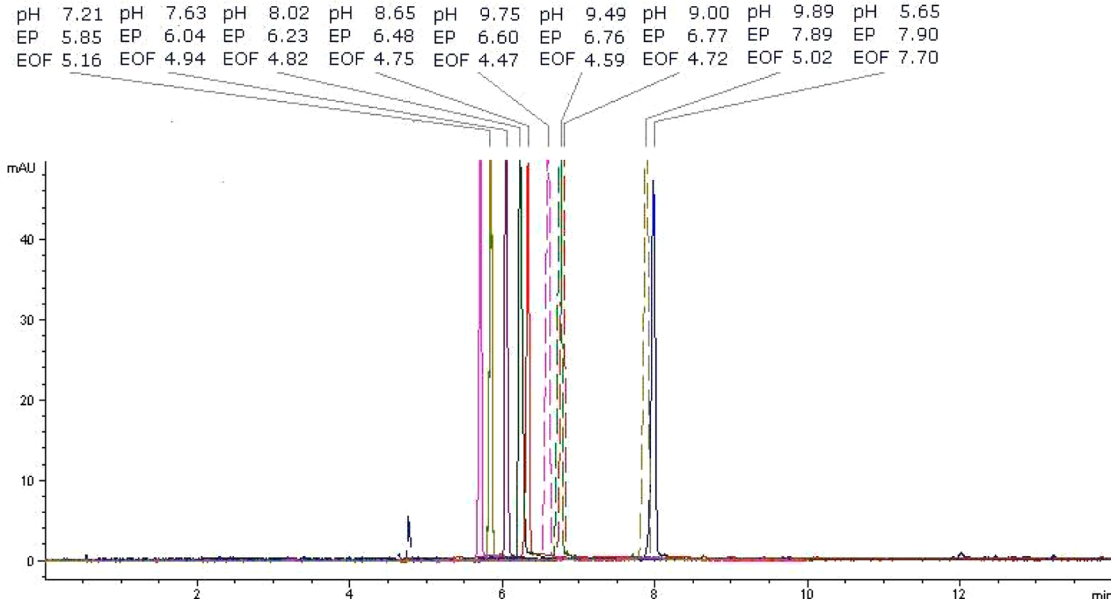

Figure 7. Overlay electropherograms of compound **10** taken under at pH values: 5.65, 6.03, 6.42, 6.81, 7.21, 7.63, 8.02, 8.65, 9.00, 9.49, 9.75, 9.89, and 10.30. Some of the peaks were indicated by giving the migration time of the peak (EP) and the migration time of the electroosmotic flow (EOF) determined by initial injection of neutral marker (compound **3**).

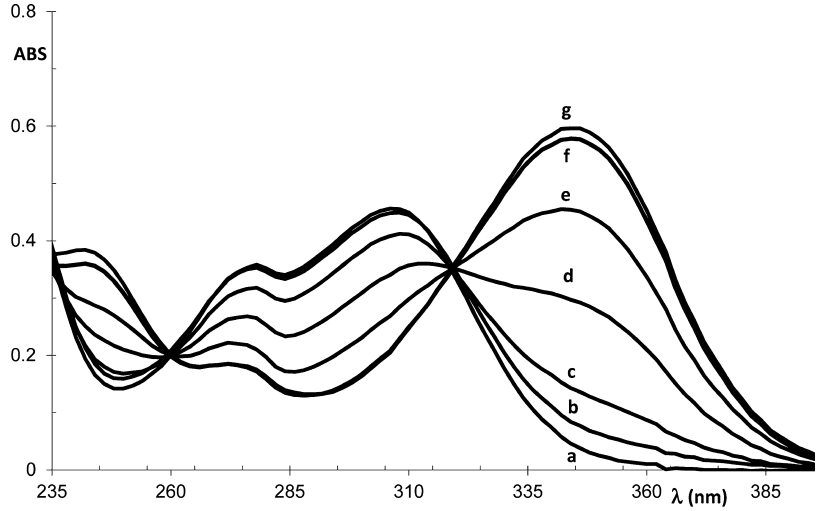

Figure 8. UV spectra obtained at various pH values for compound **11**. pH values: (a) 5.60, (b) 6.30, (c) 6.60, (d) 7.20, (e) 7.80, (f) 8.35, and (g) 8.60.

position of the main 2-(3*H*)-benzoxazolinone ring. When the acidity constants determined for compounds **6** (bearing acetyl group at the sixth position of benzoxazolinone ring) and **7** carrying a benzoyl group with that of 2-(3*H*)-benzoxazolinone (compound **2**) were compared, it was observed that the presence of a carbonyl group increased the acidity of these two compounds. Yet, the value given in the literature for compound **7** ($pK_a = 7.76 \pm 0.03$)[27] and the pK_a values that were obtained in this work are compatible and coherent.

Compounds **8**, **9**, and **10** have similar structures to compound **7**. The only difference is the different position of the fluorine atom on the benzene ring. Substitution of the fluorine atom on the ortho, meta, or para position of the benzene ring causes a decrease in the acidity, as the position changes from ortho to para. Figure 7 shows the electropherograms taken at various pH values (between 5.65 and 10.30) for compound **10**.

The structure−analgesic activity relationship of compounds **8** and **9** was determined to be 71 % and 50 %, respectively[36] (the reference compound is ASA (analgesic activity is 21 %)). As the pK_a values of these compounds increase from 7.40 to 7.65, a decrease in analgesic activity is observed. Also, the anti-inflammatory activity of these compounds drops from 59 % to 54 %[36] as the pK_a values increase (the reference compound is indometacin (anti-inflammatory activity is 71 %)). These results suggest that there is a relationship between the analgesic/anti-inflammatory activities and the acidity of the compounds. If the pK_a value increases, both types of activities decrease. Moreover, the analgesic activity and pK_a value of compound **5** are 32 %[41] and 8.92, respectively. Again, the increase in pK_a value results in a decrease in the analgesic activity.

After compounds with a single fluorine atom on 6-benzoyl ring were studied, the acidity constants of compounds

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containing two fluorine atoms (compounds **11** to **14**) were examined. Among these compounds, compound **14** did not dissolve in acetonitrile; hence, to avoid variables by using another organic solvent for dilution, assignment of a pK_a value was not attained for it. The calculated pK_a values for the rest of the compounds are listed in Table 2. It can be seen from this table that when the fluorine atoms are substituted on both the second and sixth positions of the 6-benzoyl ring, the molecule becomes more acidic when compared to the ones that bear the fluorine atoms on the second and fifth or third and fourth positions. As an example, the spectra obtained for compound **11** are given in Figure 8 and the absorbance values measured at 344 nm were plotted as a function of pH to determine the pK_a value.

The analgesic activity[15] was correlated to the pK_a values of compounds **11**, **12**, and **13** with comparison to ASA. The analgesic activities of these compounds dropped from 76 % to 20 %,[15] when the pK_a values of the compounds increased from 7.35 to 7.61 in the same order. This result points out that the acidity is an important factor, suggesting an explanation for the structural effects on the analgesic activity of a drug candidate.

When the hydrogen atom on the second position of the 6-benzoyl of compound **7** is replaced by a fluorine atom as in compound **8**, the acidic stability is increased. Similarly, when a chlorine atom is present at the same position (compound **15**), the acidity is also increased. This finding can be attributed to the electron-withdrawing property of these halogen atoms. The difference in pK_a values of compounds **8** and **15** when compared to that of compound **7** can be explained by the difference in electronegativity of the fluorine and chlorine atoms. Since the chlorine atom is known to be less electronegative, the ability to share its electron density can be expected for compound **15**. Thus, this compound can stabilize the unprotonated form of the benzoxazolinone ring more effectively than the fluorine substituent.

The acidity constant found for compound **16** indicates that this compound is more acidic than compounds **1** and **2** (Table 2). This result can be explained by the presence of a fluorine atom at the ortho position which is the reason for the stability of the basic form due to its inductive effect, causing the deprotonation on the nitrogen atom to be difficult. Consequently the electrons stay on the molecule much longer. Also the analgesic activity of this compound was found to be 82 %[36] and the pK_a value of this compound is 7.15 (see Table 2). Its analgesic activity is higher than that for compound **8** (pK_a value is 7.40), which does not have chlorine at the fifth position of the benzoxazolinone ring. Therefore, when the acidity of the compound increases, the analgesic activity decreases.

The acidity constant value could not be determined for compound **17** due to its methyl group on the nitrogen as in compounds **3** and **4**. Besides, the spectrum of the compound was time-dependent in the pH range studied. Similar studies as for previous compounds were carried out for compound **18** as well, and the calculated pK_a value was compared with that of compound **13** bearing difluorine atoms. It was observed that compound **13** is more acidic than compound **18**. This result can be attributed to the higher electronegativity of the fluorine atom compared to that of the chlorine atom, and accordingly the fluorine atom causes the nitrogen to be more unstable with its partial positive charge.

4. CONCLUSION

In this study the pK_a values of 2-(3*H*)-benzoxazolinone and its 17 derivatives were determined by UV−vis spectrophotometry, potentiometry, and capillary zone electrophoresis techniques, and the values were compared with those given in the literature.[27] It was assumed in the present literature that the removal of a proton from the molecule occurred at the nitrogen atom of the 2-(3*H*)-benzoxazolinone ring. Our results strongly indicate that the protonation occurs at the nitrogen atom in the main structure of benzoxazolinone, as the acidity constants could not be calculated for compounds **3**, **4**, and **17**. The fact that the pK_a values for these three compounds that bear a methyl group on the nitrogen could not be obtained shows that the protonation of the molecule occurs only at the nitrogen atom of the main benzoxazolinone heterocyclic group. The protonation reaction given in Figure 6 is proposed based on this observation.

In order to understand the reactivity of the studied compounds relative to each other, studies regarding the main structure and its derivatives prepared by substitutions on the third, fifth, and sixth positions were carried out. As a result, it is proven that the 6-benzoyl derivatives are more reactive than the main compound (compound **1**). The analgesic effect of the compound becomes much stronger than that of the unsubstituted ones when a chlorine atom is present on the benzoxazolinone ring. In light of our results related to 2-(3*H*)-benzoxazolinone and its derivatives, relevant information can be obtained regarding the availability of the groups that can ionize, as well as the biological activities and solubility of these compounds at a given acidity. Our results also suggest that the analgesic/anti-inflammatory activities of benzoxazolinones decrease when the pK_a values of the compounds increase.

The UV−vis spectrophotometry method can be counted as one of the best methods to determine pK_a values as shown in this study. In order to obtain reliable results with this technique, the margin of error must be minimized and buffer solutions that are the most appropriate for the pH ranges studied should be selected. On the other hand, for the potentiometric titration technique, which is used as well in this study, the volume of the added base (KOH) must be selected carefully and the pH values must be recorded frequently by adding in small amounts. Consequently, the capillary zone electrophoresis technique has the advantage of having the highest distinction capacity for samples with very small volumes. However, the working period of the capillary electrophoresis device was too long for each compound, and this was encountered as a disadvantage of this technique when compared to the others.

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