YEDITEPE UNIVERSITY
INSTITUTE OF MEDICAL SCIENCES
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DETERMINATION OF ACID DISSOCIATION CONSTANT VALUES OF SOME BENZOXAZOLINONE DERIVATIVES BY USING SPECTROPHOTOMETRIC, POTENTIOMETRIC, AND CAPILLARY ELECTROPHORETIC METHODS IN AQUEOUS BUFFERED SOLUTION

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RESUME

She was born at Bakırköy, İstanbul in 1989. She graduated from department of chemistry at Trakya University in 2001. She had done her internship at quality control laboratories at Mustafa Nevzat Pharmaceutical Company and Ustubec Dye Company. Currently, she is a chemistry teacher at a private high school.

ABSTRACT

Acid dissociation constant (pK_a) values of fourteen structurally related 3-(2/4-pyridylethyl) benzoxazolinone derivatives have been found by using UV-Vis spectroscopy, and the results have been verified by using potentiometry and capillary electrophoresis methods. The position of the equilibrium reaction was suggested based on the experimental results. Under the light of data, the electro with-drawing groups (chlorine atom) on 6-benzoyl derivatives or on the main structure of benzoxazolinone ring showed more basic character in comparison with electron donating groups. Our results suggest that analgesic/anti-inflammatory activities of benzoxazolinones decreased when the pK_a values and / or solubility of the compounds increased. Advantages and limitations of using UV-Vis spectroscopic, potentiometric, and capillary zone electrophoretic methods for determination of acid dissociation constants were discussed.

ÖZET

14 adet yapıca benzer 3-(2/4-piridiletil) benzoksazolon türevinin asit iyonizasyon sabiti değerleri (pK_a) UV-Vis spektroskopisi kullanılarak bulunmuş ve sonuçlar potansiyometri ve kapiler elektroforez yöntemleri kullanılarak doğrulanmıştır. Denge reaksiyonunun pozisyonu deneysel sonuçlara dayanarak öne sürüldü. Veriler ışığında; 6-benzoil türevlerinin ve benzoksazolon halkasının ana yapısı üzerindeki elektron verici gruplar (klor atomu) elektron alıcı gruplar içeren bileşikler ile karşılaştırıldığında daha bazik özellik gösterdi. Sonuçlarımıza göre bileşiklerin pK_a değerleri ve / veya çözünürlükleri arttığında, benzoksazolon türevlerinin analjezik / antienflamatuar etkilerinin azaldığı gözlemlendi. Asit iyonizasyon sabitlerinin belirlenmesinde UV-Vis spektroskopisi, potansiyometri ve kapiler elektroforez kullanımının avantajları ve sınırlamaları tartışıldı.

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TABLE OF CONTENTS

SYMBOLS AND ABBREVIATIONS

A	Absorbance
a	Ion activitiy
α	Selectivity
AO	Atomic Orbital
b	Beam
C	Concentration
CE	Capillary Electrophoresis
CZE	Capillary Zone Electrophoresis
CGE	Capillary Gel electrophoresis
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
COX	Cyclooxygenase enzyme
δ	Diffusion layer thickness
DMF	Dimethylformamide
E	Electric field
ε	Dielectric constant
f	Activity
q	Charge
Vis	Visible
I	Ionic strength
K_a	Acidity constant

MEKC	Micellar electrokinetic chromatography
MO	Molecular orbital
μ	Mobility
η	Viscosity
π	Pi Orbital
P	Power
PG	Prostaglandine
PGI2	Prostacycline
PPA	Polyphosporic Acid
R	Reference pattern
σ	Sigma Orbital
t	Time
T	Transmittance
UV	Ultraviolet
V	Voltage
υ	Speed
w	Peak width
ξ	Zeta Potential

TABLE OF FIGURES

LIST OF TABLES

LIST OF SCHEMES

1. INTRODUCTION

Analgesic drugs can be devided into two main categories according to their chemical structures as non-steroidal anti-inflammatory drug (NSAID) and opioid (narcotics). Since NSAIDs do not cause drug dependency, tolerance and addiction are more preferential. When hypnotic effects of nonsteroidal anti-inflammatory drugs (non-narcotic analgesics) are explained, benzoxazolinone and its derivatives increase their importance in the medicinal chemistry (1).

Non-stereoidal anti-inflammatory drugs (NSAID) efficacy mechanisms are based on inhibition of the cyclooxygenase (COX) enzyme (2). COX is the catalyst of arachidonic acid metabolism also synthesis of prostaglandin (PG) and prostacycline (PGI2). Inhibition of prostaglandin synthesis triggers NSAID's anti-inflammatory and analgesic effects. They have two well-known isoforms (COX-1 and COX-2). COX-1 enzyme exists in human body cells which protect gastric mucosa and regulate blood flow of the kidney; on the contrary COX-2 is the base enzyme which reveals response of the pain and inflammation. Third cyclooxygenase isoform (COX-3) have been claimed to exist in recent research but its functions have not been clearly identified (3). NSAIDs are frequently used to treat inflammatory conditions such as arthritis, bursitis and tendonitis (4). Frequent uses of these drugs occur to gastric ulcer and cardiovascular diseases. Nowadays, COX-2 selective drugs development are more interest in pharmaceutical industry.

The first studies on 2-(3H)-benzoxazolinone derivatives started with the synthesis of 2-(3H)-benzoxazolinone from o-hydroxy phenylurethane (5). Chemical and biological properties of benzoxazolinone derivatives were determined. These derivatives possess hypnotic (6), analgesic (7), antirheumatic (8), muscle relaxant (9), antibacterial (10) and antifungal (11) activities.

Structural modification on 2-(3H)-benzoxazolinone derivatives (See the Figure 1), were mostly done at the 3rd, 5th and 6th positions of the molecule. These modification on benzoxazolinone derivatives increased activities. 6-acyl-2-(3H)-benzoxazolinone derivatives showed analgesic activities to be higher than 2-(3H)-benzoxazolinone ring and aspirin. (12-15).

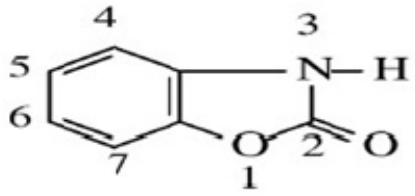


Figure 1. 2-(3H)-benzoxazolinone

Lespagnol and his co-workers were the first to indicate that compounds of the benzoxazolinone may affect on the central nervous system. 3, 5 and 6 substitue showed varying biological actitivites such as hypnotic, analgesic and anti-inflammatory (15, 16).

The muscle relaxant effect of 2-(3H)-benzoxazolinone and its derivatives was determined at the time of studying the metabolites of 2-amino-5-chlorobenzoxazole which have urisosuric and muscle relaxant effects. Amine group of this drug was observed to be the cut off the body as oxidative and transformed into 5-chloro-2-(3H)-benzoxazolinone (9).

The ionization ability of the chemical compounds is measured by the acid ionization constant (K_a), which is also called the protonation constant, equilibrium constant, or (acid) dissociation constant. pK_a (negative logarithm of the K_a) is also an important physicochemical constant of a given compound. pK_a values of some benzoxazolinone derivatives were determined and acidity constants were confirmed with three different techniques. Knowing the acidity constant of the concerning compound is essential for understanding the chemical interactions and its pharmacological effects. Celik and his co-workers investigated relationship of some 2-(3H)-benzoxazolinone derivatives between structure and activity (17).

Indicating the acidity constant of a drug molecules is quite significant parameter for the understanding of the chemical interaction between the concerning compounds and their pharmacological effects. The relationship between acidity constant (K_a) and the compound is used in the studies of new drug synthesis and in the description of the biopharmaceutic properties of these substances (17). Many biological active molecules can be ionized in physiologic pH partially or wholly, and the existence of groups that can ionize is known to be quite significant regarding biological activity and/or solubility. This requires that

appropriate techniques are used in finding the acidity constants (K_a) the newly generated molecules.

The main purpose of this study is to determine the pK_a values of fourteen structurally related 3-(2/4-pyridylethyl) benzoxazolinone derivatives 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 (17). Furthermore, the relationship between the acidity constant and analgesic/anti-inflammatory activities of the drug candidates will be discussed.

2. GENERAL INFORMATION

2.1 General Structure of Benzoxazolinone

2-(3H)-benzoxazolinone compound is composed of benzene and 2-oxazolone rings. Classical numbering of the molecule is shown in Figure 1.

2.2 Synthesis Methods

2-(3H)-benzoxazolinone was first produced by Groenvik with the dry distillation of ethyl-N-(o-hydroxyphenyl) urethane in 1876 (18) and then some researchers synthesized 2-(3H)-benzoxazolinone with o-hydroxyphenyluretane to try different reactives such as sodium hydroxide, metallic sodium or sodium ethoxide (19).

The most frequently used technique in the synthesizing of 2-(3H)-benzoxazolinone is to react o-aminophenolune with urea or phosgene. The following years, yield was increased up to 76-96% from 35% to regulate reaction time (20, 21) and degree. 2-(3H)-benzoxazolinone was also synthesized o-nitrophenol and salicylic acid derivatives. Different techniques are improved for increasing yield (22-24).

2.3 Chemical Properties

2-(3H)-benzoxazolinone is a heterocyclic ring formed by the combination of five-member 2-oxazolon ring containing an oxygen and nitrogen atom, with benzene. It is a white compound and its melting point is 139-140 °C; and is reported to be non-ionized in pH < 5 and ionized in pH >11.5 in aqueous solutions.

3-non substitiued 2-(3H)-benzoxazolinones derivatives were reported to be in lactam-lactim balance due to the free hydrogen in nitrogen atom. In the first studies, 2-(3H)-benzoxazolinones were claimed to be found in two tautomeric structure as 2-benzoxazolinone (A) and 2-benzoxazolinone (B) (25)

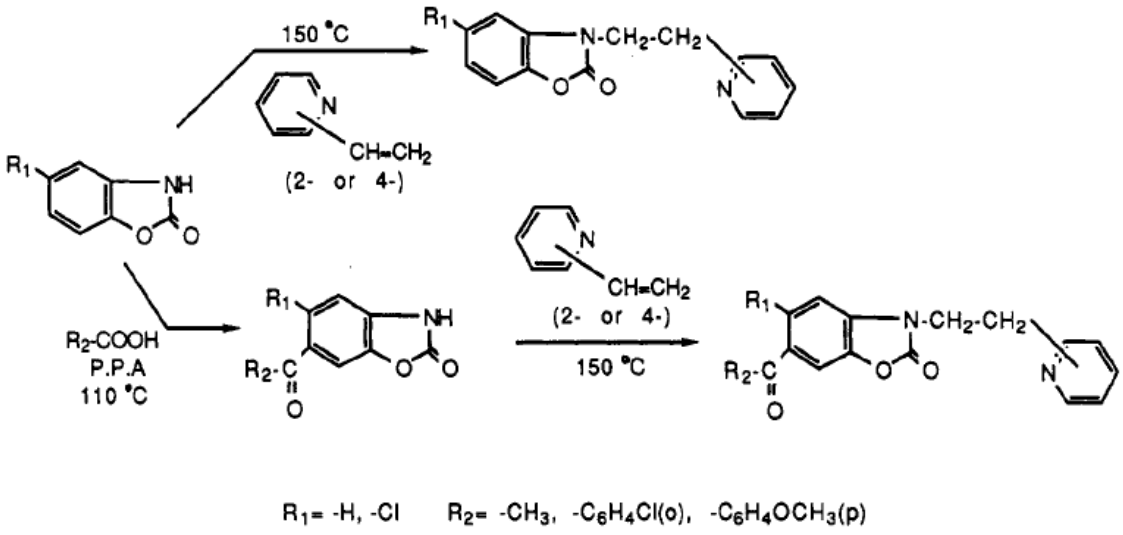
Figure 2. 2-Benzoxazolinone tautomeric structure

In subsequent studies, different 2-(3H)-benzoxazolinone derivatives of the ring were react with diazomethane in order to detect the lactam and lactim forms of 2-benzoxazolinone, and only N-methyl derivatives were reported to be formed (26). 2-(3H)-benzoxazolinone molecule was reported to show a special relationship towards classical electrophilic substitution reaction.

Three methods were reported for the preparation of 6-acyl-2-benzoxazolinones. In the first method; polyphosphoric acid (PPA) was used as the solvent and catalyst, and carboxylic acids were used as the reactive (10, 14). In the second method, acid halogenure or anhydrides (Friedel-Crafts reaction) were used in the presence of aluminum trichloride ($AlCl_3$) - dimethylformamide (DMF) (27). An alternative way in the preparation of 6-acyl-2-benzoxazolinones was formed through the application of Chattaway to 2-benzoxazolinones. 3-acyl-2-benzoxazolinones were prepared with the reaction of acid anhydrides or acyl halogenures with 2-benzoxazolinone in pyrite. When these were heated in PPA at 110-130 °C for 1.5-3.5 hours 6-acyly-2-benzoxazolinone derivatives were gained through acyl migration (28). Different researchers obtained a similar reaction when they put N-acyl derivatives into reaction with acid anhydrides or acid chlorides in tetrahydrofurane (THF) along with the presence of triethylamine (TEA). When these were heated in the presence of aluminum chloride at 165 °C for three hours, 6-acyl-benzoxazolinones were gained with high efficiency (29).

Researchers were synthesized some benzoxazolinone derivatives which were studied in this project (30). Fourteen 3-(2/4-pyridylethyl) benzoxazolinone derivatives

were synthesized (Scheme 1) and evaluated for their analgesic and ulcerogenic activities (30).



Scheme 1. Synthesis of 3-(2/4-pyridylethyl) benzoxazolinone derivatives

They have proved high analgesic activities of some benzoxazolinone derivatives. Benzoxazolinone and chlorzoxazone were acylated in polyphosphoric acid with appropriate carboxylic acid and then treated with 2- and/or 4-vinylpyridine. To 10 mmol of 6-acyl-2-benzoxazolinone (2-benzothiazolinone) was added 8 mL of 2/4-vinylpyridine, and the reaction mixture was heated under reflux in an oil bath until molten and then for a further 2 hours at 80 ˚C. By adding a cold ethanol-water mixture, the product was separated, and the resulting precipitate was collected by filtration. The crude product was recrystallized from different solvents. They have found six potent anti-inflammatory derivatives which have inhibiting prostaglandin E_2 synthesis and ulcerogenic activitiy (30, 31).

2.4 Acid-Base Chemistry

Ionization constant is a term used for the measurement of the power of acids and bases (32). All acids and bases can be distinguished in this way, and their differences can be assessed. This also allows comparisons and qualitative evaluations.

Organic compounds can contain acidic or basic groups, which assign the chemical, physical and biological properties of the compound. In such compounds, the ratio of molecular, anionic and cationic types to each other can be calculated by using pK_a values. Moreover, distinguishing substances that are too much alike chemically can be made with the help of pK_a.

The most useful description of acid and base ionization was made by Bronsted-Lowry. According to this description, acid is a substance that can give proton, and base is a substance that can take proton (33). Acid ionization constants are expressed as follows:

$$HA \rightleftharpoons H^+ + A^- \quad [1]$$

$$K_a = (\{H^+\}\ \{A^-\}) / \{HA\} \quad [2]$$

Ionization and ionization constant for bases are expressed as follows:

$$B + H_2O \rightleftharpoons BH^+ + OH^- \quad [3]$$

$$K_b = (\{BH^+\}\ \{OH^-\} / \{B\} \quad [4]$$

The bracket { } sign shows the activity of each ionic type. These equations are known as the thermodynamic ionization constant for any given temperature, and are independent from the concentration as the terms are of the activity type. The term concentration is more frequently used than the term activity while measuring the ionization constants. Ionization constant for acids and bases with respect to concentration is expressed as follows:

$$K_a = ([H^+]\ [A^-]) / [HA] \quad [5]$$

$$K_b = ([BH^+]\ [OH^-] / [B] \quad [6]$$

General equations are as expressed below:

$$pK_a = pH + \log\frac{[A^-]}{[HA]} \quad [7]$$

$$pK_b - pOH + \log\frac{[BH^+]}{[B]} \quad [8]$$

The square brackets in these expressions are used as the concentration of the ionic type. There is a **" *a*= f × C "** relation between the activity of the ions of a substance solved in a solution and concentration. The ***a*** in the equation refers to the activity of ion, **C** refers to concentration and **f** refers to the activity coefficient, which for an ion with Z charge is given through Debye-Hückel equation for dilute solutions (34).

$$-\log f_X = \frac{0.51 \times Z_X^2 \times \sqrt{\mu}}{1 + (3.3 \times \alpha_X \sqrt{\mu})} \quad [9]$$

Here μ refers to ionic strength and given as $\mu = \frac{1}{2}([A]Z_A^2 + [B]Z_B^2 + [C]Z_C^2 + ...)$. In order to use Formula 7 easier, constants must be assigned in solutions not more concentrated than 0.01 M; and only the ions with same values must be used.

Activity of a neutral molecule does not change significantly due to a change that may appear in the concentration due to any dilution. It is an easier and a better way to measure the H^+ ion activity (***a***, thus pH) instead of H^+ ion concentration (35).

Non electrolyte substances do not increase the electrical transmittance of water when dissolved in water. They reduce the freezing point of water in proportion with the amount of dissolution. Benzene, ether and chloroform are among such substances. On the other hand; acids, bases and salts increase the electrical transmittance of water when dissolved in water. These are called electrolyte substances (36). As opposed to salts, many acids and bases do not get totally ionized in solution. Strong acids and bases get ionized totally between 0-14 pH. Weak acids and bases get partially ionized (34).

In any given pH with the help of ionization constants, concentrations of different types of ions formed by the ionization of a substance can be calculated. Different types of ions have different types of UV-Vis spectrums. Significant spectroscopic studies can be made merely with this information. Ionic types of a given substance are different from each other regarding chemical, biological and physical properties. Ionization constants

define the pH range in which a substance is least ionized, and show the best conditions maximum product can be gained. This is quite significant for the scale-up chemistry (32).

Ionization constants are small and impractical numbers. Therefore, it is more practical to use the negative logarithms of acid constants (pK_a). The sum of the negative logarithms of the multiplication of water's ions is, 14.00. This is expressed as follows:

$$pK_a + pK_b = 14.00 \qquad [10]$$

Expression of pK_b values used for bases in terms of pK_a can be found easily through the way shown in the equation above. Thus, pK_a values are used as an appropriate way for the comparison of the strength of acids and bases (36). The stronger the acid, the pK_a value is lower; the stronger the base, the pK_a is higher. Most chemical reactions happen in aqueous solutions. Water and likewise solvents affect the ionization of a given compound. Also, the temperature must be specified with ionization constant because it can alter the value of the ionization constant.

2.5 Ionization Constant Assignment Methods

Three main methods are used in the assignment of ionization constants (K_a). These are;

1. Ultraviolet- Visible Region Spectroscopy (UV-Vis)
2. Potentiometry
3. Capillary Zone Electrophorese (CZE)

UV-Vis spectroscopy method is suitable for the ultra-high and ultra-low pH range that are out of the operations limits for the glass electrode and especially in the assignment of slightly soluble compounds' ionization constants. This method is only used for substances absorbing the visible light and UV.

Potentiometric titration method is used as one of the best methods to assign the ionization constant. While it takes 30-40 minutes to assign an ionization constant using the potentiometric method, UV-Vis spectroscopy method generally lasts a whole day. In potentiometric method, H^+ ions not bound by the sample are measured. In spectroscopic method, the spectral shift at the time H^+ ions are kept by the sample is measured.

Capillary zone electrophorese method is another method used for the ionization constant, and is widely used as it has a high capability of separation in rather fast and small volume samples.

2.5.1 Ultraviolet – Visible Region (UV-Vis) Spectroscopy Method

Spectroscopic analysis for organic substances occurs with the measurement of the frequency and intensity of the absorbed radiation. Devices used to obtain absorption spectra are called spectrophotometer or spectrometer.

It has two types as single or double light beam. In single light beam spectrophotometers, zero transmittance adjustment is made by closing the beam path to the same wave length solvent, and 100% transmittance adjustment is made by opening beam path. Absorbance of the solution containing analyte at the same wave length is absorbed. In devices with double beam paths, instead of making separate 0 and 100 adjustments for each wave length, the light coming out of the monochromator is divided into two beams at the same frequency. One of them is sent to the sample to be measured and the other is sent to the pot containing the solvent, thus, measurement time is shortened. In this way, transmittance value of the sample is continuously compared with the solvents.

When a molecule is exposed to electromagnetic radiation, if the energy of the radiation is equal to the electronic energy level of the molecule, this radiation is absorbed by the molecule, and electrons go up to the next level. Thus; UV-Vis spectroscopy is also called as spectroscopy branch studying electronic transitions among molecular orbitals [34].

Molecular absorption spectroscopy is based on the measurement of the transmittance of the solvent in a cell possessing the b beam path of the light between 160-780 nm wave lengths (T) or absorption (A). This absorption mostly arises from the stimulation of the bonding electrons in molecules, and as a result, molecular absorption spectroscopy is used in the identification of the functional groups in a molecule and in the qualitative assignment of the compounds carrying functional groups.

Molecular orbitals (MO) consist of atomic orbitals (AO). Two molecular orbitals are formed by the linear-combination of two atomic orbitals. As molecular orbitals occur at a lower energy level than atomic orbitals, formation of molecule is preferred and molecules

are formed. The molecular energy level which has a low energy is called as the ground state.

Electrons can be present at two levels as the bond and (the orbital in which the electron is present before taking energy) and opposite-bond (the high energized orbital where the electron is present for a moment after taking energy). Sigma orbitals are formed when their AO overlep "point to point", i.e., with a single lop overlap. Charge intensity of the sigma orbital is symmetrical around the bond axis. It usually occurs between σ-σ, σ-σ, π- π, or σ-π orbitals in organic molecules. Pi (π) orbitals overlap when their π two AO overlap "side to side". These orbitals have a nodal plane in the direction of the bond axis. Their charge intensity is found below and above the bond axis. In non-bonding (n) orbitals, when s orbital overlaps with the two lobes of p orbital, total overlap is zero. Energy level of the non-bonding orbital is mostly between the energy levels of bond and opposite-bond orbitals. As shown in the Figure 3, four types of electronic transition occur in organic molecules: σ→ σ*, n→ σ*, n→π*, π→π*.

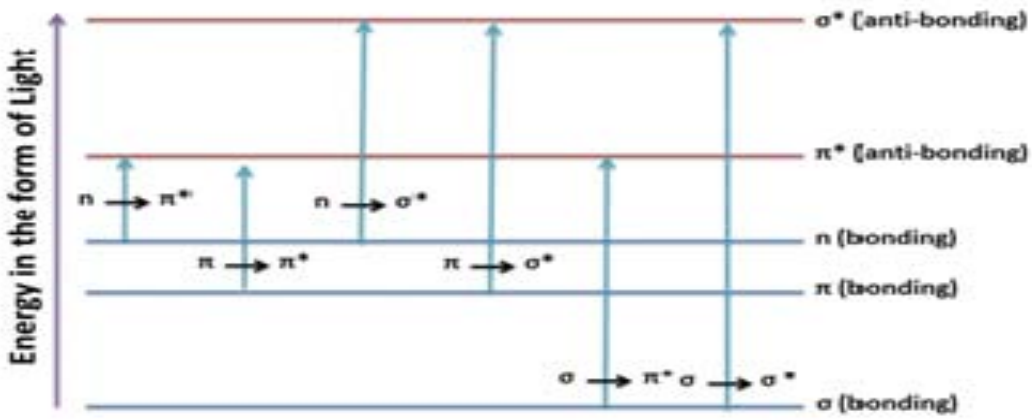


Figure 3. Electronic transitions in species

σ→σ* transitions: These are transitions that require maximum energy. The value must be λ<190 nm. They are seen in saturated hydrocarbons.

n→σ* transitions: In saturated compounds containing heteroatom, there are σ electrons

and non-partnered n electrons. They switch to σ* opposite-bond orbital. Groups such as halogens, ethers, thioether, amine, hydroxide and sulphide cause this transition. These transitions are observed in compounds containing non-partnered electron pairs (containing electron pairs found in non-bonding orbital). Most of the absorption peaks are found in the region between range 150-250 nm.

n→π* and π→π* transitions: π→π* transitions require little energy and is seen in hydrocarbons. These transitions occur around 200 nm. n→π* transitions are formed when free electron pairs on heteroatom in compounds carrying C=O, C=S, C=N groups switch to π* orbitals. These are transitions requiring minimum energy, and is around 250-300 nm. As they make absorption in the region within 200-700 nm, they are the most frequently seen transitions in UV-Vis spectroscopy. Both these transitions are observed in organic compounds containing non-saturated functional groups as they progress to π* orbitals. These non-saturated absorbing centrers are called chromophore. Whereas the molar absorptivity of n→π* transitions are mostly low (10-100), values belonging to π→π* are higher (1000-100000). Another characteristic difference between these two absorption types is the effect of the solvent on the wave length of peaks. In molecule orbital approach, delocalization of π electrons increases through conjugation. Thus; energy of π* orbitals gets reduced and has less opposite-bond character. In organic compounds, it is difficult to study transitions absorbing beams below 185 nm wave length, because vacuum goes to UV region.

UV-Vis Spectroscopy is used in qualitative and quantitative analysis. Quartz (silica) cells are generally used for analysis. In order for the best sensitive measurement in double beam path spectrophotometers, a pair of cells must be totally identical, some must be filled with pure solvent, the other with sample solution, and must be put into the places of the reference and sample. A solvent to be used for ultraviolet analysis must not make absorption in the same region with the compound whose spectrum will be taken. If the difference spectrum deviates from the line present in times when the places for the sample and reference are empty, cells become non coherent. Thus; cells must be calibrated for once, and the same cell must be used for the sample every time. Optic surfaces of the cells used must be well preserved. Touch of hand must be avoided, papers designed specifically for the cleaning of lens must be used, and paper towel must not be used. Cells must be filled and emptied with the help of a pipette; the content must not be poured from the cell.

Once used, cells must be rinsed with water or ethanol. Cells must not be used if they have burrs and scratches on their surfaces.

Assignment of ionization constant with UV-VIS Spectroscopy is based on the fact that types in balance make absorption in quite different wave lengths (38).

Organic acids and bases give absorption spectrums according to the pH value of the environment. As HA is an organic acid, the balance and balance equality is given in Formula 5.

In terms of concentration, the equation formed when negative logarithms of both are taken is like the one given in Formula 7. In this equation, the value of pK_a can be measured if pH, [HA] and $[A^-]$ are known. This measurement can also be made without assigning the three unknowns separately. In the equation given in Formula 7, when $[HA] = [A^-]$, pK_a = pH. Therefore, the value of the acidity constant (K_a) can be found using the change of absorption with pH. Spectra formed by any compound according to pH can be obtained, and pH-absorption chart can be drawn with the help of these spectra (Figure 4).

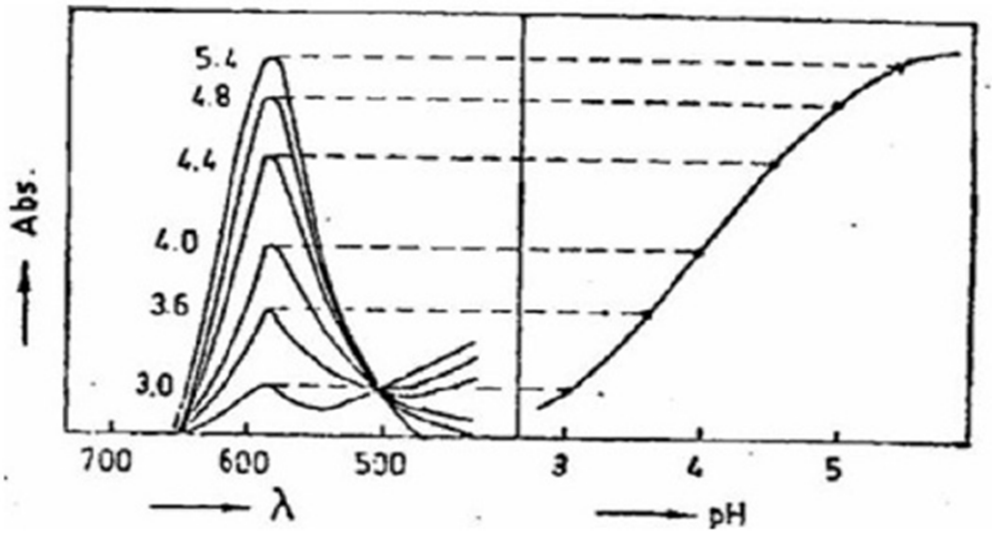


Figure 4. Different pH values spectrums of sample and creating pH – Abs. graphic in these spectrum.

pH-absorption chart given in Figure 4 is in sigmoid wave shape (S shape), and [HA] = $[A^-]$ at the middle point of this chart. The lowest and highest absorptions of the chart

must be determined to find the middle point. This point can be understood from the condition that, absorption with pH does not change any more. A parallel is drawn to the pH axis from the middle point, and then, a perpendicular is drawn from the point where this line cuts the sigmoid curve to the pH axis. The value showed by the perpendicular on pH axis is equal to pK_a. $[HA] = [A^-]$ in the middle of the absorption and $\log [HA] / [A^-] = 0$. (35)

In another method used in pK_a assignment with spectrophotometric method, $\log [(A-A_{min}) / (A_{max}-A)]$ values on a chosen wave length are put into chart against the corresponding pH values. Thus, pK_a value is obtained directly with the place where the curve obtained by this chart cuts the abscissa axis (39).

2.5.2 Potentiometric Method

Potentiometry gives information about the electrochemical change in the solution based on the potential measurement, when there is no current (there is a high resistance) in the electrochemical cell formed by a comparative electrode (for instance, glass electrode) and a suitable reference electrode (for instance, hydrogen or Ag/AgCl electrode). Potentiometric titration is a system which depends on the potential or pH measurement after the addition of each reactive. It can be carried out in different ways depending on the working environment. If the working area is totally watery, pH or the potential of a formed battery are measured after the addition of each reactive. If the area does not include any water or only includes little water, it is advised to measure just the potential difference, as pH measurements give wrong results in non-watery environments, especially in the alkali region.

As a principle in potentiometric titrations; reactive is added abundantly at first to the solution stirred well with a mechanic stirrer, then addition of reactive is reduced towards the turning point. It can be understood from the amount of change in the potential or pH measured after each reactive addition that, turning point is close.

To find the exact turning point, titration must be kept on for a while beyond the turning point. Reactive is added slowly especially when close to the turning point. Measurements must be repeated for a few times. If the mL values chart is drawn for the

reactive to which the potential differences or pH values are added after each reactive addition, an S shape titration curve is obtained. pK_a or pK_b value of the analyzed substance is determined through this chart.

Glass electrode is among the electrodes used to assign the concentration of a hydrogen ion found in an environment. Hydrogen ion activity and pH can be best assigned by glass electrode. Its various advantages make this electrode useful. Glass electrodes can be easily supplied and used for a long time. With such electrode pH values of strong oxidants, strong reducers, gases (such as H_2S, AsH_3) and proteins can be measured. Even the pH of an environment in gel state can be measured by this method. With the latest micro glass electrodes, it is possible to measure the pH of a solution in drip state. Below are the main issues to be considered when using a glass electrode:

1. The part which is submerged into water for measurement must not be touched by hand. Electrode must be washed by the water.
2. Glass electrode must not be submerged into concentrated sulfiric, nitric acid, and concentrated alkalis. Absolute alcohols and water attractive substances must also be avoided.
3. Glass electrode must not be submerged into solutions above pH 12.
4. pH values of acid solution with a concentration above 0.1 M cannot be measured. Because the figure obtained appears higher than the actual value (acidic error).
5. As the pH meter is adjusted frequently for buffer solutions, buffer solution must be well preserved.
6. Measurement of the pH values of solutions close to neutral must be made very carefully, as well. Because balance is obtained rather late in such solutions.
7. Glass electrode must not be left open to dry. Before using a dry electrode, it must be submerged into pure water for a long time and the water must be replaced from time to time. Electrode must be kept in 3M KCl environment when not in use.

The ion selective electrode used in acid-base reactions is the glass electrode. In neutralization titrations, a great change occurs in pH value suddenly at equivalence point. When the strength of acid or base decreases, i.e., values of pK_a or pK_b increase, the amount

and sharpness of pH change observed at turning points gets reduced. The same state is observed when the concentration of the titrant used or a weak acid is titrated by a weak base instead of a strong base. In neutralization titrations, analysis can be made when concentration is 3×10^{-4} M or above for strong acids, and when the multiplication of concentration and acidity constant is 10^{-7} or above for weak acids. The procedure below is used to find out the turning point (equivalence) on titration curve:

1. The closest curve is drawn according to experimental points.
2. The steepest tangent of the curve is drawn.
3. Ordinates of the beginning and end points of the tangent are drawn.
4. Ordinate of the last point is drawn between the middle points of two ordinates. The point where the last ordinate cuts the curve is the turning point.

2.5.3 Capillary Electrophorese (CE)

A capillary electrophorese system (CE) consists of a high voltage power source, inlet and outlet vials, capillary, a detector and integrator or a computer. There is thermostat in many systems in order to cool the heat in capillary and check capillary temperature. Schematic illustration of a CE system is given in Figure 5.

Basic steps of a CE analysis includes; the pre washing of capillary, filling the capillary and inlet and outlet vials with working buffer, conditioning capillary with buffer, injecting the sample to capillary, and applying electrical field. Substances found in the sample migrate inside capillary. They are observed while passing through the detector, data is sent to the integrator or computer. Data is obtained in electropherograms which is the chart of the detector's reply versus against time.

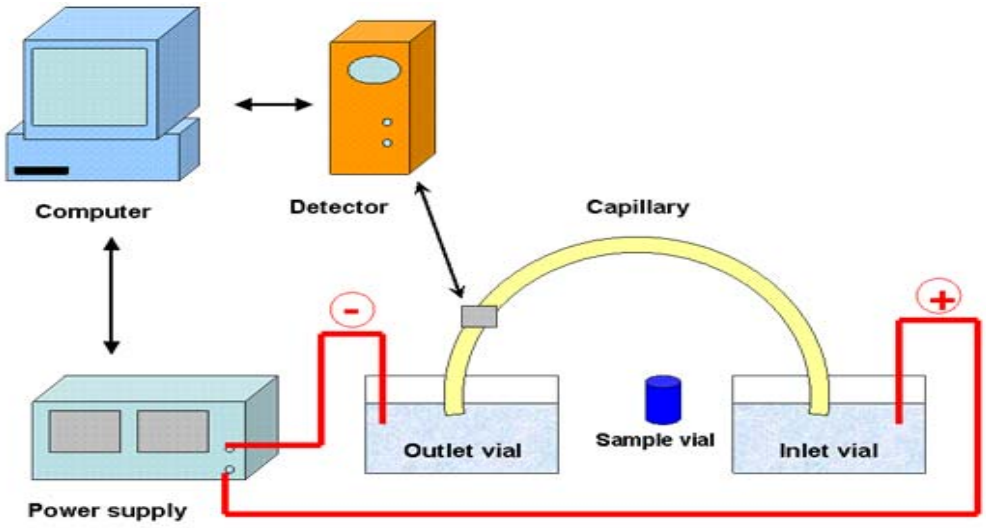


Figure 5. Capillary electrophoresis machine schematic illustration

Sample injection inside capillary is performed as hydrodynamic or electro-kinetic. Hydrodynamic injection is the most frequently used method, and can be performed in three different ways. The first one is the application of pressure to the sample vial placed at the inlet section of the capillary. A pressure around 50 mbars is generally applied for 1-5 seconds. Then, sample vial is removed, working buffer vial is placed and analysis continues by applying voltage. The second way for hydrodynamic injection is the application of vacuum to the buffer vial placed at the outlet section when the sample vial is placed at the inlet section of the capillary. When the desired amount of sample enters into the capillary by vacuum, vacuum is stopped, sample vial is removed and working buffer vial is placed, analysis continues by applying voltage. The siphon effect is used in the third hydrodynamic injection method. The sample vial placed to the inlet section of the capillary is placed to a higher location than the buffer vial at the outlet section. Sample enters into the capillary with gravity. Then, the sample vial is removed, working buffer vial is placed, and analysis continues by applying voltage (Figure 6).

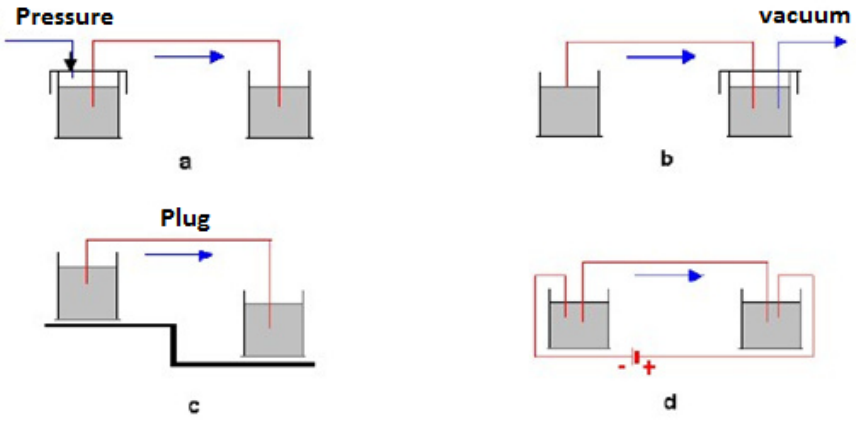


Figure 6. Injection systems in capillary electrophoresis : (a, b, c) Hydrodynamic injection (d) Electrokinetic injection

When the sample vial is placed to the inlet section of the capillary is electro-kinetic injection, voltage is applied to the place between the sample vial and the working buffer vial at the outlet section. Ions in the sample start emigrating into capillary with the effect of the electrical field formed. After the application of this voltage, sample vial is removed and buffer vial is placed, analysis continues by applying voltage (Figure 6d).

The type and size of the capillary to be used in electrophoresis is elected according to the method to be used, sample to be analyzed, desired selectivity and appropriate analysis time. Capillary is expected to perform sufficient selection in a short analysis time. The material from which capillary is produced must have no electrical conductivity, must be chemically inert and be in harmony with the detector (for instance for UV detector, capillary must not absorb the beams).

Melted silica is the most frequently used capillary material today. As it breaks easily, its outer surface is strengthened by polyimide. If UV detector is to be used, polyimide covering is removed not to block the beam path (Figure 7).

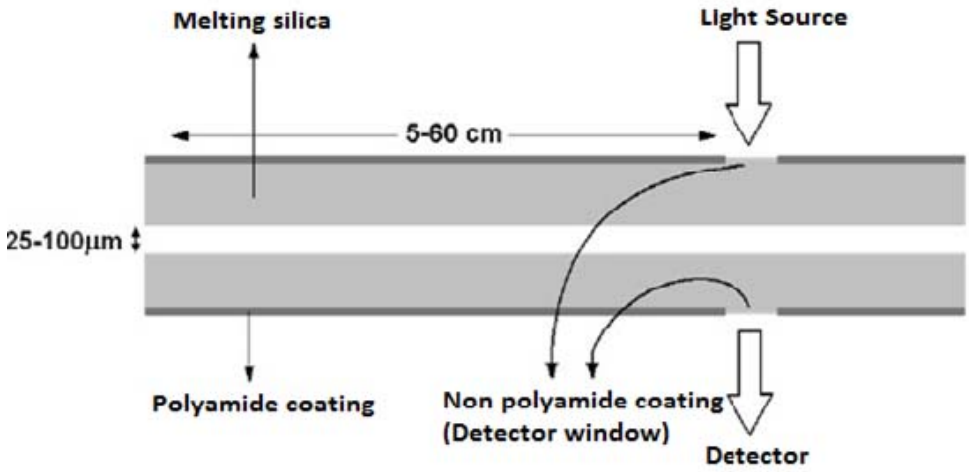


Figure 7. Longitudinal section of melting silica capillary

Though Pyrex capillary is stronger than melted silica capillary and does not require the opening of a detector window, there is one thing that, it cannot be operated below 280 nm. Homogenous inner diameter cannot be obtained in Teflon capillary, and high voltage cannot be used due to low heat conductivity. Thus; melted silica is a capillary substance that is more frequently used than Pyrex and Teflon. Melted silica capillary with an inner diameter of 25-100 µm is generally used. High temperatures come out in big diameter of capillary, and the difference of temperature between the inside wall of capillary and its centre is great. When small diameter capillary is used, UV-Vis absorbance decreases or absorbance decreases in detectors such as fluorescence, due to the narrowing of beam path, and peak height is lowered. Furthermore, it is likely that, small diameter capillary are blocked by particles.

In order to get repeatable results from capillary, the conditioning process must be applied at the first use and before each analysis. State of the surface of silica is influential on the electro-osmotic flow (EOF) which is one of the two forces affecting the speed of particles in CE. With conditioning, ionization ratio of the cylanol groups at the inside wall of capillary, thus EOF happens to be the same in each analysis. If the capillary is new; first, 1 N NaOH, next, 0.1 N NaOH, water and finally the working buffer is treated and conditioned. Also 0.1 N NaOH, water and working buffer is treated before each analysis to

ensure that capillary is conditioned the same as much as possible. Water must be treated at the end of work. If no work will be done for a few days, air should be vacuumed and capillary should be left dry as microorganisms will grow inside.

It is important to check the temperature of capillary in order to get repeatable results in CE. Activity, migration time, injection volume and detector's reply change with the change in temperature. It might also cause sample degradation. Temperature of air is adjusted to the desired value using a thermostat system, and it is inflated into the capillary inside the cartridge, using a fan. Control of capillary temperature is ensured in this way.

The purpose of high voltage power source is to provide the voltage, current or power needed for electrophorese. Though many CE separations occur at a constant voltage, the power source must be able to apply a constant current and constant force. Voltage can be applied up to 30 kV, current up to 300 μA and force up to 6 W. When needed, the direction of the electric current (inlet vial cathode, outlet vial anode) can also be changed.

The most frequently used detectors in CE are; UV-Vis absorbance (direct and indirect), fluorescence (direct and indirect), fluorescence induced by laser, mass spectrometry, conductivity, amperometry (direct and indirect), radiometric and refractive index detectors.

Absorbance detectors are widely used as they can be applied to CE. Observation range is generally between 10^{-5} – 10^{-7} M range. Absorbance can be attained at a single wave length or at many waves length using a number of diode detectors. In absorbance detectors, capillary works as the detector cell itself. For this, some of the polyimide covering at the outer part of the melted silica capillary is removed through burning, resolving or scraping. However in that condition, length of the beam path cannot be above the capillary inner diameter (50 – 100 μm), and this reduces the limit of observation in terms of concentration. Using "z" or capillary possessing detector cell in bubble shape can increase sensitivity.

In capillary electrophorese, the working buffer also moves along the capillary with the effect of the electrical field applied. This is called as electroosmotic flow (EOF). Inner surface of melted silica capillary is covered by cylanol groups (SiOH) and is ionized to SiO^- above pH 3.0. Capillary is tried to be treated by NaOH or KOH. The cause for electroosmotic flow is that, electrical double layer is formed between the working buffer and SiO^- groups inside capillary. Positively charged ions in the working buffer get close to

capillary wall. Among these, the ones that are too close to the wall are immovable.

However, as they drift away, the electrostatic pull force between the wall and the positively charged ions gets weak; the force of the electrical field on ions become predominant, and these positively charged ions emigrates to the negatively charged electrode (cathode) all together. This mass migration causes the buffer in the capillary to slow towards the cathode. This flow is called EOF (Figure 8).

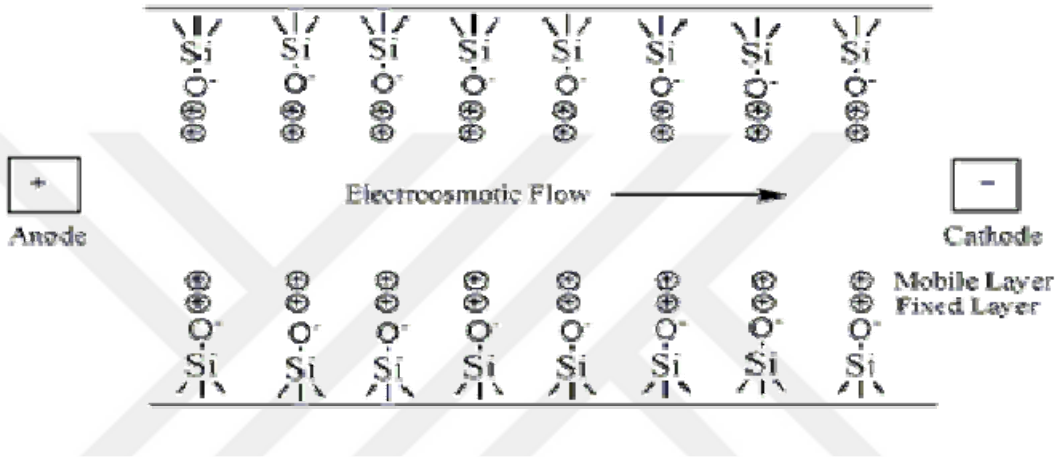


Figure 8. Illustration of electroosmotic flow in capillary

Electromagnetic flow speed (υ_{EOF}), is explained according to Formula 11.

$$\upsilon_{EOF} = E \cdot \mu_{EOF} \qquad [11]$$

In this formula; υ_{EOF} refers to the electroosmotic flow speed (cm s^{-1}), E: Electrical field (V cm^{-1}), μ_{EOF} :Electroosmotic mobility (cm^2 V^{-1} s^{-1}). The double layer formed between the immobile layer close to capillary wall and the mobile layer close to the center is called zeta potential (ξ) , and is shown in Formula 12.

$$\xi = 4\pi\delta e/\varepsilon \qquad [12]$$

δ:refers to the thickness of diffusion layer, e: the charge on the surface area, ε: dielectric constant of the buffer. Electroosmotic flow speed (υ_{EOF}) can be shown with

Formula 13.

$$\upsilon_{EOF} = \varepsilon \, \xi \, E / 4 \, \pi \, \eta \qquad [13]$$

η refers to the viscosity of the buffer.

As the electrical field (voltage/length) depends on the voltage, EOF can be easily controlled by voltage (Formula 11). EOF increases with the increasing voltage, and time for migration gets shortened. Furthermore, voltage increase allows high efficiency.

However; high voltage and thus the high flow also cause capillary to heat (Joule heating) (Formula 14).

$$P = V \times I \qquad [14]$$

In the formula P refers to Power, V: Voltage and I: Current. If the heating in capillary is not cooled soon, capillary heat increases. This increase might cause widened peak, non-repeated migration time, sample degradation or content change or prevention of electrical conductivity by causing buffer fusion. Thus; narrow peak and the voltage needed for a short analysis time (maximum voltage) must be chosen. Maximum applicable voltage can be chosen from voltage flow chart (Ohm rule chart – Figure 9).

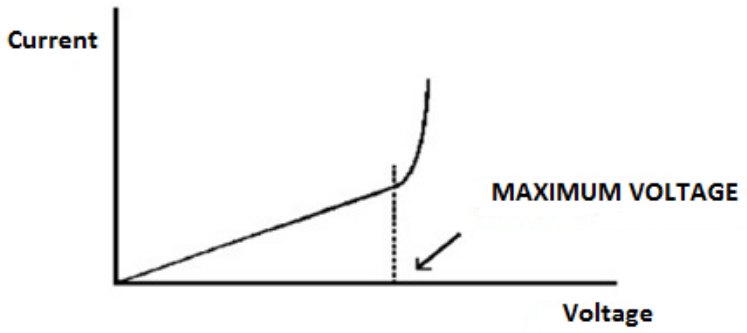


Figure 9. Voltage – current graphic (Ohm Principle)

Electrical field can be used instead of voltage in this chart. In the chart, the last voltage before the disfiguration of linearity gives maximum voltage value. Maximum voltage depends on the buffer compound, pH and concentration, and also the inner diameter and length of capillary.

Another parameter which has a significant effect on EOF is buffer pH. When pH increases, EOF increases, too. Because in high pHs, ionization of SiOH to SiO^- is abundant in capillary inner wall. As seen in Formula 12, the increase in the surface charge of capillary wall cause an increase in zeta potential. Thus; pH increase causes overcharged SiO^- group, and this causes high zeta potential and increase in electroosmotic flow speed (Formula 13).

As the increase in the ionic power or concentration of the buffer will decrease zeta potential, it decreases EOF, as well. Low buffer concentration gives a short analysis time, yet, too low buffer concentration should be avoided. If buffer concentration is not more than sample concentration, there might be some differences in the conductivity causing disruption in the electrical field between the sample and buffer. This disruption causes peak widening or peak tailing. As a general rule, concentration of the buffer must be minimum 100 times more of the sample concentration. Increase in temperature decreases buffer viscosity, and thus increases EOF. Temperature must always be under control. Because overheating might cause sample degradation, zone widening, buffer joining or non-repeatable analysis time.

Addition of organic solvent to the buffer might be influential on viscosity, dielectric constant, zeta potential, the effect of organic solvent changes according to the type and amount of it. When 0-50% methanol is added, viscosity of solution increases. When more than 50% methanol is added, viscosity decreases. On the other hand; when acetonitrile is added up to 100%, viscosity of solution decreases.

EOF can be reduced or prevented if capillary surface is covered by a material (polyacrylamide or methylcellulose) preventing the ionization of cylanol groups. It can also be reversed by applying EOF counter phase viscosity or adding various materials to the buffer.

The easiest way to measure EOF is to inject an uncharged compound to the system. For this, the compound chosen must be pure, non-interacting with the capillary wall, uncharged and can be dissolved in buffer pH and can be identified with the detector used

(such as methanol, mesityle oxide, formamide, phenol).

Under electrical field, electric charged materials in the buffer move in electrophoretic speed. Electrophoretic speed (υ_{EOF}) is measured according to Formula 15.

$$\upsilon_{EP} = E\ \mu_{EP} \qquad [15]$$

In the formula, υ_{EP} refers to electrophoretic speed, and μ_{EP} is electrophoretic mobility. Separation occurs as the materials move in different speeds along the capillary. Electrophoretic mobility is given according to Formula 16.

$$\mu_{EP} = q / 6\ \pi\ \eta\rho \qquad [16]$$

In the above formula; q refers to the charge of the ionized material, and r: radius of the sample.

Inside the capillary small and overcharged molecules move fast, big and less charged molecules move slowly. As the charge of neutral molecules is zero, their electrophoretic mobility is zero, too. Increase in buffer viscosity decreases electrophoretic mobility as well as EOF.

Speed of materials is bound to the electrophoretic speed and electroosmotic flow speed. The observed electrophoretic speed (υ_{obs}) is given according to Formula 17.

$$\upsilon_{obs} = \upsilon_{EP} + \upsilon_{EOF} \qquad [17]$$

In normal CE, where the detector part of the capillary is negatively charged and EOF is towards the detector, the observed speed of anions is lower than the electroosmotic speed as they have electrophoretic mobility in the reverse direction of EOF (υ_{obs} anion < υ_{EOF}). In cations, the observed speed is higher than the electroosmotic speed (υ_{obs} cation > υ_{EOF}). Because their mobility is in the same direction as the EOF, neutrals only move with EOF inside the capillary and thus, the observed speed is equal to EOF (υ_{obs} neutral < υ_{EOF}) (Figure 10).

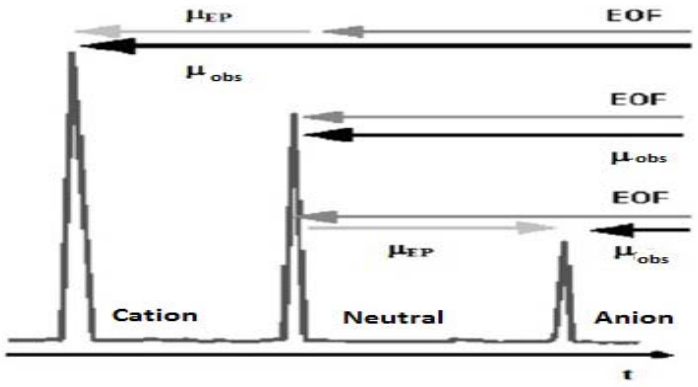


Figure 10. Effect of electrophoretic and electroosmotic speeds on migration time. (EOF:electroosmotic flow, μ_{EP}: electrophoretic mobility, μ_{obs}: observed mobility, t: time).

$$\mu_{obs} = l / t_m \qquad [18]$$

In the formula; l refers to the active capillary length (the length from the point where the samples enters into capillary to the detector), and t_m is migration time of the material. Electrophoretic separation parameters are time, activity and selectivity. Migration time of materials in active capillary length (t_m) is measured according to Formula 19.

$$t_m = l / (\mu_{EP} + \mu_{EOF})\ V \qquad [19]$$

As seen in Formula 3.19, short analysis time is attained with high voltage, short capillary and high EOF. Activity is determined according to the number of theoretical layers (N) and can be estimated by measuring the migration time and peak width (Formula 20).

$$N = 16\ (t_m / w)^2 \qquad [20]$$

In the formula; w refers to peak width at the ground line. High activity is obtained with narrow peak and long analysis time according to Formula 20. Selectivity (α) shows the distance between two peaks while passing through the detector (Formula 21).

$$\alpha = (t_2 - t_0) / (t_1 - t_0) \qquad [21]$$

t_1 refers to the migration time of the preceding material, t_2 is migration time of the following material, t_0 is migration time of the material only moved by electroosmotic flow. The most efficient way of change in selectivity is to make change in buffer pH. Selectivity

(R_s), which is the most significant separation parameter, can be measured with Formula 22.

$$R_s = 2\,(t_2 - t_1) / (w_1 + w_2) \qquad [22]$$

In the formula; w_1 and w_2 refer to peak width of neighbor peaks at the ground line. Selectivity can be improved by increasing the voltage applied, changing buffer pH and compound, increasing capillary length, and optimizing EOF.

Types of capillary electrophoresis can be categorized as the following:

1) Capillary zone electrophoresis (CZE)
2) Micellar electrokinetic capillary chromatography (MEKC)
3) Capillary gel electrophoresis (CGE)
4) Capillary isoelectric focusing (CIEF)
5) Capillary isotachophoresis (CITP)

As the analysis are performed using the capillary zone electrophoresis method in this thesis study, only this method will be discussed. Capillary Zone Electrophoresis (CZE) is a widely used electrophoretic method. In CZE, separation takes place in capillary tube filled with buffer solution. Points of capillary are submerged into inlet and outlet vials containing the same buffer solution. This buffer solution is called as the work buffer. Electrodes submerged into both vials are connected to an external power source. If sample is injected to capillary filled with buffer and voltage is applied, particles move along the capillary in zones (Figure 11)

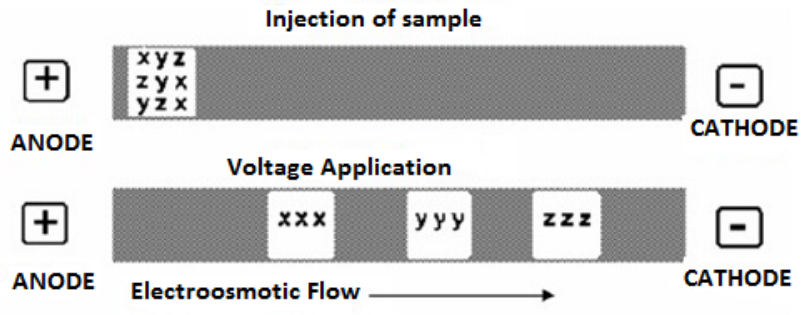


Figure 11. Seperation of sample zones in capillary zone electrophoresis

In CZE, electrophoretic and electroosmotic two forces affect the speed of particles. Cations migrate towards the negatively charged electrode (cathode), and anions migrate towards the positively charged electrode (anode). Electrophoretic migration speed of ions depends on their charge/size (charge/diameter) ratio. In ions with the same charge, the smaller one moves faster than the big one; in ions with the same size, the more charged one moves faster. There is also a movement by electroosmos inside the capillary under the electrical field. EOF moves the particles from anode to cathode. Due to electroosmotic and electrophoretic effects, order of elution in CZE is a cation, neutral and anion. As ions are separated according to their charge/size ratios, neutral compounds cannot separate from each other (Figure 12).

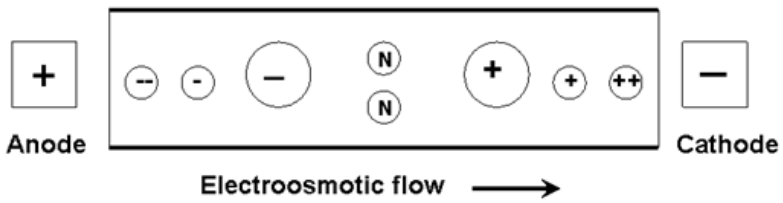


Figure 12. Separation in capillary zone electrophoresis

CZE can be used in the separation of almost any ionized compound that can be dissolved in a buffer. Small inorganic anions and cations, big molecules, and also compounds that do not dissolve in water can also be separated with CZE using non watery buffers. The time needed for a material inside capillary to reach at the detector is called as the migration time of that material. Migration time of the material (t_m) is in direct relation with the speed of the material inside capillary (μ_{obs}) and the active length of capillary (l : length to the detector) according to the Formula 18 ($\mu_{obs} = l / t_m$). Speed of material inside the capillary depends on the electrophoretic speed and electeroosmotic flow speed according to Formula 17 ($\mu_{obs} = \mu_{EP} + \mu_{EOF}$).

Acidity constant for a weak acid (HA) can be expressed as the following:

$$HA + H_2O \rightleftharpoons H_3O^+ + A^-$$

$$K_a = \frac{f_{A^-}[A^-]\,[H_3O^+]}{[HA]}$$

f_A^- refers to the activity coefficient constant for A^- ion (H_3O^+): H_3O^+ ion activity. According to Debye-Hückel equality, log f_{A^-} can be expressed as in Formula 9. Thus, f_{A^-} will be constant for solutions with same ionic strength. A number of solutions which have an ionic strength of 0.02 M, can be prepared with appropriate dilution rates for different pH values.

In weak acid types, when pH switches to basal values, $[A^-]$ / [HA] value increases based on pKa = pH – log $[A^-]$ / [HA] equality. Thus; as pH increases, values of HA concentration become trivial compared to A^- concentration. In that case, almost all HA is accepted to get ionized and transformed into A^-.

In capillary zone electrophoresis method, for weak acid type material, electrophoretic mobility reaches maximum values as the ratio of type A^- increases, and minimum values as the ratio of type HA increases. Because according to Formula 16, electrophoretic mobility is directly related to the material charge. For materials in weak acid type, electrophoretic mobility in any pH (Formula 23) can be given as follows:

$$\mu_{EP} = (\%A^-)\,\mu A^- \qquad [23]$$

If this equality is broken, it turns into the state given below (in Formula 24), and can be related to K_a (Formula 25).

$$-\mu_{EP} = \frac{[A^-]}{[HA]+[A^-]}\mu A^- \qquad [24]$$

$$\alpha = \frac{K_a}{f_{A^-}[H_3O^+]+K_a}\mu A^- \qquad [25]$$

According to equality 24 and 25, a sigmoidal curve is obtained when μ_{EP} chart is

drawn against the pH value. At the turning point value of this curve, pH = pK_a; as [HA] = [A^-] ve μ_{EP} = ½ μA^- when pH = pK_a.

If a non-ionizable neutral marker is used in basal pH values; in an environment where ionic strength, temperature and electrical field remain constant, migration time belonging to electroosmotic flow speed (t_0) for different pH values will be equal to the migration time belonging to neutral marker (t_m). If $_{obs}$= μ_{EP} + μ_{EOF} according to Formula 17, electrophoretic mobility (μ_{EP} : $cm^2\ V^{-1}\ s^{-1}$) can be expresses as the following according to Formula 26;

$$\mu_{EP} = (L / V) \times (1/t_m - 1/t_0) \qquad [26]$$

In the formula; L refers to the total length of capillary, V: application voltage, t_m: migration time of the substance, and t_0: migration time of EOF.

3. MEDIUM AND METHOD

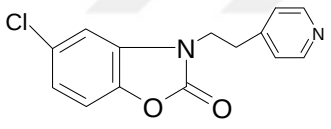
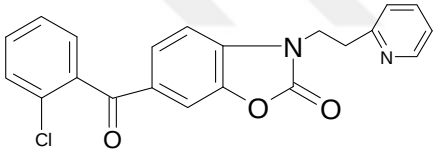
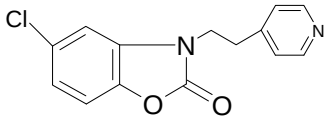
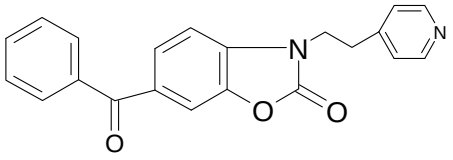
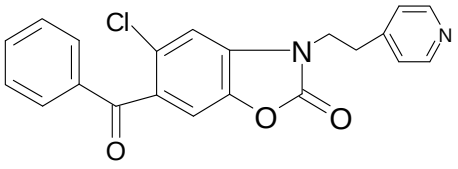
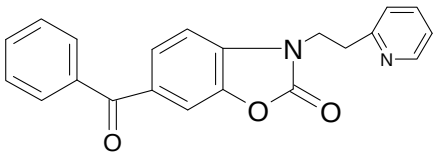
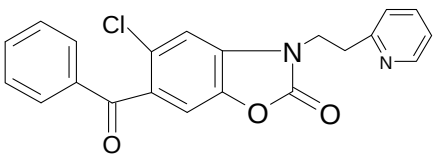
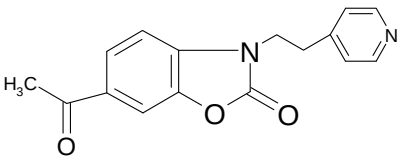
3.1 Chemical Compounds

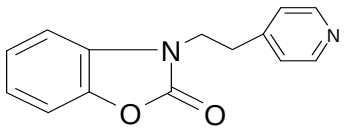
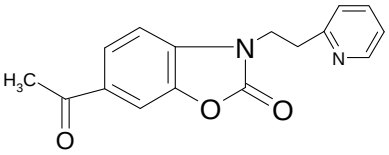
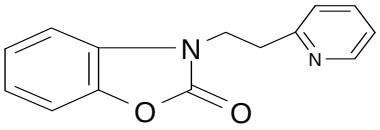
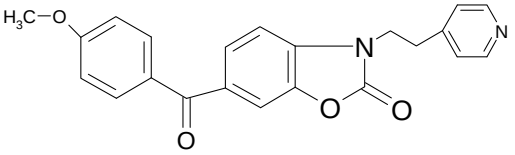
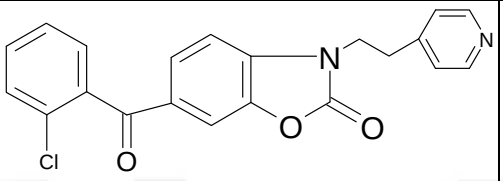
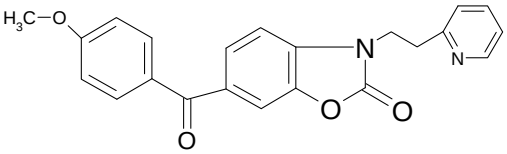
All chemical compounds used in studies are in analytic purity. All chemical materials are used without being exposed to any extra purification process.

3.1.1 Types of Benzoxazolinone Derivatives

Below (Table 1) are the open formulas of the 3-(2/4-pyridylethyl) benzoxazolinone derivatives studied with three different methods for the determination of acidity constants:

Table 1. Structural formulas of the 3-(2/4-pyridylethyl) benzoxazolinone derivatives

	Structural Formula	#	Structural Formula
1		**8**	
2		**9**	
3		**10**	
4		**11**	

5		**12**	
6		**13**	
7		**14**	

3.1.2 Chemicals Used in Analysis

Acetonitrile, methanol, acetic acid, boric acid, sodium chloride, potassium hydroxide, sodium hydroxide were obtained by SIGMA – ALDRICH and sodium acetate, phosphoric acid, disodium hydrogenphospate, sodium dihydrogenphospate, hydrochloric acid were handled by RIEDEL.

3.2 Equipment

Evolution 300 UV-Vis Spectrophotometry (Vision Pro Software Version 4.4.1 Math Version 24.00) and Agilent Technologies 8453 UV- Vis Spectrophotometry which has agilent technologies rectangular glass cell (10 mm and 3.5 mL) were used at UV- Vis Experiments. Samples were prepared in Capp autoclavable automatic pipet (100-1000 μL) and IsoLab, IsoTherm, MüllersLab volumetric flasks (10, 25, 50, 250 mL). OHAUS Analytical Plus Balances and OHAUS Explorer 5 digits balances were used for weighting samples. Agilent 3D CE capillary electrophoresis (Waldbornn, Germany) was used at Capillary experiments. Heidolph MR 3004 magnetic stirrer and heater, Oakton pH 2100 series pHmeter were used at potentiometric titration experiments. Stock solutions (pH: 4, 7, 9) were handled by Merck.

3.3 Buffer Solutions

Phosphoric acid-sodium dihydrogen phosphade buffer within pH 1.5-3.5 range was used by adjusting molarities and in suitable amounts and pHs. Acetic acid- Sodium Acetate buffer solution within pH 3.7-5.7 range was used. Molarities of acid and conjugated base were adjusted and acetate buffer solutions in needed pHs were prepared. Phosphate buffer solution within pH 5.8-7.8 range was used. Disodium hydrogenphosphate and Sodium dihydrogenephosphate ratios in suitable molarities were mixed and phosphate buffer solutions in needed pHs were prepared.

3.4 Methods

3.4.1 Ultraviolet – Visible Region (UV-Vis) Spectroscopy Method

Procedures followed in UV-VIS spectroscopy measurements are given below:

1. Concentration is adjusted and an appropriate stock solution is prepared, then diluted in tampons having suitable pH values,
2. Pure spectrum which belongs to two ion types in balance is inquired,
3. Appropriate wave length is elected for assignment (analytic wave length),
4. Value close to pK_a value is studied,
5. Real value of pK_a is assigned

In determination of acidity constants of 3-(2/4-pyridylethyl)-benzoxazolinone derivatives with UV-Vis Spectroscopy method, Evolution 300 UV-Vis Spectrophotometer device was used. In spectrophotometric measurements, benzoxazolinone derivatives were prepared as 0.01 M in 10 mL capacity volumetric flasks, were weighed in OHAUS Explorer 5 digits balance, and then dissolved in acetonitrile. Finally; the last concentration was completed to 10 mL as 1×10^{-5} with the buffer solution preferred (Buffer solutions were preserved in capped containers to prevent CO_2 absorbance and pure water was used). After waiting for the device to heat, the buffer solution was added firstly into Agilent Technologies company's 10 mm basins, and was left to measurement at room temperature (23 °C) as blind sample. Then, samples of 2-(3H)-benzoxazolinone derivatives were added into the basin for measurement. Absorbance-wave length values obtained for each sample after the measurements were transferred to computer and put into charts.

Then, the best wave length range was chosen among all pH values, and absorbance-pH charts of that wave length were made. pK_a values of each compound were determined with these spectrums obtained within 190-400 wave length range in three independent experiments.

3.4.2 Potentiometric Method

In determination of acidity constants with potentiometric method, Oakton pH 2100 Series was used. The pH-meter was calibrated using standard pH 4.00 and pH 7.00 buffers. For titrimetric analysis, Isolab basin and Heidolph MR Hei-Standard hot-plate and Teflon stirrer were used. 0.1 mL 1 M hydrochloric acid (HCl), 7.9 mL H_2O (was preserved in capped containers to prevent CO_2 absorbance), 0.154 M 1 mL potassium chloride and the sample compounds dissolved in 1 mL acetonitrile. This mixture were placed in 25 mL beaker, and stirred at room temperature using a magnetic stirrer. The amount of acetonitrile was increased for some 2-(3H)-benzoxazolinone derivatives which do not dissolve well in water. As a result of the titrations performed with 0.2 M potassium hydroxide (KOH), the pH values corresponding to the added volumes were recorded and put into charts, and then pK_a values were found.

3.4.3 Capillary Zone Electrophoresis Method

In determination of acidity constants using capillary zone electrophoresis method, Agilent 3D device was used. Photodiode detector was used at 210 wave length. When the temperature of capillary (inner diameter: 50.0 microns, total length: 48.5 cm, effective length: 40.0 cm) made of melted silica was 25°C, 20 kV constant pressure was applied to attain the best separation peak.

10 mg was taken from each sample material, and these were diluted to 10 mL with methanol to prepare stock solutions. For the sample solution to be analyzed, 100 µL stock solution, 100 µL buffer solution and 800 µL distilled water were mixed. (N-CH_3)-2-(3H)-benzoxazolinone was also added as the neutral marker.

Sample solution added into the vial was injected to the device. Capillary was washed with 0.01 NaOH for 2 minutes before each measurement. Next, capillary was rinsed with water for 2 minutes and with buffer solution for 4 minutes. Following these washing processes, 50 mbar pressures was applied using hydrodynamic injection, and 20 kV

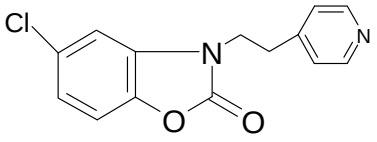
voltages was applied after the injection. The compound injection repeated for 3 times for each sample and the completion stages of the process were monitored through the signal window. Separation process was performed based on the electrophoretic mobility of the solutes and different speeds of migration for ionic types dissolved in electrophoretic buffer inside the capillary. Peaks attained as a result of this process were recorded. Electrophoretic mobility observed with pH was put into chart and pK_a values were found.

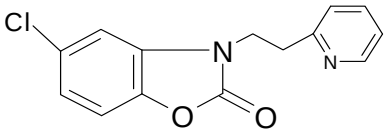
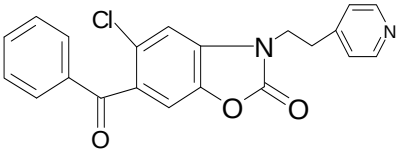
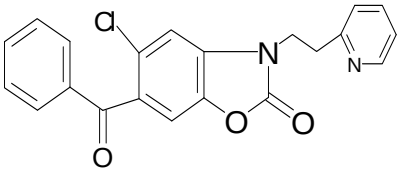
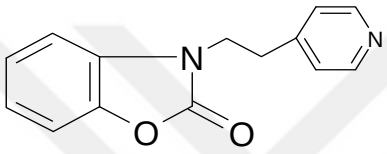
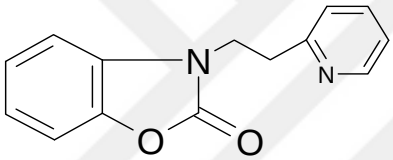
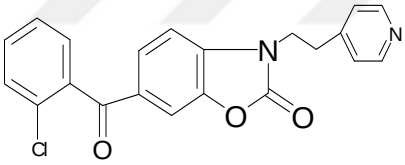
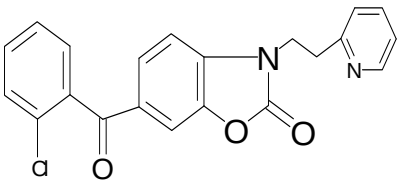
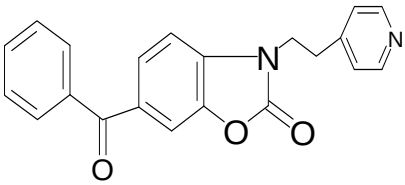
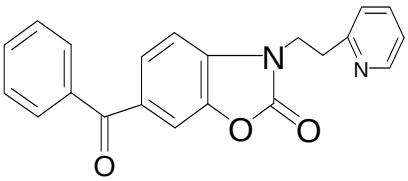
4. FINDINGS

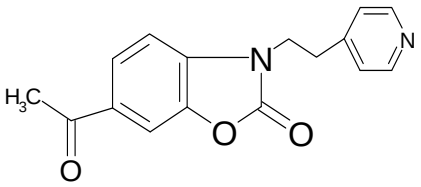
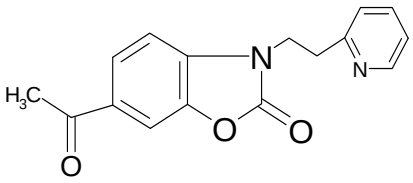
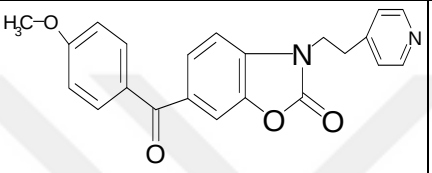
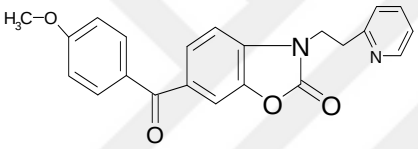
Knowing the acidity constant, pK_a, is a quite significant parameter for the understanding of the chemical interactions between the concerning compounds and their pharmacological effects. The relationship between acidity constant (K_a) and the compound is used in the studies of new drug synthesis and in the description of the biopharmaceutic properties of these substances. Many biologically active molecules can be ionized in physiologic pH partially or wholly, and the existence of functional groups on the molecules that can ionize is known to be important regarding biological activity and / or solubility. This requires appropriate techniques that are used in finding the acidity constants (K_a) the newly generated molecules. Therefore, the purpose of this study is to determine the pK_a values of fourteen 3-(2/4-pyridylethyl)-benzoxazolinone derivatives 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.

First, we have studied Compound ***5*** and ***6*** which are structurally based on the rest of the twelve compounds. At the end of our studies, acidity constant value of Compund ***5*** found as 5.10 with UV-Vis spectroscopy, 5.04 with the potentiometric method and 5.20 with the capillary zone electrophoresis method. In this study, pK_a values obtained for all the compounds were given in Table 2.

Table 1. The experimental pK_a values determined by UV-Vis spectrophotometry, potentiometry and capillary zone electrophoresis (CZE) for 3-(2/4-pyridylethyl)

#	Structural Formula	UV- Vis	Potentiometry	CZE	% Analgesic Activity[30]
01		5.05±0.06	4.92±0.09	5.16±0.08	100.00

02		4.90±0.08	4.91±0.06	5.05±0.11	96.96
03		5.50±0.09	-	5.30±0.12	92.61
04		5.25±0.12	-	5.60±0.10	72.67
05		5.10±0.05	5.04±0.07	5.13±0.08	96.06
06		5.21±0.09	5.10±0.07	5.30±0.11	92.17
07		5.37±0.05	-	5.33±0.12	84.35
08		5.14±0.05	-	5.17±0.08	91.74
09		4.89±0.08	-	4.99±0.09	85.47
10		4.96±0.04	-	4.93±0.07	79.49

11		5.40±0.08	5.28±0.06	5.31±0.09	38.46
12		5.20±0.05	5.14±0.06	5.23±0.09	48.74
13		4.85±0.08	-	5.20±0.13	75.21
14		5.13±0.04	5.19±0.07	5.11±0.09	80.34
%Analgesic activity of Aspirin:					58.26

Absorption spectrum attained for Compound ***5*** is given in Figure 13. Where two absorption bands 250 nm and 260 nm were observed in acidic environment (pH < 4), these absorbance values were observed to decreased at pH above 4 and remained constant after pH value 6.60. The isosbestic points were seen in spectrum means that, Compound ***5*** only gives one acid-base reaction (Figure 13).

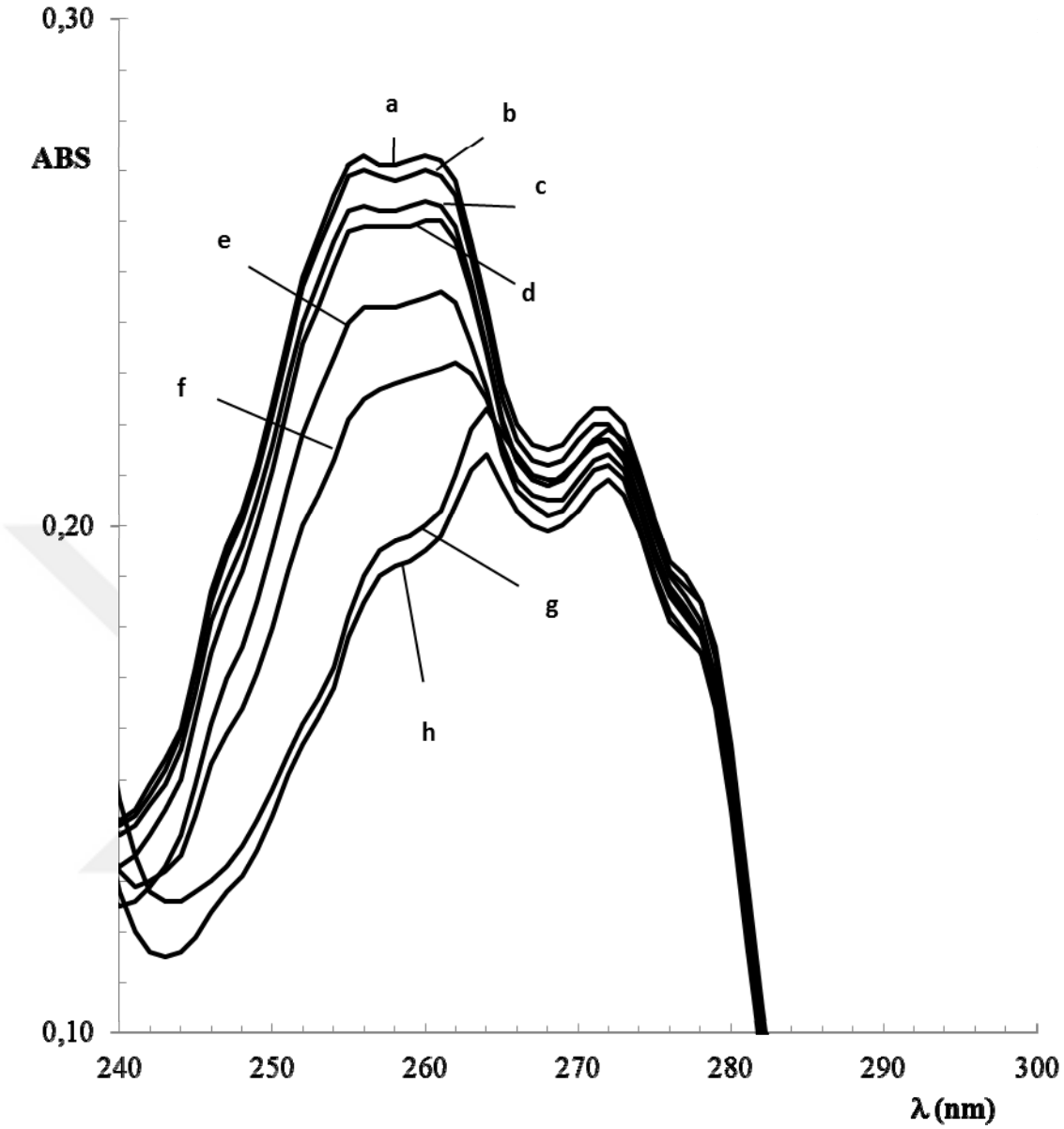


Figure 13. Absorbance vs. wavelength plot for $5x10^{-5}$ M Compound *5* in various buffered solutions. pH values: (a) 2.68, (b) 3.16, (c) 3.72, (d) 4.16, (e) 4.66, (f) 5.19,

Figure 14 shows a graphic drawn pH versus absorbance value at selected wavelengths (256 nm) for Compund ***5.***

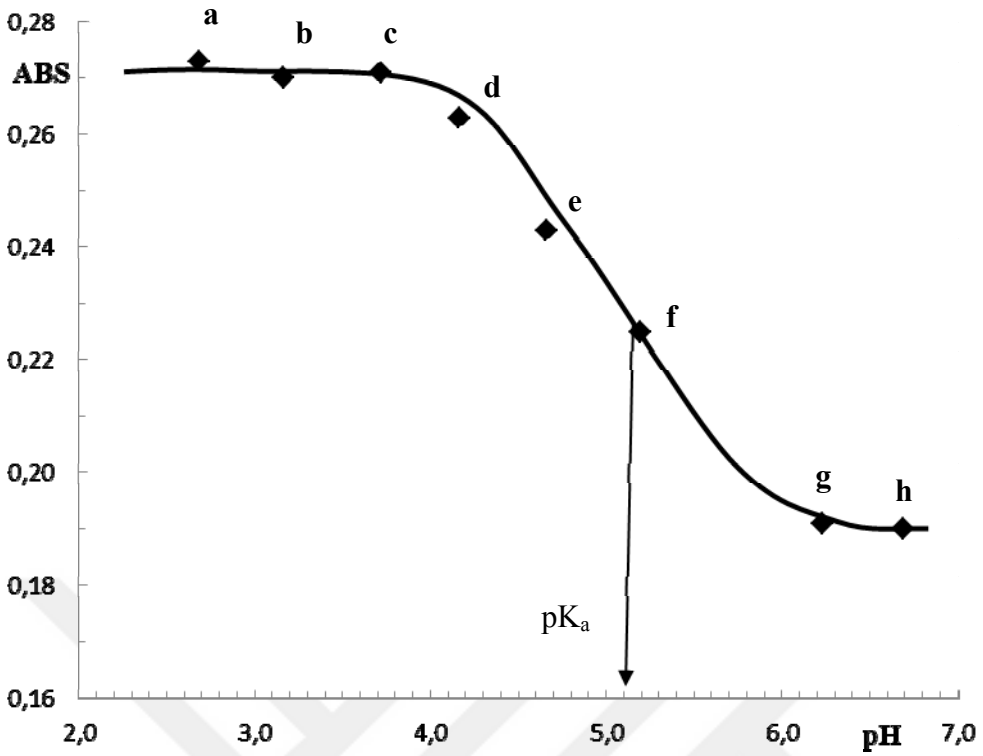


Figure 14. Plot of absorbance values as a function of pH for Compound *5* at 256 nm. pH values: (a) 2.68, (b) 3.16, (c) 3.72, (d) 4.16, (e) 4.66, (f) 5.19, (g) 6.22 and (h) 6.68.

As seen about Figure 14, it has an S shaped and pK_a value of Compound ***5*** in the middle point of this curve is 5.10. Determination of pK_a value was repeated three times. On the other hand, the same and/or similar pK_a values are obtained from similar graphics at different wave lengths.

In order to check the correctness of the obtained pK_a values of Compound ***5*** by UV-Vis spectroscopy, the potentiometry and capillary zone electrophorese methods have used.

In the potentiometric experiments that have carried out, the ionic strength of the solution has fixed by potassium chloride to reach 0.154 M. However, the concentration of Compound ***5*** was used in the potentiometric measurements was higher than that of UV-Vis spectrophotometer. Due to the fact that the solubility of the some studied compounds were decreased at high concentrations, only pK_a values of some 3-(2/4-Pyridylethyl)-benzoxazolinone derivatives have obtained by this method. The acidity of the solution obtained by this method (pH)-the volume graphic of the added base is given in Figure 15.

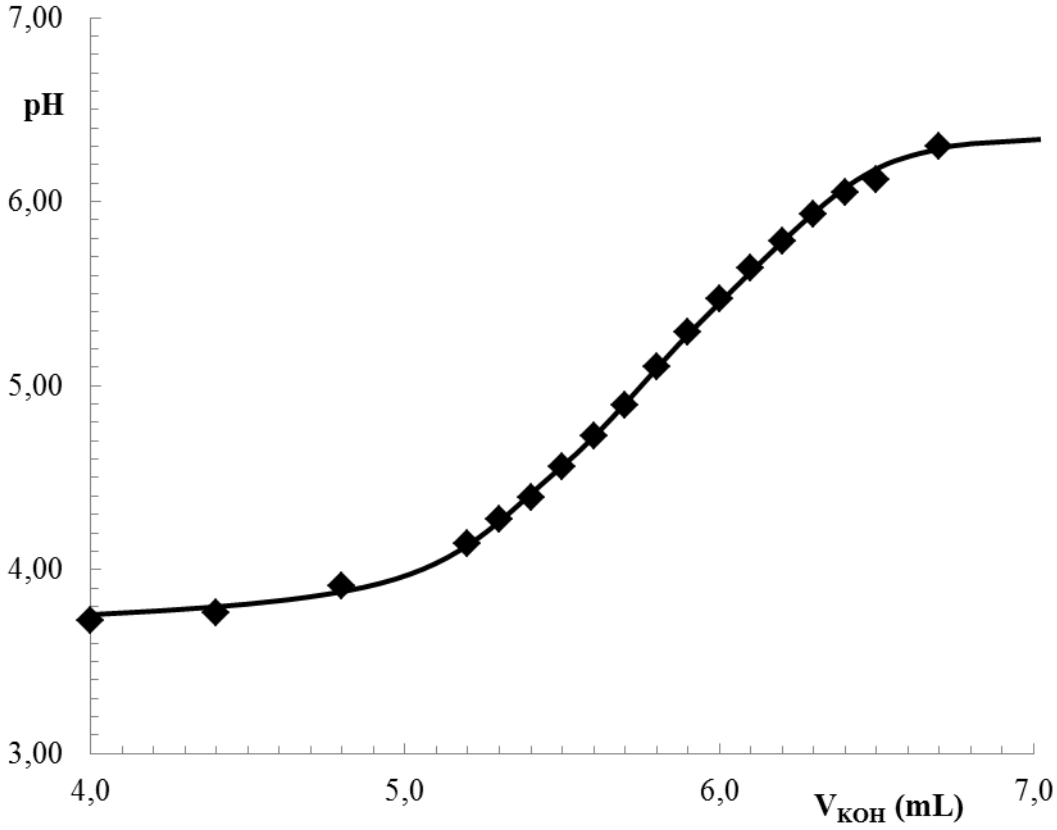


Figure 15. The graphic of Compound 5 by potentiometric method and the graphic drawn against the volume of the base (KOH) of the pH added.

The pK_a value of Compound ***5*** from Figure 15 was obtained as a result of the activity was determined as 5.04. There is no doubt that the pK_a values obtained by the potentiometric method and values obtained by UV-VIS spectroscopy are similar. The experimental data obtained by this method was repeated three times and the obtained pK_a value are provided in Table 2.

Figure 16 shows the results which are obtained by capillary zone electrophorese (CZE) that is used as the third techniques; the value of pK_a of the Compound ***5*** was assigned as 5.13. The Compound ***5*** within the buffer solution and neutral marker (5-methyl-2-(3H)-benzoxazolinone) has passed from capillary and depending on the electrophoretic movements and based on the migration of the ionic types that are dissolved within the buffer, the peaks that have composed as a result of the dissolution process has

repeated three times. The values of the electrophoretic movement values that are observed by the pH has transferred into the graphic (Figure 16) and the pK_a values have determined (Formula 24- 25).

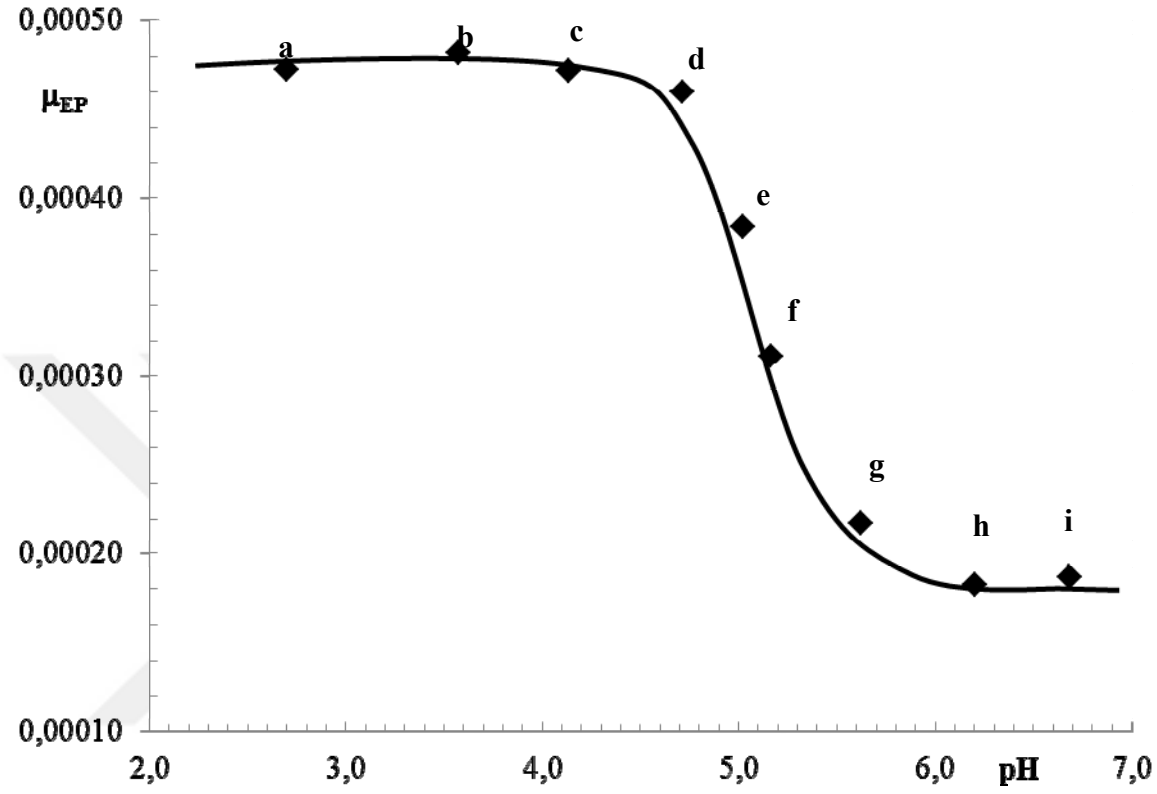
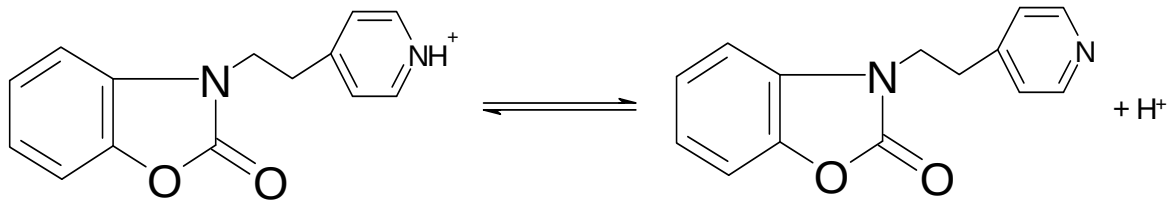


Figure 16. Electrophoretic mobility graphic in variable pH values of the compound 5. The pH values are : (a) 2.70, (b) 3.57, (c) 4.13, (d) 4.71, (e) 5.02, (f) 5.16, (g) 5.62, (h) 6.20, and (i) 6.68

It is determined that the acidity constant of 3-(2-Pyridylethyl) benzoxazolinone main structure (Compound ***5***) has relevance among the values provided by means of three variable experimental conditions and in order to understand the relationship between the structure and acidity constant (pK_a), the studies have focused on the 3-(2/4-Pyridylethyl) benzoxazolinone in similar structure.

Based on the experimental evidence, the equilibrium between protonated and unprotonated form can be given as follows (Scheme 2):



Scheme 2. Equilibrium for the protonation of 3-(4-Pyridylethyl) benzoxazolinone

In order to determine the acidity constant (pK_a) within the buffer solution for Compound ***6***, all three methods have used. The absorption spectrum that has obtained for Compound ***6*** is given in Figure 17.

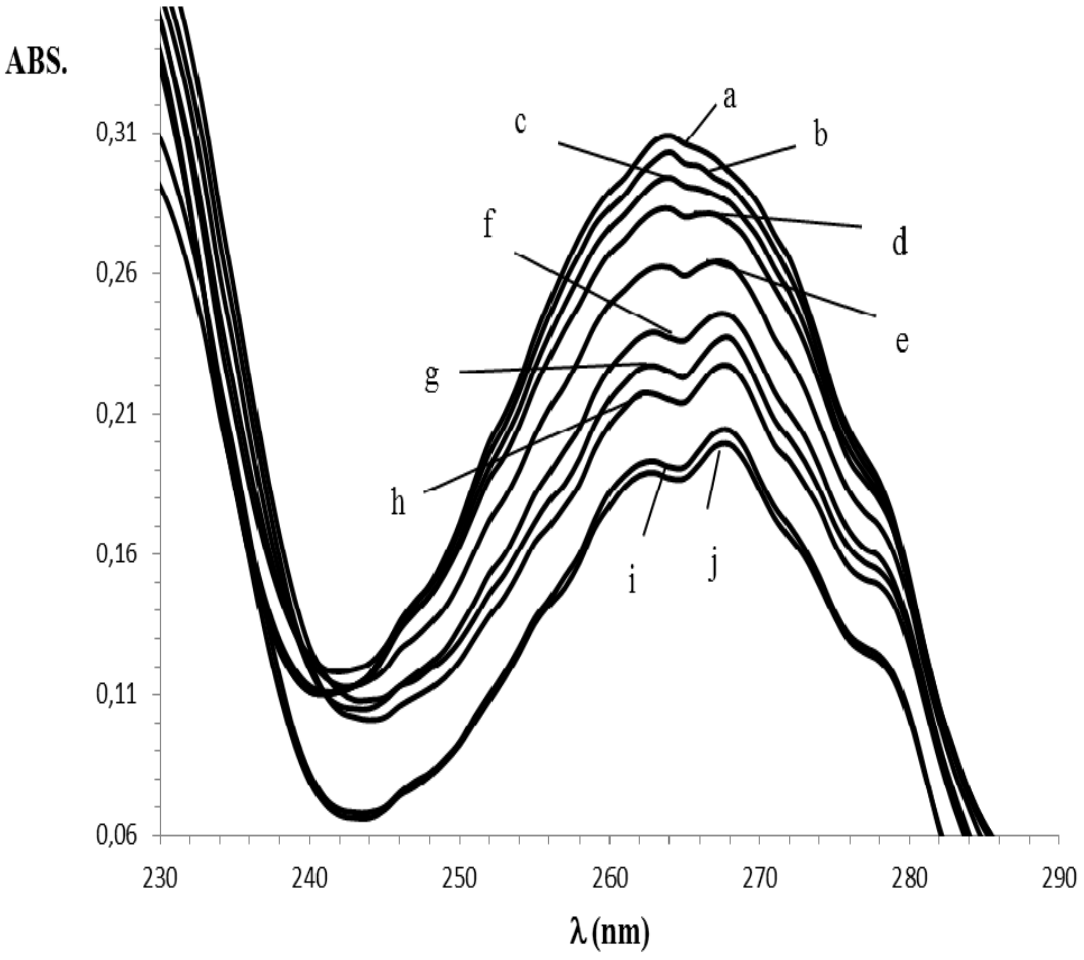


Figure 17. Absorbance – wavelength graphic obtained from variable pH values of the Compound 6. The pH values are ; (a) 2.79, (b) 3.16, (c) 3.72, (d) 4.16, (e) 4.66, (f) 5.19, (g) 5.78, (h) 6.22, (i) 6.68, (j) 7.21, and (k) 7.67.

When the absorbance values have decreased from the acidic values to neutral solutions, the compound has passed from the protonated form to neutral form, and the absorbance values were collected. The graphic related to the absorbance values obtained at 255, 261, 269, and 276 nm wavelengths versus the pH in different buffer solutions for Compound ***6*** is given in Figure 18.

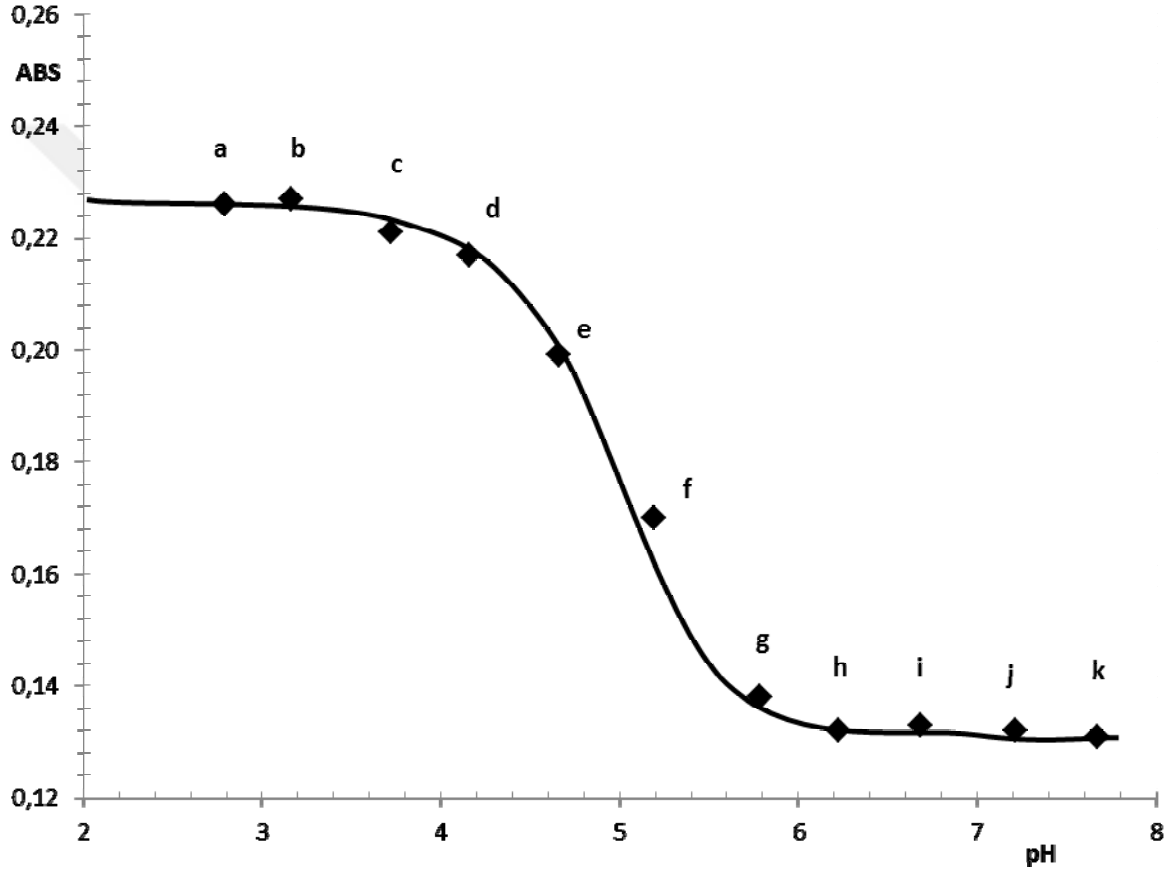


Figure 18. Plot of absorbance values as a function of pH for Compound *6* at 255 nm. pH values: (a) 2.79, (b) 3.16, (c) 3.72, (d) 4.16, (e) 4.66, (f) 5.19, (g) 5.78, (h) 6.22, (i) 6.68, (j) 7.21,

The obtained curve has a shape of S and pK_a value of Compound ***6*** from the middle point of this curve has determined as 5.21. As is seen in Figure 18, the similarity between the experimental data and theoretical curve has shown the harmony between the

experimental points and theoretical line. The pK_a values have also calculated in some other wavelengths and similar results have obtained. In order to check the acidity constant, the same test has repeated at least three times and the average pK_a values have determined (Table 2).

In the potentiometric experiments that have carried out, the ionic strength of the solution has fixed by potassium chloride to reach 0.154 M. However, the concentration that is used in the potentiometric measurements have done higher than this measure in order to read the measurement values through concentration that is used in UV-Vis spectrophotometer.

Due to the fact that the solubility of the current items that have been worked on is decreasing in higher concentration, only pK_a value of Compound ***6*** has obtained by this method. The acidity of the solution obtained by this method (pH)-the volume graphic of the added base was plotted. The pK_a value of Compound ***6*** from the middle point of the curved line obtained as a result of the activity was determined as 5.10.

There is no doubt that the pK_a values obtained by the potentiometric method and values obtained by UV-Vis spectroscopy are similar. The experimental data obtained by this method was repeated three times and the obtained pK_a value are provided in Table 3.

By means of capillary zone electrophorese method, in order to assign the acidity constant of Compound ***6***, it is benefited from the electrophoretic peaks that have been given by Compound ***6*** that was transmitted from the buffer solutions prepared in different pH values and neutral markers (5-methyl-2-(3H)-benzoxazolinone). The electrophoretic mobility observed by pH has passed to the graphic (Figure 19) and the pK_a values have determined.

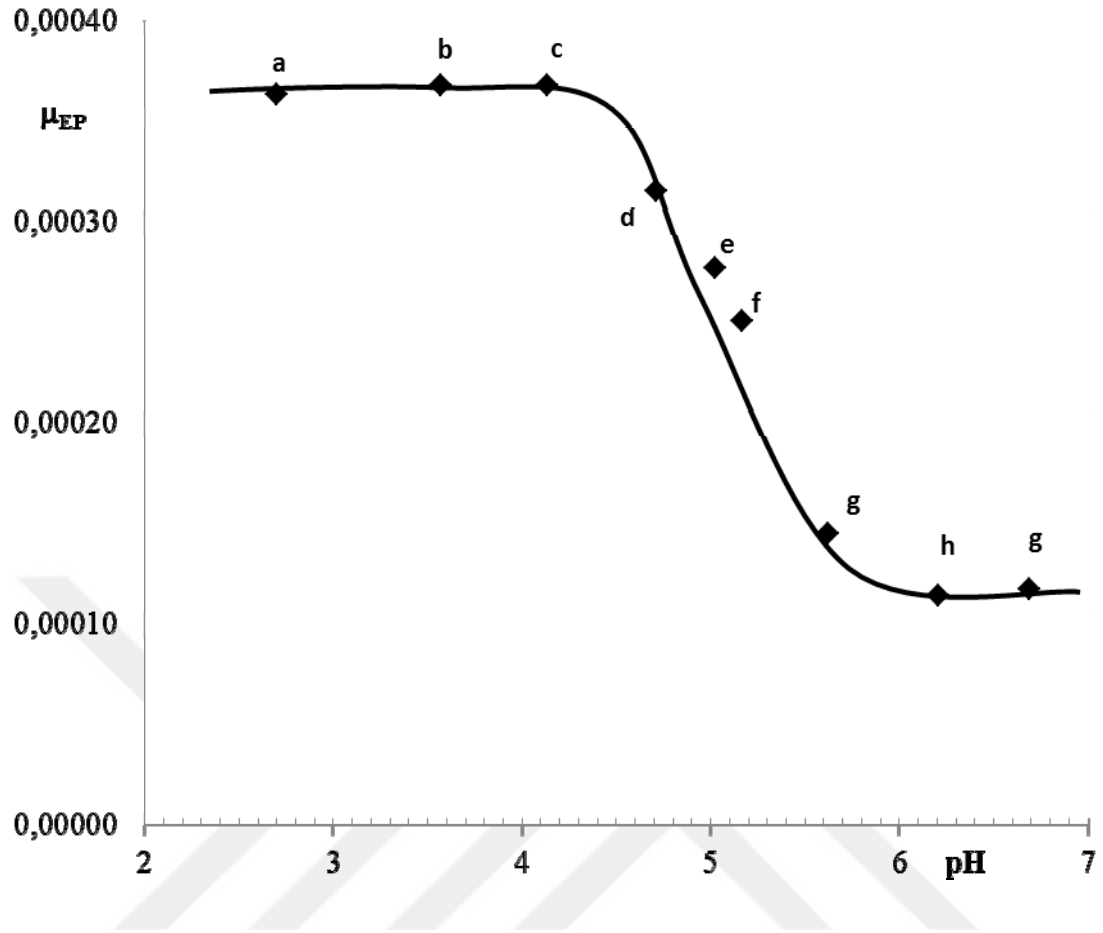


Figure 19. Electrophoretic mobility graphic in variable pH values of the Compound *6*. The pH values are : (a) 2.70, (b) 3.57, (c) 4.13, (d) 4.71, (e) 5.02, (f) 5.16, (g) 5.62, (h) 6.20, and (i) 6.68.

Absorption spectrum attained for Compound ***1*** is given in Figure 20. Where two absorption bands 255 nm and 280 nm were observed in acidic environment (pH < 4), these absorbance values were observed to decreased at pH above 3.5 and remained constant after pH value 6.5 (Figure 20).

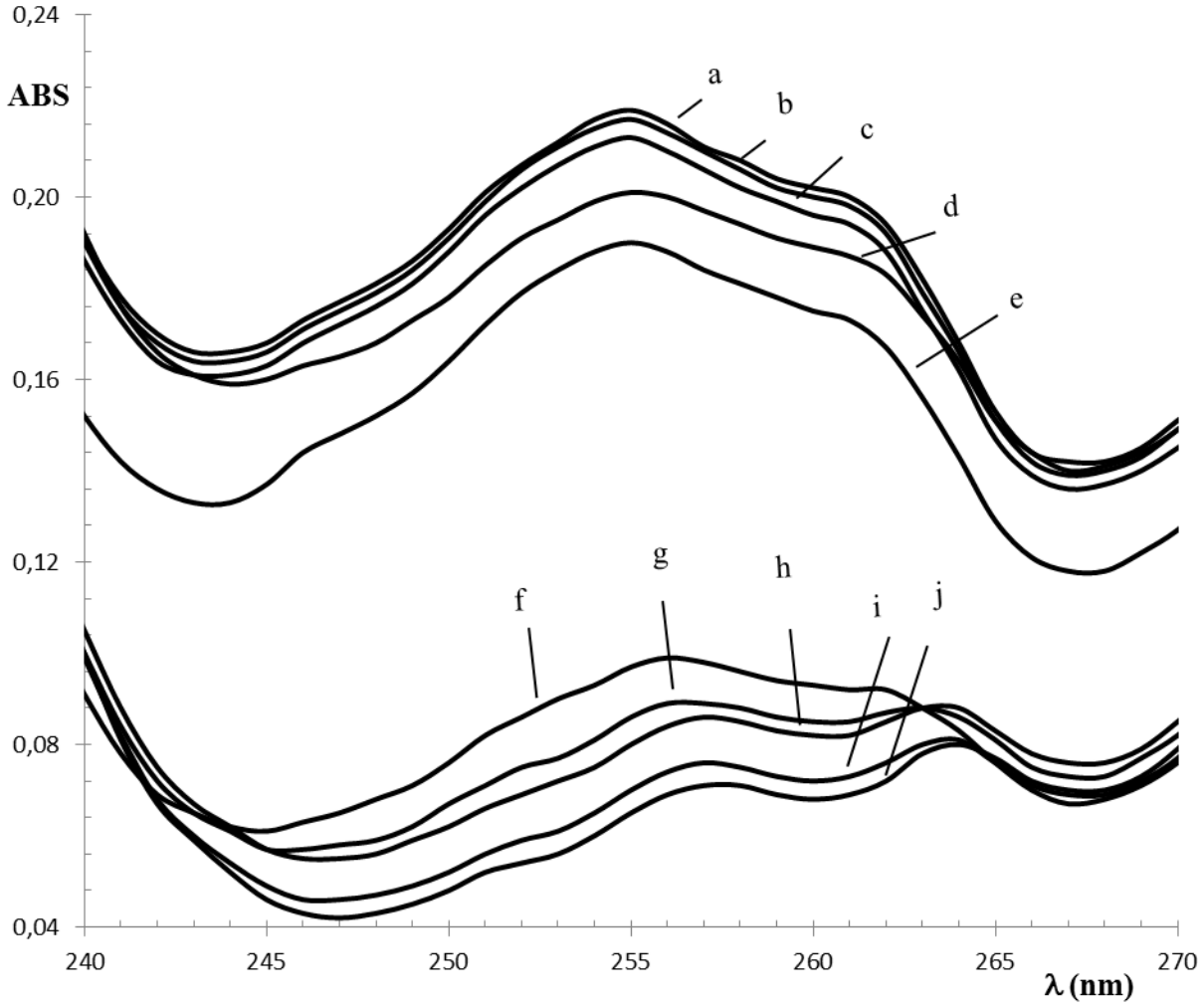


Figure 20. Absorbance vs wavelength plot for $5x10^{-5}$ M Compound *1* in various buffered solutions. pH values: (a) 2.21, (b) 3.16, (c) 2.79, (d) 4.66, (e) 3.72, (f) 5.19, (g) 5.78, (h) 6.22, (i) 6.68, and (j) 7.66

The graphic drawn against pH of the absorbance data in the selected wave lengths (256 nm) of the absorbance values obtained in variable pH values of Compound ***1*** is given in Figure 21.

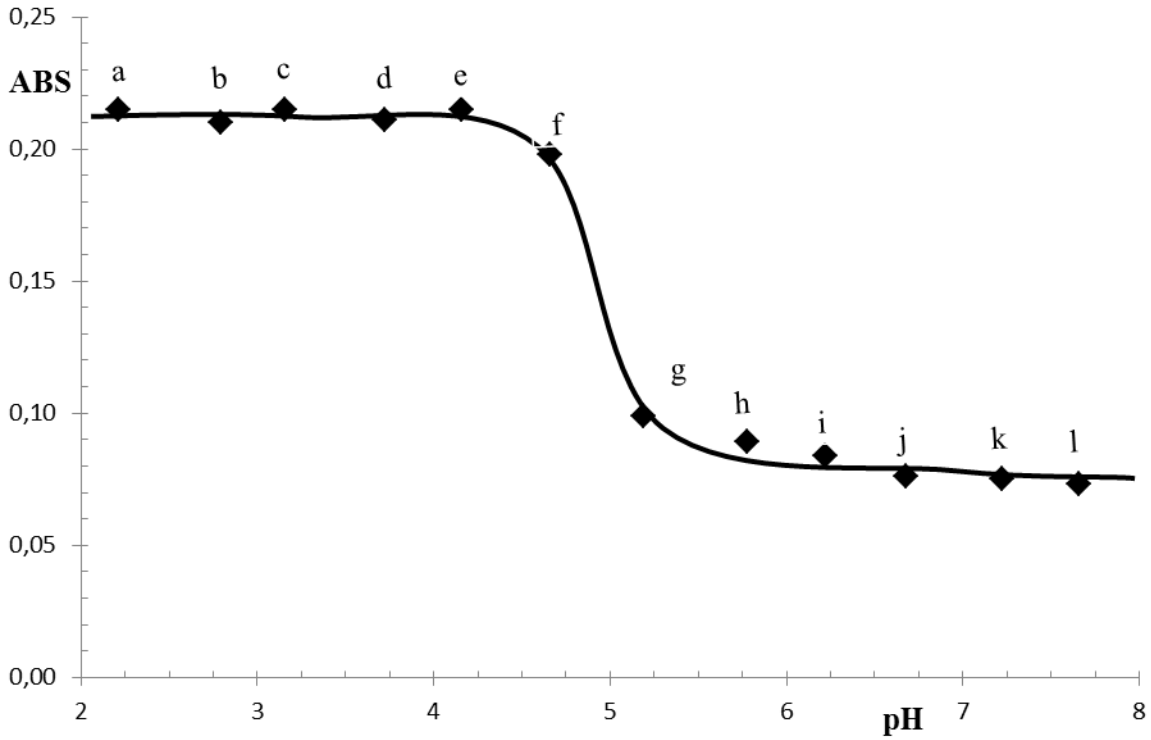


Figure 21. Plot of absorbance values as a function of pH for Compound *1* at 256 nm. pH values: (a) 2.21, (b) 2.79, (c) 3.16, (d) 3.72, (e) 4.16, (f) 4.66, (g) 5.19 ,(h) 5.78, (i) 6.22,(j) 6.68, (k) 7.22, and (l) 7.66

The pKa value of Compound 1 from the middle point of the curved line obtained as a result of the activity was determined as 5.05. The same and/or similar pK_a values are obtained from similar graphics at different wave lengths (249, 253, and 261 nm) (Figure 21).

The ionic power of the solution has fixed by potassium chloride to reach 0.154 M at potentiometric method. The obtained curve has a shape of S and pK_a value of Compound ***1*** from the middle point of this curve has determined as 4.92 in Figure 22. The experimental was repeated three times and the obtained pK_a value are provided in Table 2.

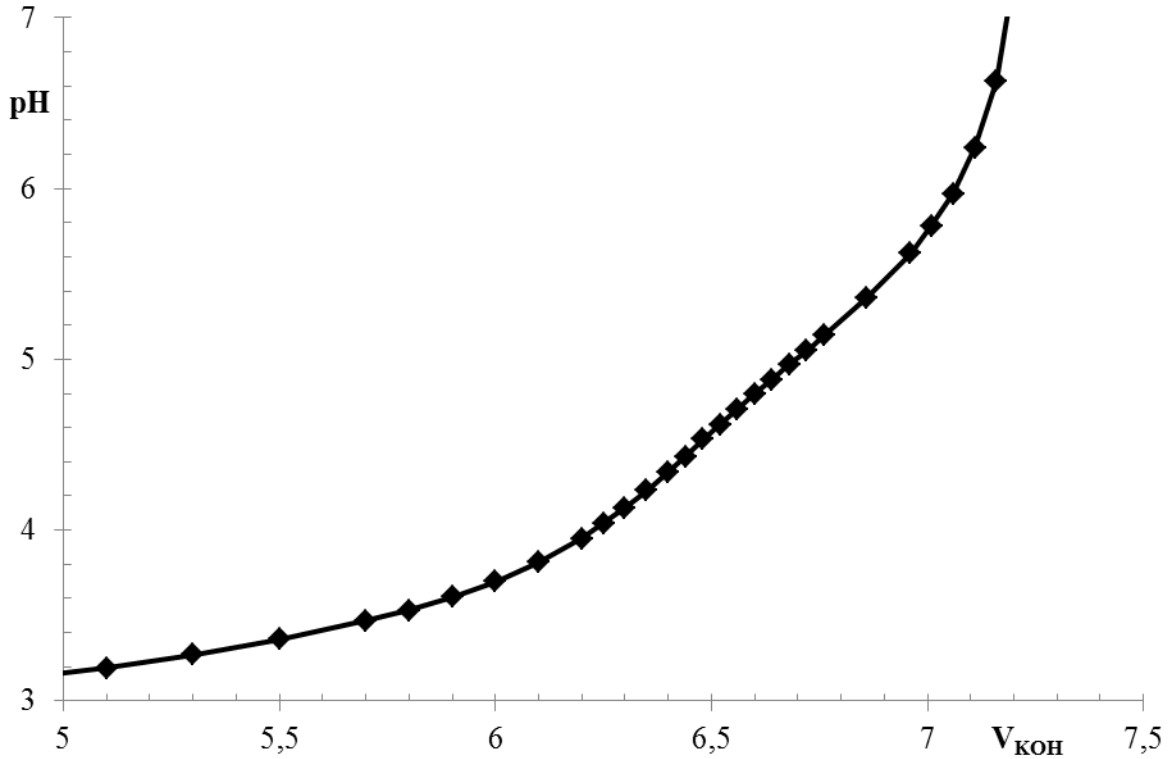


Figure 22. The graphic of Compound *1* by potentiometric method and the graphic drawn against the volume of the base (KOH) of the pH added.

By means of capillary zone electrophorese method, in order to assign the acidity constant of Compound ***1***, it is benefited from the electrophoretic peaks that have been given by Compound ***1*** that was transmitted from the buffer solutions prepared in different pH values and neutral markers (5-methyl-2-(3H)-benzoxazolinone). The electrophoretic mobility observed by pH has passed to the graphic (Figure 23) and the pK_a values have determined. The value of pK_a of the Compound ***1*** was assigned as 5.16 by Capillary zone electrophoresis

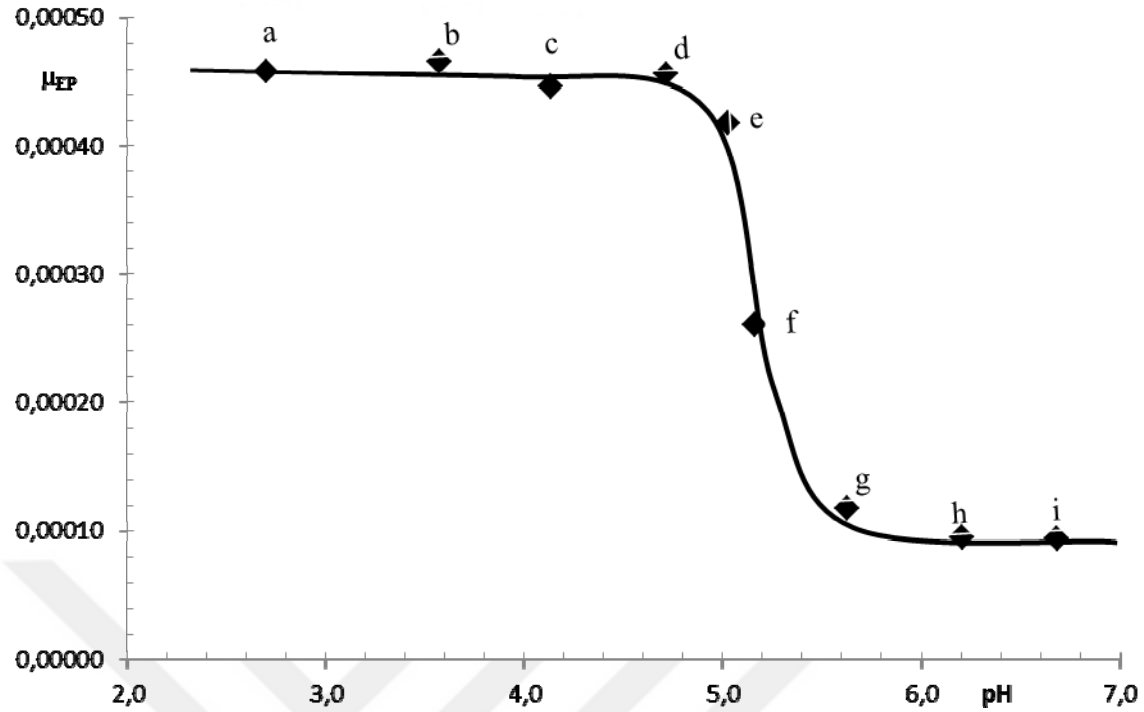


Figure 23. Electrophoretic mobility graphic in variable pH values of the Compound 1. The pH values are: (a) 2.70, (b) 3.57, (c) 4.13, (d) 4.71, (e) 5.02, (f) 5.16, (g) 5.62, (h) 6.20 and (i) 6.68.

The same experimental study has been done for Compound ***2*** to determine the acidity constant by using the same three methods. The obtained pK_a values are reported in Table 2.

After finding the pK_a values of first four compounds, the study was extended on the compounds that contain an acyl group into the 6^{th} position of the benzoxazolinone ring. The acidity constants of Compound ***11*** and ***12*** (bearing acetyl group at the 6^{th} position of benzoxazolinone ring) have been determined. When comparing these compounds with Compounds ***5*** and ***6***, it is observed that the availability of the carbonyl group has increased the acidity of these compounds (see Table 2).

We have continued to study on Compound ***9*** and ***10*** to see the effect of phenyl group on 6^{th} position of the main ring. After studying these compounds in the buffer solutions, the pK_a values were determined by UV-Vis spectroscopy and capillary zone electrophoresis methods. The acid dissociation constant values were reported in Table 3.

Potentiometry was proven to be a useless technique in this case due to the low solubility of these compounds in aqueous buffer solutions.

Our systematic studies were continued by the investigation of compounds which have substituent on the phenyl group. Compound ***7*** and ***8*** posses a chlorine atom on ortho position, respectively. After introducing an electron with-drawing group, the acidity constant values have been increased to 5.37 and 5.14 for Compound ***7*** and ***8***, respectively.

Compounds ***13*** and ***14*** have similar structures to Compound ***9*** and ***10***. The only difference is methoxy group being on para position on the benzene ring. Introducing an electron donating group of the ring did not change the acidity of the compounds when compared to Compound ***5*** and ***6*** (See Table 2).

The last compounds (Compound ***3*** and ***4***) that we have studied possess a chlorine atom on the 5^{th} position of main benzoxazolinone ring. These compounds can be compared with Compound ***9*** and ***10***. It can be seen from Table 3 that the pK_a values for Compound 3 and 4 have been increased when compared to Compound ***9*** and ***10***. These results suggest that the substitution on the main ring is much more important to change the pK_a value of the given compound than the substitution has been made on the phenyl ring at the 6^{th} position of the main structure.

5. RESULTS AND DISCUSSION

As a result of our studies related to the 3-(2/4-Pyridylethyl)-benzoxazolinone and its derivatives, the pK_a values have reported in this study in Table 2. By means of UV-Vis spectroscopy, potentiometer and capillary zone electrophorese techniques have been used to determine the acid dissociation constants for fourteen structurally related compounds. In this study, it is proven that the protonation should be on the nitrogen atom of pyridine. Based on this, the studies regarding the main structure and derivatives in 5^{th} and 6^{th} positions have been carried out and it is assigned that 6-benzoyl derivatives are more basic than main structure.

The structure−analgesic activity relationship of compounds was examined in this study. The analgesic activity [30] was correlated to the pK_a values of studied compounds with comparison to acetylsalicylic acid (ASA). Analgesic activities of the studied compounds were reported in Table 3. The analgesic activity of the compounds were screened by a "Modified Koster's Test" using ASA as a reference analgesic [30]. As seen in Table 3, the studied compounds showed analgesic activities higher than ASA except Compound ***11*** and ***12*** [30]. We have tried to correlate these finding with the acid dissociation constants and the solubility of the compounds.

When we compare the pK_a values of Compound ***5*** and ***6*** with Compound ***1*** and ***2***, the analgesic activities are similar as the pK_a values. However, when the acetyl group at the 6^{th} position of benzoxazolinone ring was introduced (Compound ***11*** and ***12***), there are significant decrease in activities were observed. These observations can be attributed to either change in pK_a values and / or decrease in solubility of the Compound ***11*** and ***12***. When we calculated log P values for Compound ***5***, **6**, **9-12**, the log P values for compound ***11*** and ***12*** were lower than Compound ***5***, ***6***, ***9*** and ***10***. The log P values are reported in Table 3.

Table 2. The log P and pKa values of some 3-(2/4-pyridylethyl) benzoxazolinone derivatives

#	UV- Vis	Log P	% Analgesic Activity[30]
05	5.10	2.07	96.06
06	5.21	2.32	92.17
09	4.89	3.28	85.47
10	4.96	3.53	79.49
11	5.40	1.38	38.46
12	5.20	1.63	48.74

Based on the log P values, the decrease in analgesic activities of the Compound ***11*** and ***12*** were attributed on the increase solubility in aqueous solution. Also the pK_a values of them higher than that of Compound ***5***, **6**, ***9***, and ***10***. This finding may suggest that when the compound is more soluble in aqueous environment, less analgesic activity may be observed.

When the pK_a values of Compound ***8*** and ***10*** (pK_a values are 5.14 and 4.96, respectively), it can be seen that the decrement in pK_a values may cause a decrement in activities for these compounds from 91.74% to 79.49%, respectively. Similar behavior can be seen for Compound ***3*** and ***4***. When the pK_a values decrease from 5.50 to 5.25, the analgesic activities drop from 92.61% to 72.67. This correlation cannot be explained by only acidity of the compound. There is a combination of acidity constant and solubility of the compound on the analgesic activity.

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.

Fourteen 3-(2/4-pyridylethyl)-benzoxazolinone derivatives have been studied, in order to indicate the acidic constants (pK_a) of them. The acidity constants of all compounds were determined by using UV-Vis spectroscopy and the correctness of these results have been compared by acidic constants obtained by potentiometer and capillary zone electrophorese methods accordingly. The position of the equilibrium reaction was suggested based on the experimental results (Scheme 2). Under the light of data, the electro with-drawing groups (chlorine atom) on 6-benzoyl derivatives or on the main structure of benzoxazolinone ring showed more basic character in comparison with electron donating groups. By moving from this point of view, it was suggested that the Compound ***5*** and ***6*** have more basic character than that of Compound ***9*** and ***10***. Meanwhile, analgesic activities of Compound ***5*** and ***6*** are higher than that of Compound ***9*** and ***10***. Similar behaviors have observed for other structurally related derivatives (see Table 2).

Because of the acidic and / or basic character of the compounds that show different activities, the ionization degrees of them could be determined by pH values and acidity constant with regarding its medium. Under the light of our results related to 3-(2/4-pyridylethyl) benzoxazolinone and its derivatives, relevant information can be obtained about the availability of the groups that can ionize, as well as the biological activities and prediction of solubility of these compounds at a given acidity. Our results also suggest that analgesic/anti-inflammatory activities of benzoxazolinones decrease when the pK_a values and / or solubility of the compounds increase.

The compounds can be in the form of cationic, anionic or neutral form which affects the solubility, permeability, UV absorptions, and its reactivity. The ionize form is usually more water soluble, while the neutral form is more lipophilic and has higher membrane permeability.

From the acid dissociation constants, the major species (cationic, anionic or neutral form) of pharmaceuticals present in environment can be estimated. For these reasons, it is very important to know dissociation constants for environmentally relevant active pharmaceutical ingredients in order to estimate their occurrence, fate and effects.

Moreover, the acid-base property of a drug candidate molecule is a key parameter for drug development progress because it governs solubility, absorption, distribution, metabolism as well as elimination. Particularly for developing new active pharmaceutical ingredients, the pK_a has become of great importance because the transport of drug into cells and across other membranes is a function of physicochemical properties and of the pK_a of the drugs. Hence, the discovery of new active pharmaceutical ingredients (drugs) requires accurate methods for determination of acid dissociation constant values. Several advanced analytical methods have been used for the determination of dissociation constants for active pharmaceutical ingredients.

Besides from UV-Vis spectroscopy, potentiometry, and capillary zone electrophoresis; there are many other analytical methods such as liquid chromatography, NMR, and capillary electrophoresis with mass detectors which are being used in the literature. Even though these methods have brought some advantages for finding the pK_a values, limitations such as expensive instrumental setup, sensitivity, and solubility of the compounds have been reported [17]. The UV−Vis spectroscopic 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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