Piroksikamın fizikokimyasal özelliklerinin belirlenmesi

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GİRİŞ ve AMAÇ: Bu çalışmanın amacı, HPLC ve UV-VIS Spektrofotometrisi kullanılarak piroksikamın asit ayrışma sabiti (pKa) ve piroksikamın ayrılma katsayısı (Log P), dağılım katsayısı (Log D) ve "Log kw" değerlerini HPLC kullanarak belirlemektir.
YÖNTEM ve GEREÇLER: HPLC çalışmaları, 1.0 mL min-1 akış hızında ters faz kromatografisinde ACE C18 (150 x 4.6 mm ID, 5 um) kolon kullanılarak gerçekleştirildi. Detektör 360 nm'ye ayarlandı. Farklı pH değerlerinde (3.0 - 6.5) log D değeri, hareketli faz olarak fosfat tamponu (20 mM): asetonitril (30: 70 h/h) karışımı ile incelendi. pKa'nın belirlenmesi için HPLC çalışmaları, 3.50 ve 6.00 pH aralığında fosfat tamponu (20 mM): metanol karışımı ile gerçekleştirildi. Log kw ölçümleri, pH 3.50 ila 6.00 arasında bir pH aralığında fosfat tamponu (20 mM): MeOH (20: 80 h/h ile 10: 90 h/h) karışımları ile yapıldı. UV-GB Spektrofotometrik pKa ölçümleri 285 nm dalga boyunda gerçekleştirildi.
BULGULAR: Piroksikamın pKa değeri sırasıyla HPLC ile 5.3 ve UV-VIS spektrofotometresi ile 5.7 olarak bulundu. Deney şartlarımızda piroksikamın log P değeri 1.58 olarak bulundu. Log D değerleri, sırasıyla 3.17, 3.79, 4.44, 5.42 ve 6.56 pH değerleri için 1.57, 1.57, 1.44, 1.13 ve 0.46 olarak bulundu.
TARTIŞMA ve SONUÇ: Literatürde, piroksikam için farklı Log P (3.1, 2.2 ve 0.6) ve pKa (6.3 ve 4.8) değerleri bildirilmiştir. Çalışmamızda piroksikam için elde edilen Log P (1.58) ve pKa (5.3 ve 5.7) değerleri literatür değerleri arasındaydı. Tüm bu sonuçlar, fizikokimyasal özelliklerin belirlenmesinde kullanılan farklı deneysel yaklaşımların farklı değerler sağlayabileceğini göstermektedir. UV Spektrofotometrisinin uygulanması kolay olsa da, HPLC, bileşiklerin pKa, Log D ve Log P değerlerinin eşzamanlı olarak belirlenmesi için eşsiz tekniklerden biridir.

Anahtar Kelimeler: Piroksikam, fizikokimyasal özellikler, pKa, Log P, Log D, Log kw

Determination of the physicochemical properties of piroxicam

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INTRODUCTION: The aim of this study was to determine the acid dissociation constant (pKa) of piroxicam using HPLC and UV-VIS Spectrophotometry, and to determine the partition coefficient (Log P), distribution coefficient (Log D) and "Log kw" values of piroxicam using HPLC method.
METHODS: The HPLC studies were performed on a reversed-phase ACE C18 (150 x 4.6 mm ID, 5 μm) column at a flow rate of 1.0 mL min-1. The detector was set at 360 nm. Log D at different pH values (3.0 – 6.5) was examined with a phosphate buffer (20 mM): acetonitrile (30: 70 v/v) mixture as the mobile phase. For pKa determination, HPLC studies were performed with a mixture of phosphate buffer (20 mM): methanol within the pH range of 3.50 and 6.00. Log kw measurements were performed with phosphate buffer (20 mM): MeOH (from 20: 80 v/v to 10: 90 v/v) mixtures within the pH range of 3.50 and 6.00. UV-VIS Spectrophotometric pKa measurements were performed at 285 nm wavelength.
RESULTS: pKa value of piroxicam was found to be 5.3 by HPLC and 5.7 by UV-VIS spectrophotometry, respectively. Log P of piroxicam was determined as 1.58 in our experimental conditions. Log D values were 1.57, 1.57, 1.44, 1.13 and 0.46 for pH values of 3.17, 3.79, 4.44, 5.42 and 6.56, respectively.
DISCUSSION AND CONCLUSION: In the literature, different Log P (3.1, 2.2 and 0.6) and pKa (6.3 and

4.8) values were reported for piroxicam. The Log P (1.58) and pKa (5.3 and 5.7) values obtained for piroxicam in our study were within the range of literature values. All these results indicate that different experimental approaches used for determination of physicochemical properties could provide different values. Although UV Spectrophotometry is easy to apply, HPLC is one of the unique techniques for simultaneous determination of pKa, Log D and Log P values of compounds.

Keywords: Piroxicam, physicochemical properties, pKa, Log P, Log D, Log kw

Introduction

Dissolution from the dosage form is one of the factors that limits the absorption of drugs from the gastrointestinal (GI) tract. According to the Noyes-Whitney dissolution model (1), the *in vivo* dissolution rate is influenced by drug diffusivity, drug solubility in gastrointestinal contents, wetted surface area of solid by biological fluids, and the gastrointestinal hydrodynamics (2).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are are chronically used as anti-inflammatory, analgesic, and antipyretic agents (3) to reduce pain, decrease stiffness, and improve functions of patients suffering from all forms of arthritis. They are also used for the acute treatment of pain associated with headache, dysmenorrhea and postoperative pain (4). Recent studies have focused on the usage of NSAIDs for cancer treatment or prevention from cancer (5). Pharmacologic treatment of cancer pain using NSAIDs has also been investigated for a long time (6), although their use is limited to their side effects (7). The relationship between some gastrointestinal tract diseases and NSAIDs usage is also another discussion (8). Drug-drug interactions related with the usage of NSAIDs are still an issue to be investigated (9). Because of all these aspects, the physicochemical properties of drugs are one of the key points to understand their behavior inside the body. Therefore, to understand the drug's behavior in GI tract, it is important to know the lipophilicity and pKa of drugs (10).

Piroxicam belongs to the non-steroidal anti-inflammatory drug (NSAID) of the oxicam class, and is used to alleviate the symptoms of painful and inflammatory conditions such as arthritis (3). They are also used for the treatment of headache, dysmenorrhea, and postoperative pain (4).

In the literature, it is easy to find various studies for determination of the pKa values of pharmaceuticals using HPLC (11-14), UV spectrophotometry (15-17), capillary electrophoresis (18, 19) and potentiometric titrations (20-22). Identical analytical techniques have also been applied over a long period of time for calculation of Log P and Log D values (23-30). For piroxicam, the reported Log P values differ from 0.6 to 3.6, and the reported pKa values differ from 4.76 to 6.30 according to the Drugbank.

Variable results in such basic physicochemical parameters motivated us to investigate these parameters in our conditions. In this study, a simple experimental procedure based on HPLC and UV-VIS Spectrophotometry was applied for determination of physicochemical properties (pKa, Log P and Log D) of piroxicam (Figure 1). Comparison of HPLC and UV-VIS Spectrophotometry used for determination of pKa values were also carried out. A chromatographic approach based on the "Log kw" is suggested in the literature (31, 32) to determine the lipophilicity of drugs. This technique is relatively new than classical shake flask method (33), and to our knowledge, there is no report available in the literature to correlate the shake flask method and Log kw measurements with each other. So that, this is the only study to compare the Log P and Log kw measurements for an active pharmaceutical ingredient.

Materials and Methods

Chemicals

Piroxicam was supplied by Sigma Aldrich (St. Louis, MO, USA). Sodium dihydrogen phosphate ($NaHPO_4 \cdot 2H_2O$) and NaOH were purchased from Merck (Darmstadt, Germany). Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$), potassium chloride (KCl), acetonitrile (ACN), methanol (MeOH) and 1-octanol were obtained from Sigma Aldrich. Water was obtained from the Milli-Q water system (Barnstead, USA) and used for preparation of all standard solutions and buffers.

Instrumentation

The HPLC system equipped with a gradient pump (Spectra-SYSTEM P2000), a degasser (Spectra SYSTEM SCM 1000), a manual injector with 20 µL injection loop (Rheodyne), and a detector (SpectraSYSTEM UV2000, Thermo Separation Products, USA) was used. The detector was adjusted to 360 nm, and retention times were determined automatically by an online computer equipped with ChromQuest software. The separations were performed using a reversed-phase ACE C18 (125 x 4.6 mm ID, 5µm) HPLC column (Aberden, Scotland) at flow rate of 1 mL min^{-1}. The spectrophotometric measurements were carried out using a Varian Cary 50 UV-Vis Wavelength Spectrophotometer with The Xenon lamp (200 – 800 nm). The UV spectra of reference and sample solutions were determined in 1 cm quartz cells from 200 to 800 nm wavelengths.

Solutions

Standard stock solution of Piroxicam (1000 µg mL^{-1} in MeOH)

Piroxicam standard stock solution was prepared by dissolving piroxicam (50 mg) in MeOH in a volumetric flask (50 mL).

Phosphate buffer (20 mM) with potassium chloride (100 mM) solution (between pH 3.00 and 7.50) for UV-VIS Spectrophotometric pKa determination

Phosphate buffer (20.0 mM) and 100 mM KCl mixture were prepared by dissolving 3.56 g of sodium dihydrogen phosphate ($NaH_2PO_4 \bullet 2H_2O$) and 7.45 g of potassium chloride (KCl) in about 800 mL water, and then making up the volume to 1000 mL with water. The pH of the solutions was adjusted with 1 M NaOH and 0.1 M KCl mixture solution.

Phosphate buffer (20 mM): MeOH (50:50 v/v) solutions (between pH 3.50 and 6.00) for HPLC pKa determination

Phosphate buffer (20.0 mM) was prepared by dissolving 3.12 g of sodium dihydrogen phosphate ($NaH_2PO_4 \bullet 2H_2O$) in about 800 mL water, and then making up the volume to 1000 mL with water. The mobile phase was prepared as 500 mL mixture of phosphate buffer/methanol (50:50 v/v), and adjusting the pH with o-phosphoric acid/MeOH (50:50 v/v) or 20 mM disodium hydrogen phosphate (Na_2HPO_4)/MeOH (50:50 v/v) mixtures for different pH values in range of 3.64-5.94.

Phosphate buffer (20 mM) solutions (between pH 3.00 and 6.60) for Log D determination

Phosphate buffer (20.0 mM) was prepared by dissolving 3.56 g of disodium hydrogenphosphate ($Na_2HPO_4 \bullet 2H_2O$) in about 800 mL water, and then making up the volume to 1000 mL with water, and adjusting the pH with o-phosphoric acid to different pH values in a range of 3.17-6.56.

Phosphate buffer (20 mM, pH 3.0): ACN (30:70 v/v) solutions as the HPLC mobile phase on Log D determination

Phosphate buffer (20.0 mM) was prepared by dissolving 3.56 g of disodium hydrogenphosphate ($Na_2HPO_4 \bullet 2H_2O$) in about 800 mL water, and adjusting the pH with o-phosphoric acid to 3.0, and then making up the volume to 1000 mL with water. The mobile phases were prepared as 500 mL mixture of phosphate buffer (20 mM, pH 3.0): ACN (30:70 v/v).

Phosphate buffer (20 mM): MeOH (from 20:80 v/v to 10:90 v/v) solutions for Log kw determination (between pH 3.00 and 6.00)

Phosphate buffer (20.0 mM) was prepared by dissolving 3.56 g of disodium hydrogenphosphate ($Na_2HPO_4 \bullet 2H_2O$) in about 800 mL water, and then making up the volume to 1000 mL with water. The mobile phases were prepared as 500 mL mixture of phosphate buffer (20 mM)/MeOH in a range of 20:80-10:90, v/v. The pH of the mobile phases were adjusted with o-phosphoric acid/methanol (20:80 – 10:90, v/v) or 20 mM disodium hydrogen phosphate (Na_2HPO_4)/methanol (20:80 – 10:90, v/v) mixtures for different pH values (3.00 and 6.00) and different mobile phase compositions (phosphate buffer (20 mM)/MeOH in a range of 20:80 – 10:90, v/v).

Procedures

UV-VIS Spectrophotometric pKa determination

Standard stock solution of piroxicam was diluted to 20.0 µg mL^{-1} by using Phosphate buffer (20 mM) potassium chloride (100 mM) solutions for UV-VIS Spectrophotometric pKa determination (pH 3.09 – 7.48). Spectra were taken from 200 to 400 nm wavelength. The shifts on absorption were considered. A sigmoidal curve was constructed between pH of the solutions and absorption at 285 nm. The pKa of piroxicam was determined according to the sigmoidal relationship given by Microsoft Excel between pH 3.09 and 7.48.

HPLC pKa determination

Standard stock solution of piroxicam was diluted to 5.0 µg mL^{-1} with phosphate buffer (20 mM): MeOH (50:50 v/v) solutions for HPLC pKa determination (pH 3.64 –5.94). These solutions were injected into the HPLC system with the mobile phases in which they were dissolved. A sigmoidal curve was constructed between the pH of the mobile phases and capacity factor (k') of piroxicam. The pKa of piroxicam was determined according to the sigmoidal relationship between pH 3.64 and 5.94.

Log D determination

The experimental system was a modified shake-flask method to meet the requirements of OECD guideline for testing of chemicals (33). For this purpose, standard stock solution of piroxicam was diluted to 250 µg mL^{-1} with MeOH. 50.0 µL of this solution was added into aqueous biphasic systems containing phosphate buffer (20 mM)

solutions for Log D determination (pH 3.17 – 6.56) and 1-octanol (50:50 v/v, 950 µL). After the extraction at ambient temperature, the amount of piroxicam remaining in buffer was determined by HPLC. Phosphate buffer (20 mM, pH 3.0): ACN (30:70 v/v) mixture was used as mobile phase and the buffer phase of the extracted sample was diluted 5-fold with the mobile phase before injection into the HPLC system. Peak areas were compared with 5.0 µg mL^{-1} piroxicam solution to determine the piroxicam amount that partition into the octanol phase.

Log P determination

The lipophilicity of a compound can be expressed by its partition coefficient (Log P) which is the concentration ratio of a non-ionized compound in a mixture of two immiscible phases (aqueous and organic phases) at equilibrium. For measurement of the Log P value of a weak basic drug, piroxicam, the equation given below is used for different pH values. The average of the obtained results were calculated to find the Log P value.

$$\text{Log}\, D = \text{Log}\, P - \text{Log}(1 + 10^{pKa-pH}) \qquad \text{Equation 1}$$

Log kw determination

For determination of *Log* k_w values, piroxicam was diluted to 5.0 µg mL^{-1} using phosphate buffer (20 mM): MeOH (from 20:80 v/v to 10:90 v/v) solutions (pH 3.00 and 6.00). The relationship between Log k' and methanol concentration in the mobile phase is described with Log k' = Log k_w – Sφ (31, 34). In this equation, k_w is the k' value for a compound when aqueous phase is used as the eluent, S is the slope of the regression line, φ is the volume percentage of methanol in the mobile phase. If φ is zero (no methanol in the mobile phase), the mobile phase is consisted of only the phosphate buffer, the Log k' will be equal to the Log k_w.

Measurements on Sigmoidal Curves

The data measured from sigmoidal curve for pKa determination were found using two different approaches. The first one was to print the whole graph in a A4 paper and finding the pKa values by drawing tangents. The second one was to use the derivative of the actual graph.

Results and Discussion

In this study, we focused on the pKa, Log P and Log D values of piroxicam because the reported values are very different from each other. Log kw values of piroxicam were also evaluated and the values were compared with the Log D values.

UV-VIS Spectrophotometric pKa determination

Absorption of organic compounds including most of the drugs is based on transitions of n or π electrons to the π* excited state. The absorption peaks for these transitions fall in 200 - 700 nm range which is experimentally convenient region of the spectrum. Referred transitions require an unsaturated group in the molecule to provide the π electrons. The solvent in which the absorbing species are dissolved may have serious effects on the spectrum (35). In the present study, the spectra of piroxicam in the phosphate buffers having identical ionic strengths (adjusted with 0.1 M KCl) but at different pH values from 3.09 to 7.48 were recorded. The values between 3.09 and 7.48 were used to construct the sigmoidal curve at 285 nm wavelength. The characteristic of absorption spectrum for piroxicam was changed by changing the pH of the buffered aqueous media (Figure 2). The changes in the absorbance values are usually followed by overlaid plots of recorded spectra, and the greatest change occurs when the acidity of the aqueous solution is equal to the pKa of the studied compound. The sigmoidal curve was constructed and pKa value of piroxicam was estimated as 5.7 (Figure 3.)

HPLC pKa determination

The capacity factor of a drug in a reversed phase HPLC system is related with its lipophilicity (36, 37). Since weak acidic drugs like piroxicam are ionized at basic pH, they tend to be eluted rapidly from a lipophilic C_{18} stationary phase with basic mobile phases. This situation is vice versa for acidic mobile phases. Our results are in a good agreement with this statement. Piroxicam was eluted at 7.44 min at acidic pH (pH 3.64) and eluted at 4.34 min at relatively basic pH (pH 5.94) values (Figure 4). When the pH value of mobile phase was plotted against k' values, a sigmoidal relationship was obtained between pH and capacity factor (Figure 5). The pK_a value of piroxicam was calculated as 5.3 from this relationship.

Log D and Log P determination

In drug discovery and development, lipophilicity of a compound is usually expressed by its partition between water and 1-octanol. The concentration of a non-ionized

compound in the organic and aqueous phases refers to Log P. The log P of any ionizable solution can be measured in the aqueous phase in which the pH is adjusted to the non-ionized form. The concentration ratio of non-ionized solute in the solvents is calculated according to the Log P value which is a measure of lipophilicity, and is not pH dependent (Equation 2).

$$\text{Log}\, P_{oct/wat} = \text{Log}\left(\frac{[solute]_{octanol}}{[solute]_{un-ionized\ water}}\right) \qquad \text{Equation 2}$$

As described above, piroxicam is a weak base, and it is partially ionized when dissolved in water. Log D is the ratio of the sum of the concentrations of all forms of the compound (ionized plus non-ionized) in each phase, and is pH dependent. The distribution coefficient is defined as a function of the ratio of total concentration of the solute species in each phase (Equation 3) (38).

$$\text{Log}\, D_{oct/wat} = \text{Log}\left(\frac{[solute]_{octanol}}{[solute]_{ionized\ water} + [solute]_{neutral\ water}}\right) \qquad \text{Equation 3}$$

For a non-ionized drug, Log P is equal to Log D at any pH value, but the effective lipophilicity at any specific pH value is directly related to its pKa value for any ionized drug. In our experimental conditions, Log D of piroxicam were investigated at pH 3.17, 3.79, 4.44, 5.42 and 6.56. Table 1 summarizes the Log D values of piroxicam at the investigated pH values. Log P values given in Table 1 were calculated according to the Equation 1 where pKa of the piroxicam was accepted as 5.7 and 5.3 by the results of UV Spectrophotometric and HPLC measurements, respectively.

Since piroxicam is an acidic drug, it is ionized at basic pH values and tends to be dissolved in aqueous phases. As it was expected, solubility of piroxicam in aqueous phase (phosphate buffer) was increased about 13 fold by the changes at pH from 6.56 to 3.17. This situation was in a harmony with the capacity factor of piroxicam on HPLC for different mobile phase pH values. Although Log D values differ by pH values, calculated Log P values should be identical according to the theory. When the mean and standard error of Log P values were calculated it was found that Log P was 1.58 ± 0.04 if pKa is taken as 5.3, and 1.46 ± 0.05 if pKa is taken as 5.7. This situation shows us that the pKa value of 5.3 determined by HPLC, provides less standard error for the

calculated Log P values. Therefore, Log P value for piroxicam was accepted as 1.58 in our experimental conditions.

Log kw determination

Since the retention time of an analyte in RP-HPLC is directly related with its lipophilicity, this relationship can be used to show how the lipophilicity of an analyte will be affected by pH changes. In our study, initially we have calculated the Log k' values for all mobile phase compositions (80:20, 85:15, 90:10 MeOH:phosphate buffer (20 mM); (v/v)) for pH values below (3.0) and above (6.0) the calculated pKa (5.3). The Log k' values were calculated for the mobile phase not containing any organic phase (0 % MeOH). Figure 5 shows an example of this application for pH 6.0. When there was no MeOH in the mobile phase, the kw' values were calculated for pH 3.0 and 6.0 and found to be 37.5 and 7247.7, respectively.

Conclusion

In this study, spectrophotometric and chromatographic analytical approaches were examined to determine the physicochemical properties of piroxicam. Since, piroxicam is a well-known compound, the results were easily compared with the ones in Drugbank (https://www.drugbank.ca/drugs/DB00554). According to our experimental results, pKa value was found to be 5.7 and 5.3 for spectrophotometric and HPLC experiments, respectively. The value found by UV-VIS Spectrophotometry is close to experimental value of 6.3 given in Drugbank. However, the predicted value of 4.76 given in Drugbank is close to the one calculated by the results of HPLC analysis. Experimental Log P value in Drugbank for piroxicam is 3.06 where predicted values are 0.6 and 2.2. Our experiments show that piroxicam Log P value is 1.58. This value is between the predicted and experimental values reported in Drugbank. The differences for pKa and Log P between experimental and predicted values in Drugbank, indicate the experimental data found in this study is novel for piroxicam. Based on all these results, we can conclude that use of different experimental approaches for determination of physicochemical properties can clearly provide different values for drugs.

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Table 1. Experimental Log D and calculated Log P values of piroxicam

pH	D	Log D	Log P (pKa is accepted as 5.3)	Log P (pKa is accepted as 5.7)
3.17	37.17	1.57	1.57	1.57
3.79	37.38	1.57	1.58	1.58

4.44	27.65	1.44	1.50	1.46
5.42	13.58	1.13	1.50	1.32
6.56	2.89	0.46	1.74	1.38

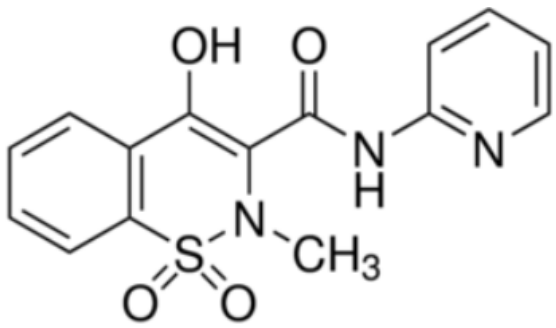

Figure 1. Chemical structure of piroxicam

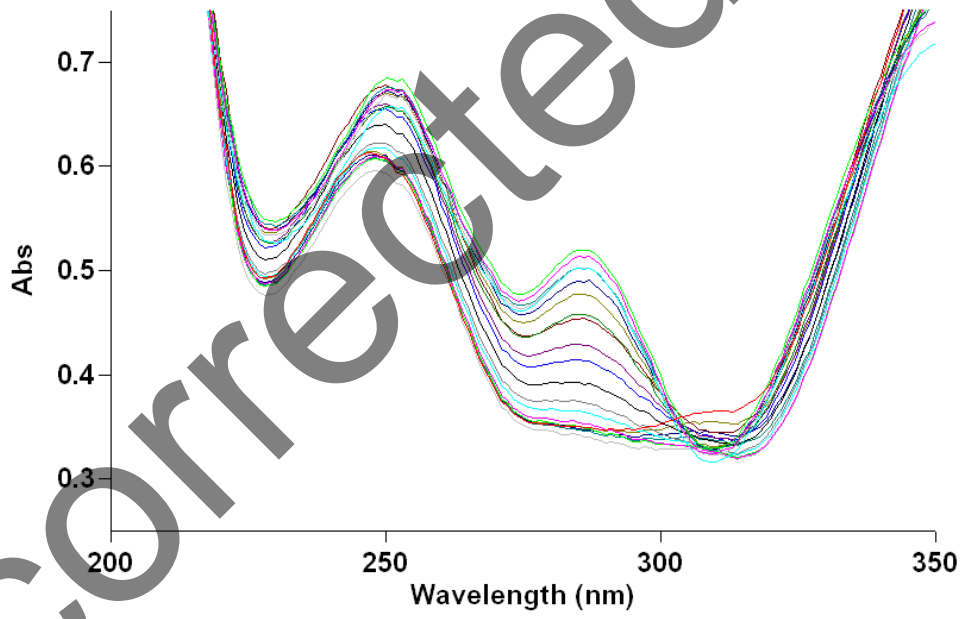

Figure 2. Representative overlaid spectra (200 – 400 nm) of piroxicam under optimum conditions at various pH values (pH 3.09 – 7.48)

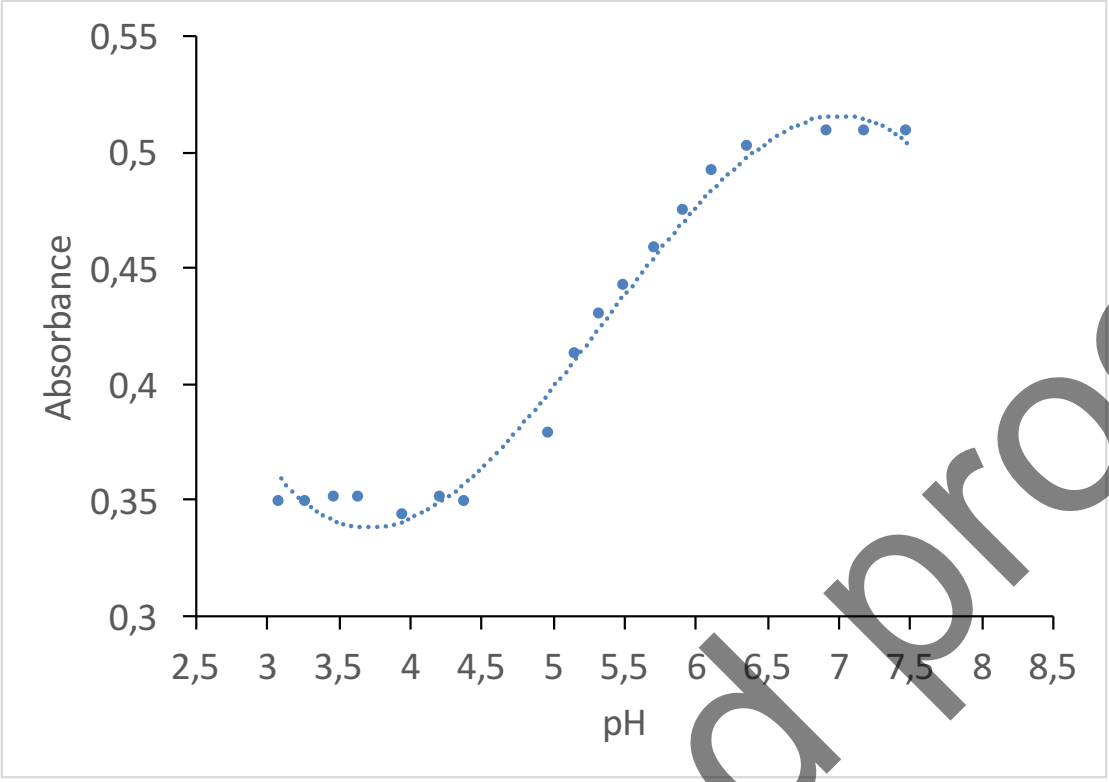

Figure 3. The plot of the absorbance values of piroxicam at 285 nm obtained as a function of pH (3.09 – 7.48)

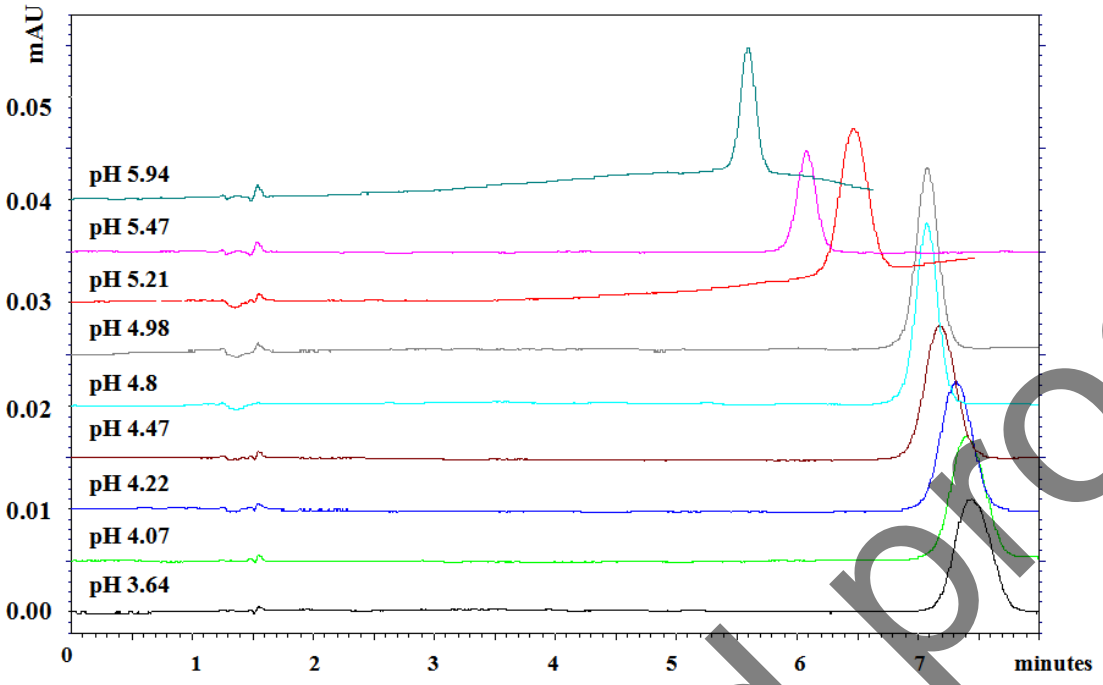

Figure 4. Representative overlaid chromatograms of piroxicam taken under optimum conditions at various pH (3.64 – 5.94). pH values were given on the top of each chromatogram peak.

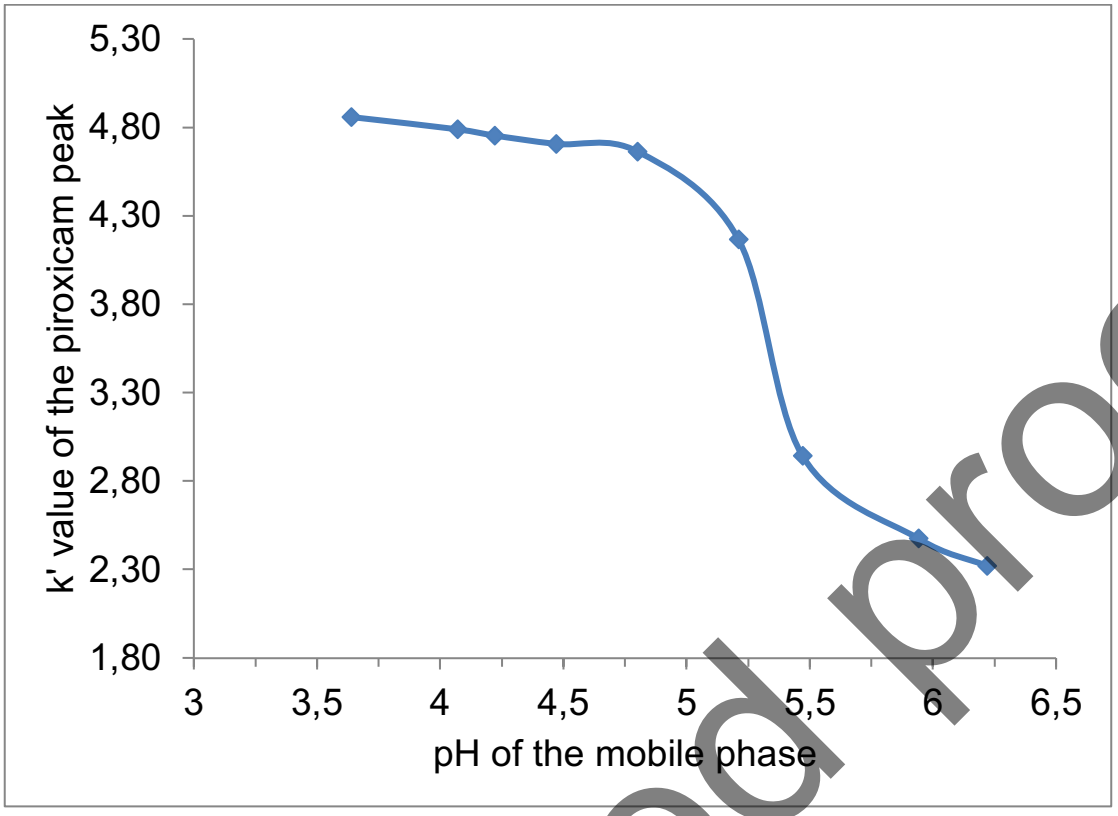

Figure 5. Sigmoidal relationship between pH (3.64 – 6.22) and k' of the piroxicam peak