J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(2): 405-417, 2022
Doi: 10.33483/jfpau.1081989

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF CHROMATOGRAPHIC PROTONATION CONSTANTS OF SOME IMIDAZOLE ANTIMYCOTIC DRUGS IN ACETONITRILE-WATER BINARY MIXTURES

ASETONİTRİL-SU İKİLİ KARIŞIMLARINDA BAZI İMİDAZOL ANTİMİKOTİK İLAÇLARIN KROMATOGRAFİK PROTONASYON SABİTLERİNİN BELİRLENMESİ

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ABSTRACT

Objective: *In this study, the thermodynamic protonation constant ($^{s}_{s}pK_a$) of antifungal azole derivative miconazole and tioconazole were investigated by the reversed-phase liquid chromatography (RPLC) method. Although classical stationary phases are the most commonly used column-packed materials in the analyses of these compounds by the RPLC method, the determination of the pK_a value of miconazole and tioconazole with a cyano-based column has not been reported so far.*

Material and Method: *Chromatographic behavior of the selected compounds was determined with Ultra Cyano column (Restek®, 150×3.0 mm I.D, 3µm) which is compatible with hydrophobic compounds. The dependence of the retention times on the acetonitrile content of the mobile phase and the effect of the mobile phase pH on the chromatographic retention was determined for each compound by changing the pH in the range of 3.0-7.50.*

Result and Discussion: *The $^{s}_{s}pK_a$ values were determined by a nonlinear regression program. The thermodynamic aqueous pK_a ($^{w}_{w}pK_a$) value of the studied compounds was calculated from the $^{s}_{s}pK_a$ value by using the macroscopic parameters (dielectric constant and mole fraction) that play an important role in the solvent properties. The calculated $^{w}_{w}pK_a$ values were compared with literature values.*

Keywords: *Combined effect, Imidazole, Nonlinear regression, RPLC*

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Gönderilme / Submitted: 02.03.2022 **Kabul / Accepted:** 19.04.2022

ÖZ

Amaç: *Bu çalışmada, antifungal azol türevi mikonazol ve tiokonazolün termodinamik protonasyon sabiti ($^{s}_{s}pK_a$) ters faz sıvı kromatografisi (RPLC) yöntemi ile araştırılmıştır. Bu bileşiklerin RPLC yöntemi ile analizlerinde klasik sabit fazlar en yaygın olarak kullanılan kolon dolgulu malzemeler olmasına rağmen, siyano bazlı bir kolonla şimdiye kadar mikonazol ve tiokonazolün pK_a değerinin tayini rapor edilmemiştir.*

Gereç ve Yöntem: *Seçilen bileşiklerin kromatografik davranışları hidrofobik bileşiklerle uyumlu Ultra siyano kolon (Restek®, 150×3.0 mm I.D, 3µm) ile belirlenmiştir. Alıkonma sürelerinin mobil fazın asetonitril içeriğine bağımlılığı ve mobil faz pH'ının kromatografik alıkonma üzerindeki etkisi, pH 3.0-7.50 aralığında değiştirilerek her bir bileşik için belirlenmiştir.*

Sonuç ve Tartışma: *$^{s}_{s}pK_a$ değerleri, doğrusal olmayan bir regresyon programı ile belirlenmiştir. Çalışılan bileşiklerin termodinamik suda pK_a ($^{w}_{w}pK_a$) değeri, çözücü özelliklerinde önemli bir rol oynayan makroskopik parametreler (dielektrik sabiti ve mol fraksiyonu) kullanılarak $^{s}_{s}pK_a$ değerinden hesaplanmıştır. Hesaplanan $^{w}_{w}pK_a$ değerleri literatür değerleri ile karşılaştırılmıştır.*

Anahtar Kelimeler: *Birleşik etki, İmidazol, Nonlineer regresyon, RPLC*

INTRODUCTION

Worldwide, more than 150 million cases of serious fungal infections occur each year. About 1.7 million deaths occur due to these infections each year [1]. Azole antifungals are the most commonly used drug molecules in therapeutic applications [2]. These are synthetic compounds and generally classified as imidazoles or triazoles which include two or three nitrogen atoms, respectively, in the five-membered ring systems. Two important members of imidazole class are Miconazole (MICO) and Tioconazole (TIO) [3]. These antimycotic imidazole compounds have a broad spectrum of activity against different types of microorganisms [4,5]. Due to its non-polar chemical structure and low solubility in blood plasma, tioconazole is used only in skin infections. Miconazole is widely used in the treatment of dermal, oral, and vaginal mycosis [6].

The acid-base dissociation constant, typically represented as pK_a, is a very important physicochemical parameter to be aware of in pharmaceutical research and other fields including the discovery and evaluation of newly synthesized compounds. The 77.5% of the drug-like molecules that are involved in the WHO essential medicine list have ionizable functional groups with a pK_a in the range of 2-12 [7]. The neutral form of these drug molecules is generally water-insoluble, but highly membrane-permeable and lipophilic whereas ionizable forms are vice versa. Thus, lipophilicity, permeability, and solubility of drug molecules all can be influenced by their pK_a value [8]. Furthermore, this type of information can be valuable to evaluate the absorption, distribution, metabolism, and excretion (ADME) properties of drug molecules [9,10].

Analytical methods used to determine pK_a values involve exposing a compound to a changing pH environment and monitoring the changes that occur due to the ionization state of the drug molecule [11]. Although potentiometric titration and spectrophotometric analyzes are routinely used for pK_a determination among these methods, these techniques have disadvantages such as poor solubility,

absence of UV chromophore, and different absorption spectra of neutral and ionized forms [12]. Reversed-phase liquid chromatography (RPLC) is the other preferred method to determine the pK_a value of ionizable substances. This method is based on the different retention behavior of the ionized and neutral forms of drug molecules [13,14]. Among the advantages of this method is its necessity for a very small amount of the analyte and high purity of the sample is not required for the LC assays [15].

In the analyzes performed in this method, the compound must be dissolved with a solvent. To eliminate the harmful effects of organic solvents, water is the first solvent preferred in the analysis. However, in cases the solubility of the compounds in water is insufficient, it is necessary to use an organic solvent-water binary mixture to dissolve the compound. On the other hand, in most of the analyses conducted in a 100% water environment with the RPLC method, problems such as non-repeating retention times (t_R), and phase precipitation are observed in the RPLC column [16]. For these reasons, it is necessary to add a small amount of organic modifiers to the mobile phase to eliminate these problems [17,18]. Acetonitrile (ACN) is the most preferred solvent among organic solvents. The determinations made using binary mixtures formed with water have excellent chromatographic properties [19,20]. The activity coefficients of molecular and ionic species in ACN-water mixtures can be calculated from the Debye-Hückel equation (Eq. 1) [21].

$$\log \gamma = \frac{-AI^{1/2}}{1 + a_o BI^{1/2}} \qquad \text{(Eq. 1)}$$

where the values of the Debye-Hückel A and B constants and the ion size parameter, a_o, in the acetonitrile-water mixtures have been reported by J. Barbosa et.al. [22]. The ionic strength, I, of the mobile phases used can be calculated for each pH value from charge and mass balances, taking into account the pK_{a1} and pK_{a2} values of phosphoric acid at each mobile phase composition, the analytical concentration of this acid in the mobile phase, the pH values and the activity coefficients, using iterative calculation [23].

When the literature is examined, the pK_a values of two imidazole drugs were determined by different computer programs [24], potentiometric titration [25], pH-metric method [10], capillary electrophoresis [26] and there is only one study aimed at determining the pK_a values with a liquid chromatographic method [27]. In this study, the retention behavior of MICO and TIO was investigated in the microheterogeneity region (30-45 v/v%) at 37°C. The thermodynamic pK_a ($^s_s pK_a$) values of these two compounds in ACN-water binary mixtures were determined by using the t_R value determined depending on the mobile phase pH change and the calculated activity coefficients in this region. The pK_a values ($^w_w pK_a$) in zero cosolvent concentration of these two poor water-insoluble compounds were

calculated by the mole fraction-$^{s}_{s}pK_a$ relationship and Yasuda-Shedlovsky (YS) extrapolation method. In addition, the degree of ionization of the related compound was calculated using the $^{w}_{w}pK_a$ values.

MATERIAL AND METHOD

Chemicals and Standard Solutions

All the analytical chemicals have not been subjected to any purification process. MICO and TIO were supplied from Sigma-Aldrich (St. Louis, USA). Acetonitrile (ACN), o-phosphoric acid, potassium hydrogen phthalate (KHP), sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). The TIO and MICO were prepared in ACN-water binary mixtures at a concentration of 50 µg/mL. These solutions were prepared daily and stored at +4°C.

Instrumentation and Apparatus

The Shimadzu HPLC system is integrated with an SPD-20A model UV-vis detector, a CTO-20A model column oven, an LC-20AD model pump, and a DGU-20A3 model degasser. Mettler Toledo InLab 413 Ag/AgCl glass electrode combined with a Mettler Toledo MA 235 pH/ion analyzer was used for pH measurements of RPLC. Potassium hydrogen phthalate (0.05 mol/kg) was chosen for electrode calibration in binary mixtures of ACN-water [28,29]. In adjusting the mobile phase pH, the temperature was kept constant at 25 ºC. The ultrapure water purification process was carried out with the Direct Q3 UV system (Millipore-France).

Chromatographic Conditions

Experiments were carried out in different proportions of ACN-water binary mixtures (30:70; 35:65; 40:60; 45:55 v/v%) which include 25 mM orthophosphoric acid as a mobile phase component in this study. Due to the appropriate pK_a values (pK_{a1}, pK_{a2}) and buffering capacity, orthophosphoric acid is sufficient to adjust the mobile phase pH ($^{s}_{s}pH$). The $^{s}_{s}pH$ from 3.0 to 7.5 is set by 1 M NaOH [30]. The separation was carried out in the Ultra Cyano column (Restek®, 150 ×3.0 mm I.D, 3µm) at a constant temperature of 37 °C. In the HPLC system, the flow rate of the mobile phase was fixed at 0.6 mL/min. Analysis of the compounds with a UV-visible detector gave a maximum absorbance value of 204 nm. All retention data were obtained with three repeated analyses.

Evaluation of data

In this study, the $^{s}_{s}pK_a$ values of the TIO and MICO were calculated using the relationship between t_R and $^{s}_{s}pH$, taking into account the activity coefficients in the selected binary mixtures of ACN-water [22]. Data were calculated with the nonlinear regression (NLREG) program [31].

RESULT AND DISCUSSION

In the RPLC method, cyano columns allow the elution of hydrophobic compounds more quickly, using fewer organic solvents. In addition, it provides good selectivity for the determination of polar compounds and provides different selectivity than C18 and C8 columns. Cyano phase also shows enhanced retention of certain basic analytes relative to C18. In the literature, high concentration organic modifier and C18 columns are preferred for the separation of basic hydrophobic compounds in RPLC analyses [21,32]. The TIO and MICO selected in this study are also highly hydrophobic compounds (log*P*: 6.11; log*S*:-6.42 for TIO; log*P*: 5.93; log*S*:-6.26 for MICO) [33]. For the chromatographic analysis of these two drug molecules, the cyano column (Restek®) was preferred. In this study, the retention behavior of the studied compounds has been examined using different isocratic elution conditions on the selected RPLC column. The combined effects of pH and ACN percentage on the t_R values were investigated. Changes in retention time were determined by the changing of the $\frac{s}{s}pH$ and concentration of the selected organic modifier (ACN) in the mobile phase. All experiments were performed at least three times. The relative standard deviation is below 2%. A chromatogram showing the change of t_R value of MICO and TIO depending on the mobile phase pH change is given in Figure 1.

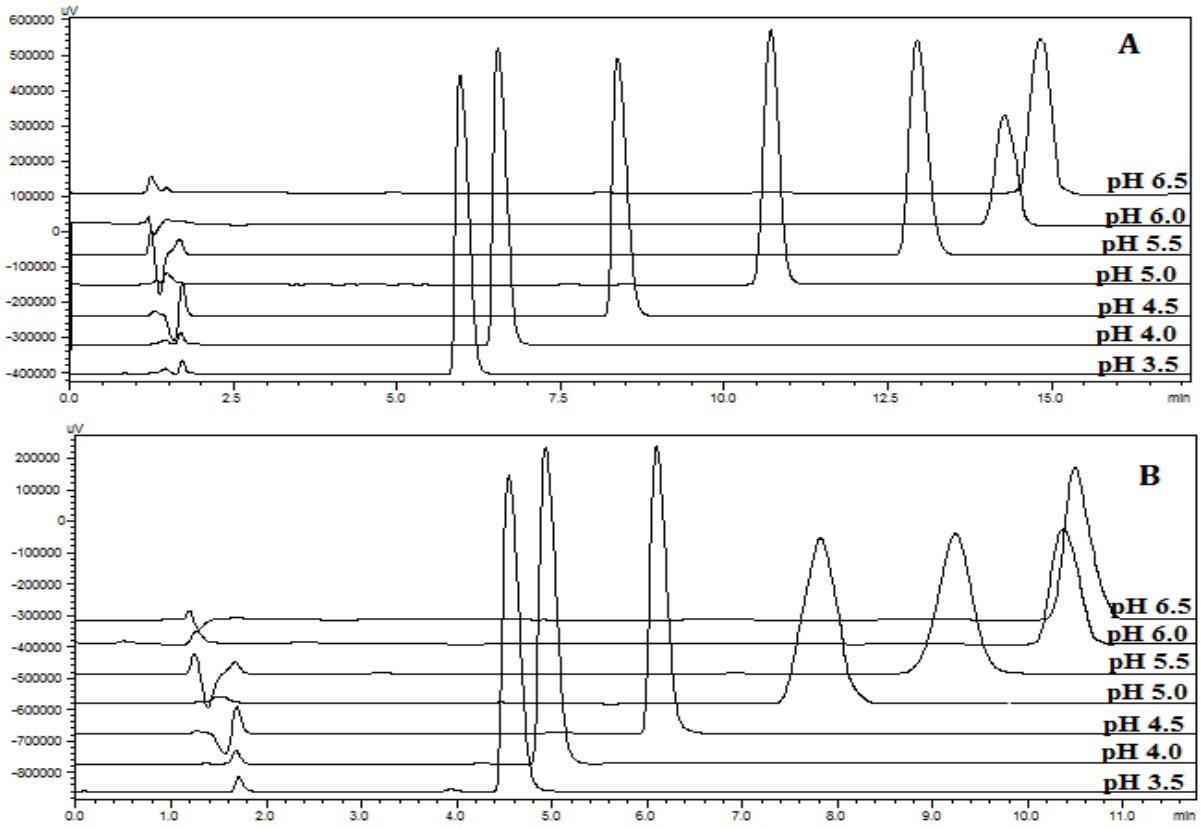

Figure 1. Overlaid RPLC chromatograms of ionized and molecular forms A) MICO B) TIO (ACN-water binary mixture containing 40 v/v% ACN)

The percentage of solvent, column temperature, and pH of the mobile phase are major factors to determine the retention time of ionizable molecules. The retention times were obtained over a pH range of 3.0-7.5, to determine pK_a of MICO and TIO using the RPLC method. In Figure 2, data pairs of t_R/ ${}^{s}_{s}pH$ for investigated compounds in different percentages of acetonitrile-water binary mixtures are shown, together with the corresponding experimental and calculated retention times. The t_R values of the studied compounds are plotted as a function of the mobile phase pH using a nonlinear regression program (Figure 2).

In the calculations made in this program, the thermodynamic ${}^{s}_{s}pK_a$ values were calculated by taking the activity coefficients into account. The ${}^{s}_{s}pK_a$ value, the retention time of the molecular (t_{RB}), and ionized (t_{RBH^+}) form of the compounds were calculated by the NLREG program (Table 1). The activity coefficients of the ionic and molecular species in the studied hydro-organic mixtures were computed in each mobile phase and constant ionic strength with the Debye-Hückel equation [22].

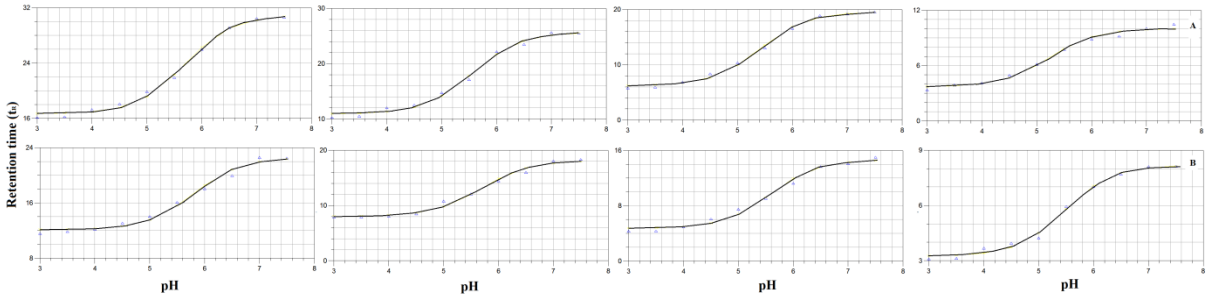

Figure 2. Plots of the retention times versus the pH of the mobile phase for A)30% B)35% C)40% D)45 v/v% ACN. The solid lines indicate the predicted retention times

Table 1. The thermodynamic pK_a values and the retention times of ionic and neutral species of the studied antifungal drugs

Compounds	**ACN (v/v%)**	$\boldsymbol{pK_a}$	$\boldsymbol{t_{RBH^+}}$	$\boldsymbol{t_{RB}}$
Miconazole	**30**	5.684 (0.061)*	16.661 (0.274)	30.939 (0.371)
	35	5.553 (0.075)	10.892 (0.366)	25.661 (0.452)
	40	5.411 (0.058)	6.188 (0.270)	19.595 (0.302)
	45	5.227 (0.089)	3.690 (0.207)	10.021 (0.302)
Tioconazole	**30**	5.801 (0.098)*	12.121 (0.310)	22.566 (0.458)
	35	5.688 (0.094)	8.055 (0.300)	18.130 (0.408)
	40	5.584 (0.088)	4.718 (0.286)	14.672 (0.361)
	45	5.466 (0.067)	3.248 (0.112)	8.182 (0.130)

* standard deviation

MICO and TIO show basic retention behavior in RPLC and the experimentally determined ${}^{s}_{s}pK_a$ values belong to a nitrogen atom at position 3 (N3) in the imidazole ring. As the concentration of acetonitrile in the mobile phase increases, the ${}^{s}_{s}pK_a$ value of the compounds decreases. The pK_a values demonstrate the equilibrium between the positively charged, protonated nitrogen (N3) atom of the studied drug molecules and their neutral conjugate bases.

The ${}^{s}_{s}pK_a$ of the two hydrophobic antifungals were determined in studied binary mixtures since they are insoluble in water. Because of this, the thermodynamic aqueous pK_a (${}^{w}_{w}pK_a$) values at zero cosolvent concentration were computed by Yasuda-Shedlovsky (YS) extrapolation method. In this method, the calculated dielectric constant (ε) values in the studied ACN (v/v%) were used. The ε values at the different compositions of binary mixtures of ACN and water were obtained from Barbosa et al. [34]. The ${}^{w}_{w}pK_a$ values of these two antimycotic drugs were calculated by using the linear equations which were obtained by plotting ${}^{s}_{s}pK_a$+ log[H_2O] data against 1/ε values. The ${}^{w}_{w}pK_a$ values at zero cosolvent concentration are given in Table 2.

Table 2. The ${}^{w}_{w}pK_a$ value of the MICO (A) and TICO (B) by using the Yasuda-Shedlovsky extrapolation method and X_{ACN}-${}^{s}_{s}pK_a$ relationship

Yasuda-Shedlovsky extrapolation method								**X_{ACN}-${}^{s}_{s}pK_a$ relationship**	
Compounds	**ACN (v/v%)**	**ε**	**1/ε**	**[H_2O]**	**log[H_2O]**	**pK_a**	**${}^{s}_{s}pK_a$+log[H_2O]**	**Equation**	**r**
Miconazole	30	62.220	0.0161	53.889	1.731	5.684	7.415	${}^{s}_{s}pK_a$ = -5.172X_{ACN} + **6.336**	0.999
	35	60.230	0.0166	53.611	1.729	5.553	7.282		
	40	58.240	0.0172	53.333	1.727	5.411	7.138		
	45	56.250	0.0178	53.056	1.725	5.227	6.952		
	0	74.180				**6.374**			
Tioconazole	30	62.220	0.0161	53.889	1.731	5.801	7.532	${}^{s}_{s}pK_a$ = -3.781X_{ACN} + **6.269**	0.999
	35	60.230	0.0166	53.611	1.729	5.688	7.417		
	40	58.240	0.0172	53.333	1.727	5.584	7.311		
	45	56.250	0.0178	53.056	1.725	5.466	7.191		
	0	74.180				**6.296**			

Figure 3, shows a straight line in all cases according to the YS plots. The slopes of the linear lines in the graphs are different from each other (Figure 3). Basic functional groups containing two drugs have negative slopes. These straight lines could be clarified by taking into account the structural features of ACN-water mixtures.

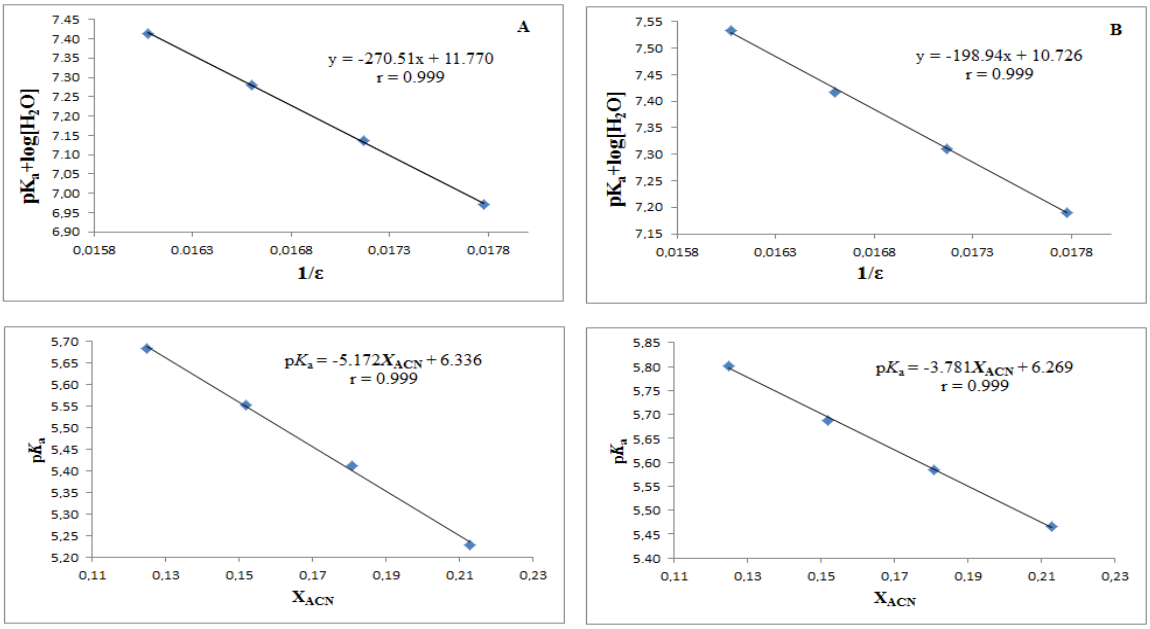

Figure 3. Yasuda-Shedlovsky extrapolation and X_{ACN} -$^{s}_{s}pK_a$ relationship

A negative slope is indicative of decreasing basicity with increasing organic solvent concentration in the mixture. Another approach to determine the $^{w}_{w}pK_a$ values of the compounds in the aqueous medium is to determine the mole fraction of the ACN -$^{s}_{s}pK_a$ relationship. The calculated $^{s}_{s}pK_a$ values in this study were plotted against the X_{ACN} value. The intercept value of the linear graph plotted using the X_{ACN} -$^{s}_{s}pK_a$ relationship gives the $^{w}_{w}pK_a$ value of the compound (Figure 3). The $^{w}_{w}pK_a$ values calculated by these two approaches are given in Table 2. The negative slope is due to the basic functional group of the compound. It is seen that the $^{w}_{w}pK_a$ values calculated using these two macroscopic constants are compatible with each other.

An active drug substance can cross the cell membrane in a non-ionized form. Therefore, the degree of ionization must be known. The degree of ionization is necessary for the determination of ADME properties and can be calculated using the Henderson-Hasselbach equation [35]. In this study, the studied compounds with the basic functional group, whose $^{w}_{w}pK_a$ value was determined, become 50% completely ionized at the $^{w}_{w}pK_a$ value of MICO and TIO. Molecular and anionic compounds were determined by using the % ionization values calculated in the pH 1.5-14 ranges (Figure 4). In basic compounds, two units above the pK_a value represent the non-ionized state of the compound.

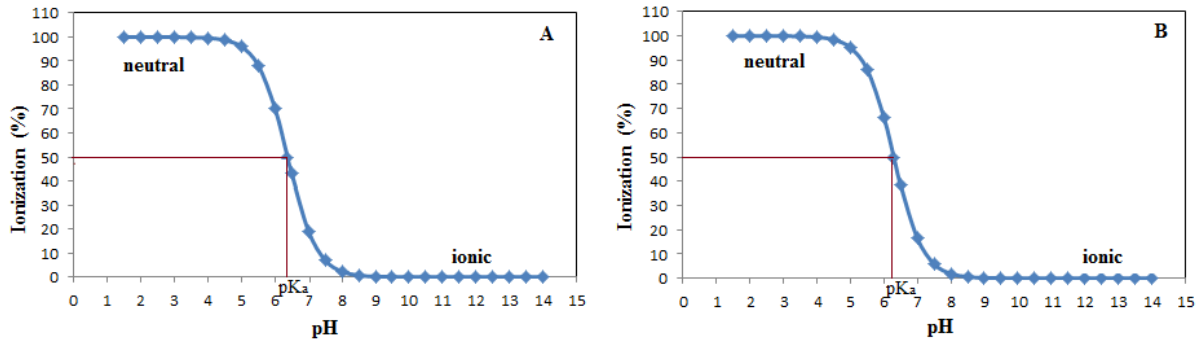

Figure 4. Percentage ionization of studied compound as a function of pH A) MICO, B) TIO

When the literature studies are examined, the experimental study (mostly for miconazole) is limited to determining the pK_a values of MICO and TIO. The pK_a values of the compounds were estimated using different computer programs [24,36]. According to the data obtained from these programs, pK_a values for these two compounds were calculated between 5.99-6.48 (Table 3). There is only one experimental study in which the pK_a value of tioconazole was determined. In this study performed with the RPLC method, the pK_a value of miconazole was also determined [27]. In this study, MICO and TIO were analyzed at 62.5%, 65%, and 70% ACN-water binary mixtures with X-Terra RP-18 column at 25 ºC. High ACN concentration was used in the analysis of these two compounds by the RPLC method. In addition, a hybrid-based C18 column was preferred. In our study, MICO and TIO were analyzed with an HPLC column containing the cyano phase, showing different selectivity compared to C8 and C18 columns. Because this type of column was chosen, compounds could be determined with low ACN concentration. In the conducted study by Sanli et al. [27]. The pK_a value of MICO was determined by a potentiometric method in a binary mixture of methanol-water (50:50 v/v%) [25]. In a capillary electrophoresis study [26], the pK_a value of MICO was determined by the pressure-assisted capillary electrophoresis method in an aqueous solution with a pH range of 2.0 to 12.4 and an ionic strength of 0.05 M. (Table 3).

Table 3. pK_a values of studied compounds obtained from different methodologies.

Compounds	**Computer programs**	**Literature values**
TIO	6.48 [36]	7.10 [27]
MICO	5.99 [24], 6.48 [36]	6.16 [27], 6.91 [25], 6.63 [26], 6.07 [37]

The present study shows that the pH of the buffer and the percentage of acetonitrile in the mobile phase were two important parameters that affected the separation and chromatographic results. Most of the determinations of these hydrophobic antifungal compounds with low solubility by the RPLC method are carried out in high organic modifiers and C18, C8 columns. This study, which took place at 37 °C,

was performed both in the microheterogeneity region and in the cyano column. Since there is only one experimental data on tioconazole and a limited number of experimental data on miconazole, the thermodynamic data calculated with this study is the first in the literature. With this study, experimentally determined physicochemical parameter thermodynamic pK_a value will provide information about the transport process of the drug molecules through biological membranes and the advancement in the use of nanostructures for the drug transport mechanism.

ACKNOWLEDGMENTS

We acknowledge Dr. Jose Luis Beltran (University of Barcelona) for kindly providing the NLREG 4.0 program.

AUTHOR CONTRIBUTIONS

Concept: *H.S., E.Ç.D., K.P.;* Design: *E.Ç.D.;* Control: *E.Ç.D., K.P.;* Sources: *H.S., E.Ç.D., K.P.;* Materials: *H.S., K.P.;* Data Collection and/or processing: *H.S., K.P.;* Analysis and/or interpretation: *H.S.;* Literature review: *E.Ç.D., K.P.;* Manuscript writing: *E.Ç.D.;* Critical review: *H.S., E.Ç.D., K.P.;* Other: *H.S.*

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that this study does not include any experiments with human or animal subjects.

REFERENCES

1. Kainz, K., Bauer, M.A., Madeo, F. Carmona-Gutierrez, D. (2020). Fungal infections in humans: the silent crisis. *Microbial Cell*, *7*(6), 143-145. [CrossRef]

2. Moraes, F., Bittencourt, S.F., Perissutti, E., Frencentese, F., Arruda, A.M.M., Shi Chen, L., Babadópulos, T., Nucci, G. (2014). Quantification of dapaconazole in human plasma using high-performance liquid chromatography coupled to tandem mass spectrometry: Application to a phase I study. *Journal of Chromatography B, 958*, 102-107. [CrossRef]

3. Shalini, K., Kumar, N., Drabu, S., Sharma, P.K. (2011). Advances in synthetic approach to and antifungal activity of triazoles. *Beilstein journal of organic chemistry, 7*, 668-677. [CrossRef]

4. Carrillo-Muñoz, A.J., Tur-Tur, C., Hernández-Molina, J.M., Santos, P., Cárdenes, D., Giusiano, G. (2010). Antifungal agents for onychomycoses. *Revista Iberoamericana de Micologia, 27*, 49-56. [CrossRef]

5. Piel, G., Evrard, B., Fillet, M., Llabres, G., Delattre, L. (1998). Development of a non-surfactant parenteral formulation of miconazole by the use of cyclodextrins. *International journal of pharmaceutic, 169*, 15-22. [CrossRef]

6. Zan, M.M., Cámara, M.S., Robles, J.C., Kergaravat, S.V., Goicoechea, H.C.(2009). Development and validation of a simple stability-indicating high performance liquid chromatographic method for the determination of miconazole nitrate in bulk and cream formulations. *Talanta, 79*, 762-767. [CrossRef]

7. Manallack, D. (2007). The pKa Distribution of Drugs: Application to Drug Discovery. *Perspectives in Medicinal Chemistry*, *1*, 25-38. [CrossRef]

8. Avdeef, A. (2012). Absorption and Drug Development, Solubility, Permeability, and Charge State, John Wiley & Sons, Inc., Hoboken, New Jersey, p.31.

9. Box, K., Bevan, C., Comer, J., Hill, A., Allen, R., Reynolds, D. (2003). High-throughput measurement of pKa values in a mixed-buffer linear pH gradient system. *Analytical Chemistry, 75*, 883-892. [CrossRef]

10. Box, K., Comer, J. (2008). Using Measured pKa, LogP and Solubility to Investigate Supersaturation and Predict BCS Class. *Current drug metabolism*, *9*, 869-878. [CrossRef]

11. Jelfs, S., Ertl, P., Selzer, P. (2007). Estimation of pKa for druglike compounds using semiempirical and information-based descriptors. *Journal of Chemical Information and Modeling, 47*, 450-459. [CrossRef]

12. Babić, S., Horvat, A.J.M, Mutavdžić, P., Kaštelan-Macan, M. (2007). Determination of pKa values of active pharmaceutical ingredients. *TrAC - Trends in Analytical Chemistry, 26*, 1043-1061. [CrossRef]

13. Canals, I., Valkó, K., Bosch, E., Hill, A.P., Rosés, M. (2001). Retention of ionizable compounds on HPLC. 8. Influence of mobile-phase pH change on the chromatographic retention of acids and bases during gradient elution. *Analytical Chemistry, 73*, 4937-4945. [CrossRef]

14. Yılmaz, H., Üstün, Z., Çubuk Demiralay, E. (2016). RPLC determination of acid dissociation constants and quantitative estimation for sulfasalazine. *Journal of the Iranian Chemical Society, 13*, 103-110. [CrossRef]

15. Manderscheid, M., Eichinger, T. (2003). Determination of pKa values by liquid chromatography. *Journal of Chromatographic Science, 41*, 323-326. [CrossRef]

16. Bocian, S., Krzemińska, K. (2019). The Separations Using Pure Water as A Mobile Phase in Liquid Chromatography Using Polarembedded Stationary Phases, *Green chemistry letters and reviews, 12*(1), 69-78. [CrossRef]

17. Kazakevich, Y., Lobrutto, Y. (2007). HPLC for Pharmaceutical Scientists, Wiley-Interscience, USA, p.232.

18. Espinosa, S., Bosch, E., Rosés, M. (2002). Retention of ionizable compounds in high-performance liquid chromatography. IX. Modelling retention in reversed-phase liquid chromatography as a function of pH and solvent composition with acetonitrile-water mobile phases. *Journal of Chromatography A, 947*(1), 47-58. [CrossRef]

19. Daldal, Y.D., Çubuk Demiralay, E. (2020). Chromatographic and UV-Visible Spectrophotometric pKa Determination of Some Purine Antimetabolites. *Journal of Molecular Liquids, 317*, 1-8. [CrossRef]

20. Bosch, E., Espinosa, S., Rosés, M. (1998). Retention of Ionizable Compounds on High-Performance Liquid Chromatography III. Variation of p*Ka* Values of Acids And pH Values of Buffers in Acetonitrile-Water Mobile Phases. *Journal of Chromatography A, 824*, 137-146. [CrossRef]

21. Poole, C.F., Poole, S.K. (1991). Chromatography Today. Elsevier, Amsterdam, p.412.

22. +Barbosa, J., Bergés, R., Sanz-Nebot, V. (1998). Retention behaviour of quinolone derivatives in high-performance liquid chromatography: Effect of pH and evaluation of ionization constants, *Journal of Chromatography A, 823*(1-2), 411-422. [CrossRef]

23. Rosés, M., Bosch, E. (2002). Influence of mobile phase acid–base equilibria on the chromatographic behaviour of protolytic compounds. *Journal of Chromatography A, 982*(1), 1-30. [CrossRef]

24. Pallicer, J.M., Calvet, C., Port, A., Rosés, M., Ràfols, C., Bosch, E. (2012). Extension of the liquid chromatography/quantitative structure-property relationship method to assess the lipophilicity of neutral, acidic, basic and amphotheric drugs. *Journal of Chromatography A, 1240*, 113-122. [CrossRef]

25. Peeters, J. (1978). Determination of ionization constants in mixed aqueous solvents of varying composition by a single titration. *Journal of Pharmaceutical Sciences, 67*, 127-129. [CrossRef]

26. Miller, J.M., Blackburn, A.C., Shi, Y., Melzak, A.J., Ando, H.Y. (2002). Semi-empirical relationships between effective mobility, charge, and molecular weight of pharmaceuticals by pressure-assisted capillary electrophoresis: Applications in drug discovery. *Electrophoresis, 23*, 2833-2841. [CrossRef]

27. Şanli, S., Başaran, F., Şanli, N., Akmeşe, B., Bulduk, I. (2013). Determination of dissociation constants of some antifungal drugs by two different methods at 298 K. *Journal of Solution Chemistry*, *42*, 1976-1987. [CrossRef]

28. Mussini, T., Covington, A.K., Longhi, P., Rondinini, S. (1985). Criteria for Standardization of pH Measurements in Organic Solvents and Water - Organic Solvent Mixtures of Moderate to High Permittivities. *Pure and Applied Chemistry*, *57*(6), 865-876. [CrossRef]

29. Rondinini, S., Mussini, P.R., Mussini, T. (1987). Reference Value standards and Primary standards for pH Measurements in Organic Solvents and water - organic Solvent Mixtures of Moderate to High Permittivities. *Pure* and *Applied Chemistry*, *59*, 1549-1560. [CrossRef]

30. Dolan, J.A. (2009). A Guide to HPLC and LC-MS Buffer Selection. New York. [CrossRef]

31. Sherrod, P. H. Nonlinear Regression Analysis Program (NLREG) Version 4.0. Nashville, TN, USA. 1998. Accessed date 20.05.2021.

32. Marchand, D.H., Croes, K., Dolan, J.W., Snyder, L.R., Henry, R.A., Kallury, K.M.R., Waite, S., Carr, P.W. (2005). Column selectivity in reversed-phase liquid chromatography. VIII. Phenylalkyl and fluoro-substituted columns. *Journal of Chromatography A*, 1062(1), 65-78. [CrossRef]

33. SwissADME program. http://www.swissadme.ch/. Accessed date 08.06.2020.

34. Barbosa, J., Sanz-Nebot, V. (1991). Autoprotolysis Constants and Standardization of the Glass Electrode in Acetonitrile-Water Mixtures. Effect of Solvent Composition. *Anaytica Chimica Acta*, *244*, 183-191. [CrossRef]

35. Tallarida, R.J., Murray, R.B. (1987). Henderson-Hasselbalch Equation. In: R.J. Tallarida, R.B. Murray (Eds.), Manual of Pharmacologic Calculations, (pp 74-75). New York: Springer.

36. http://www.chemicalize.org. Accessed date 20.07.2018.

37. Avdeef, A. (1993). Applications and Theory Guide to pH-Metric pKa and log P Measurement, Sirius Analytical Instruments Ltd. Forest Row, UK, p. 241.