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Determination of protonation constant value of thiabendazole in ethanol-water binary mixtures by green RPLC method

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ABSTRACT: In this study, an environmentally friendly analysis of an anti-infective/anthelmintic drug thiabendazole was carried out by liquid chromatography method using ethanol solvent that is not harmful to human health and the environment. Protonation constant ($^{s}_{s}pK_a$) values of thiabendazole in 15, 20, 25, and 30% (v/v) ethanol-water binary mixtures at 37 °C were obtained by using the green reverse phase liquid chromatography method. The method is based on a model that relates the chromatographic behavior of the determined analytes to pH. The linear relationship between the solvatochromic parameter E_T^N and the retention values of the compound was determined depending on the variation of the organic solvent in the mobile phase. The relationship between the calculated $^{s}_{s}pK_a$ value and the macroscopic properties of ethanol was investigated. Compatibility was observed between the pK_a ($^{w}_{w}pK_a$) values in the aqueous medium determined by this linear approach. By using the $^{w}_{w}pK_a$ value calculated for thiabendazole, the ionization percentages were calculated at different pH (1-12) values with the Henderson-Hasselbalch equation.

KEYWORDS: Benzimidazole; pK_a value; ethanol-water mixtures; modeling of retention.

1. INTRODUCTION

Nitrogen-containing heterocyclic compounds play an important role in the drug discovery process. The benzimidazole group thiabendazole, which is widely used in the treatment of human helminthic infections, is the first drug of this class [1]. The fruits are sprayed or dipped with a mixture of thiabendazole and wax. For this, thiabendazole is also analyzed as a pesticide residue [2].

Among the studies for the determination of thiabendazole in different samples, the most widely used analytical method is reverse phase liquid chromatography (RPLC) [3-6]. This method is the most popular of the liquid chromatographic techniques. Almost 80-90% of analyzes of low molecular weight samples are performed using this method [7]. By providing both retention and selectivity at the same time, the determination of acidic, basic, and neutral compounds can be performed. This chromatographic determination is carried out using chemically modified stationary phases. Silica-based filler materials are widely used in applications for RPLC separations in the pharmaceutical industry. In addition, for successful separation, the type of organic modifier in the mobile phase, the eluent composition and the change in pH, and buffer concentration should be ensured. If the determined compound is ionizable, it is affected by the pH of the mobile phase, the temperature, and the change in the organic solvent concentration in the mobile phase. The strength of the mobile phase depends not only on the concentration of the organic solvent but also on the type of solvent used. In recent years, the identification of drug candidates has often faced the problem that many new molecules in drug discovery are less water-soluble and more lipophilic. This problem can be solved by using water-organic solvent binary mixtures. In hydro-organic mixtures formed by mixing with water in different volumes, depending on the change in its amount, it plays an active role in the retention behavior of the compound analyzed in RPLC [7,8]. Although they are toxic, the most frequently used solvents as mobile phase in RPLC are organic solvents such as tetrahydrofuran, acetonitrile, and methanol. In green chemistry,

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alternative solvents are used to minimize the negative environmental impact of these toxic solvents. Ethanol is a particularly preferred solvent for green liquid chromatography as it has similar properties to acetonitrile and methanol but is less volatile and less toxic [9]. Although the elution power of ethanol is lower than that of acetonitrile, the development of high-performance column technology provides satisfactory results for ethanol. The green RPLC method using mobile phases containing ethanol has been widely reported for the analysis of active ingredients in drug formulations [10-12].

In the analysis of basic ionizable compounds, the pH of the mobile phase at least one unit away from the protonation constant (pK_a) values of the analytes can be preferred for chromatographic determination. pK_a is an important physicochemical parameter. The pK_a values of the analyte affect the solubility of ionizing compounds as well as an effective parameter in determining their pharmacokinetic (ADME-absorption, distribution, metabolism, excretion) properties. The potentiometric titrations and spectrophotometric analyses used for the determination of this physicochemical parameter can be problematic if the compound is not pure, poorly soluble in water, has no distinct chromophore group, and is low in amount. Determination of pK_a values by the RPLC method, which overcomes such disadvantages, is preferred [13-15].

Using the relationship between the studied mobile phase pH (${}_s^spH$) and analyte retention time (t_R), the pK_a (${}_s^spK_a$) value of the specific functional group in the analyte's structure can be calculated in hydro-organic mixtures. With this determination, which can be performed with the isocratic method, the pK_a value of many active pharmaceutical ingredients can be determined today.

The ${}_s^spH$ dependence of chromatographic retention for ionizable compounds in RPLC was suggested by Horvath et. al. (Eq. (1)) [16]. This equation shows the variation of the retention time for a weak monoprotic base (B) with hydrogen ion activity ($a_{H_m^+}$) in the mobile phase.

$$t_R = \frac{t_{RBH^+} + t_{RB}\frac{K_a\gamma_{BH_m^+}}{a_{H_m^+}}}{1+\frac{K_a\gamma_{BH_m^+}}{a_{H_m^+}}} \qquad \text{[Eq. 1]}$$

B and BH^+ represent the molecular base and ionized base, respectively. $\gamma_{BH_m^+}$ is the activity coefficient of the ionized base in the mobile phase, which can be calculated by the Debye-Hückel equation [17].

In RPLC, the polarity of the mobile phase has a significant effect on the retention of the analyte in the stationary phase. As the polarity of the mobile phase increases with the addition of water, the retention in RPLC increases [18]. Solvatochromic solvent polarity parameters are used to describe the effect of solvents on many physicochemical solute properties. The most widely used among these is the E_T^N parameter. E_T^N values can be correlated with solute properties such as ionization constant and retention parameters in the RPLC method. The normalized E_T^N scale of mobile phase polarity is used to examine chromatographic retention [19]. There is a linear relationship between the log t_R value of the analyte and the E_T^N parameter. This parameter has been used to estimate the chromatographic behavior of the analyte. The variation of the molecular and ionized (t_{RB}, t_{RBH^+}) species of a base with the percentage of ethanol in the mobile phase is expressed by the Dimroth and Reichardt polarity parameter (Eq. (2), Eq. (3)) [19].

$$\log t_{RB} = C_B + e_B E_T^N \qquad \text{[Eq. 2]}$$

$$\log t_{RBH^+} = C_{BH^+} + e_{BH^+} E_T^N \qquad \text{[Eq. 3]}$$

Substituting Equations (2), (3) in Eq. (1), the theoretical equation describing the dependence of retention time for basic compounds as a function of pH and E_T^N can be expressed by Eq. (4).

$$t_R = \frac{10^{(C_{BH^+} + e_{BH^+} E_T^N)} + 10^{(C_B + e_B E_T^N)} \cdot (K_a\gamma_{BH_m^+} / a_{H_m^+})}{1 + (K_a\gamma_{BH_m^+} / a_{H_m^+})} \qquad \text{[Eq. 4]}$$

C_B and C_{BH^+} are intercept values of the molecular and ionic species, respectively. Similarly, e_B and e_{BH^+} are the slope values of these species [13].

To determine the retention time, especially for water-insoluble substances, it is necessary to add an organic solvent to the mobile phase. In this study, ethanol was used instead of the toxic solvents commonly used in mobile phases in RPLC. ${}_s^spK_a$ values of thiabendazole in an acetonitrile-water binary mixture were determined by the RPLC method [20]. In this study, thiabendazole were determined in an ethanol-water binary mixture. There are no studies done with this binary mixture. These data will be the first data in the literature. In the analyzes made with the RPLC method, the pK_a determination of the compound with low solubility in the aqueous medium is made in hydro-organic mixtures and the pK_a value (${}_w^wpK_a$) in the water medium can be calculated by using the ${}_s^spK_a$ obtained in these mediums [7,21]. ${}_w^wpK_a$ values for the studied compound were calculated using macroscopic parameter values [22].

2. RESULTS AND DISCUSSION

2.1. Influence of pH on chromatographic behavior

In this study, instead of working with the classical silica-based column, the cyano-based column was preferred for the analysis of thiabendazole used in the treatment of human helminth infections. The Pinnacle DB cyano (5 µm, 250 x 4.6 mm) column was developed for the analysis of acidic, basic, and neutral compounds. Its pH stability and reproducibility are higher than conventional silica and polymeric-based columns. It is composed of spherical particles with a pore size of 140 A°. In the literature, there is no data on the retention behavior of the compound in liquid chromatographic analyses with such columns. In addition, the fact that the theoretical plate number of the column is high, and the tailing factor of the compound is lower than 2 shows that the symmetry of the compound and the column performance is good.

In the present study, the analysis of the compound was carried out in ethanol-water binary mixtures. There is no study on the analysis of thiabendazole in this hydro-organic mixture. There is only one study in which the retention behaviors of the compound were determined by the RPLC method in core-shell based particles with a bonded silica phase column and acetonitrile-water binary mixture [20]. In chromatography, ethanol has similar properties to acetonitrile and methanol. In the studies carried out on the octadecyl column, the analyzes made in the ethanol-water binary mixture and the analyzes made in the methanol-water and acetonitrile-water mixtures were compared. According to this study, it has been shown that the adsorption mechanisms of solvents are quite similar. Similar separation mechanisms can be expected using these different solvent mixtures [23].

Thiabendazole with a benzimidazole ring has nitrogen atoms. This compound has acidic and basic functional groups in its chemical structure. Since the pK_a values of the acidic group are above 12 and the pH upper limit value of the HPLC columns is 12, the pK_a value of this group cannot be determined by the RPLC method.

The ${}^{s}_{s}pH$ value is where the electrode system is calibrated with buffer-organic mixtures of the same composition as the mobile phase, and the pH of the hydro-organic mobile phase is measured after the addition of the ethanol. In this study, the mobile phase pH range was determined as 2.5-6.0. The t_R value of the compound was determined in triplicate at each mobile phase pH value studied and the average t_R value was used. The ethanol percentage in the mobile phase and the experimental t_R values obtained depending on the pH change of the mobile phase were analyzed in the NLREG program. Obtained ${}^{s}_{s}pK_a$ and t_{RBH^+}, t_{RB} values with their standard deviation values are given in Table 1.

Table 1. ${}^{s}_{s}pK_a$ and t_{RBH^+}, t_{RB} data calculated with NLREG program

Compound	Data	Ethanol concentration			
		15% (v/v)	20% (v/v)	25% (v/v)	30% (v/v)
Thiabendazole	t_{RBH^+}	21.906 (1.76.10-4)*	13.774 (1.24.10-4)	10.744 (1.84.10-4)	6.292 (7.73.10-5)
	t_{RB}	33.913 (2.52.10-4)	25.239 (1.61.10-4)	19.727 (2.14.10-4)	13.632 (7.73.10-5)
	${}^{s}_{s}pK_a$	4.606 (4.42.10-5)	4.517 (3.10.10-5)	4.401 (5.56.10-5)	4.315 (2.73.10-5)

*Standard deviation

The ${}^{s}_{s}pK_a$ values, which change depending on the change in each ethanol percentage examined, belong to the N-3 nitrogen of the benzimidazole ring (Figure 1). As can be seen in Table 1, the decrease in the ${}^{s}_{s}pK_a$ of thiabendazole in ethanol-water has been attributed to the breaking of the water structure by the addition of ethanol which consequently changes its ionization equilibrium. This is in harmony with the chromatographic behavior of this basic moiety. Basic compounds interact more in the HPLC column in ionized form than in the molecular form, depending on the ${}^{s}_{s}pH$ value of the mobile phase [7].

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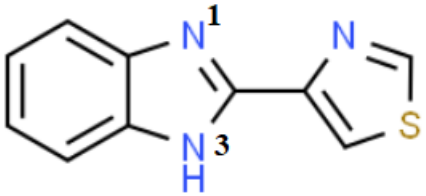

Figure 1. Chemical structure of Thiabendazole

The chromatograms showing the change in the t_R value of the compound in a molecular and ionized form in ethanol-water binary mixtures containing 20%, and 25% (v/v) ethanol were given in Figure 2. The t_R relationship obtained against the mobile phase pH values was shown in Figure 3 for the binary mixtures studied.

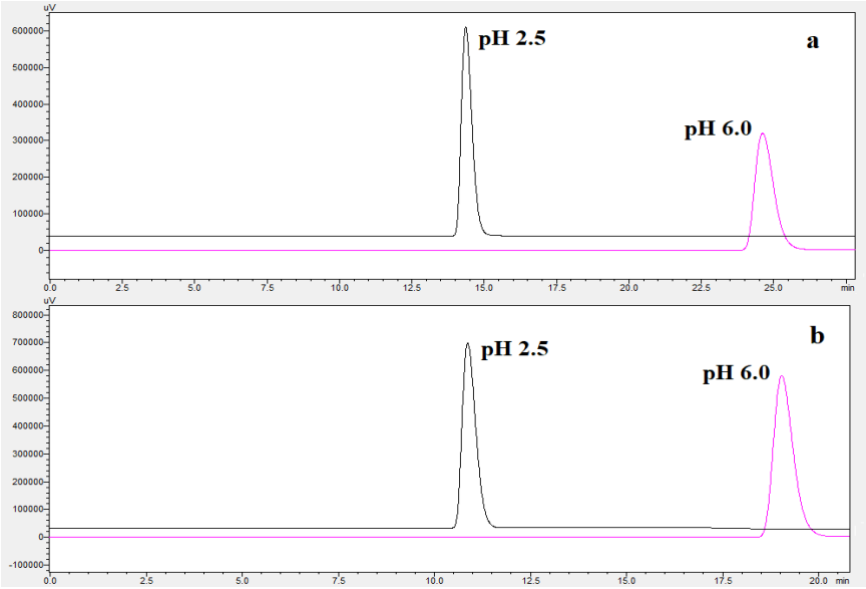

Figure 2. RPLC chromatograms of ionized and molecular forms of thiabendazole in the ethanol-water binary mixture containing **a** 20%(v/v), **b** 25% (v/v) ethanol.

Obtained nonlinear curves were drawn with Originlab Pro 2015 program [24]. According to the nonlinear model Boltzmann equation, ${}^{s}_{s}pK_a$, initial t_R (A_0), and final t_R (A_1) values were also calculated. It is seen that these values calculated from the Boltzmann equation, and the values calculated from the NLREG program are the same.

In this study, the theoretical t_R values for the liquid chromatographic behavior of the compound were also calculated, and the compatibility of the experimental and theoretical values is shown by calculating the residual sum of squares (RSS). Theoretical values were calculated using Eq. (5) [25].

$$t_{Rtheoretical} = t_{RBH^+}\left(\frac{[H^+]}{[H^+]+K_a}\right) + t_B\left(\frac{K_a}{[H^+]+K_a}\right) \qquad \text{[Eq. 5]}$$

Using this equation, the $t_{Rtheoretical}$ values of the compound were calculated in the hydro-organic mixtures containing 15%, 30% (v/v) ethanol, and at varying mobile phase pH values. The data obtained are given in Table 2.

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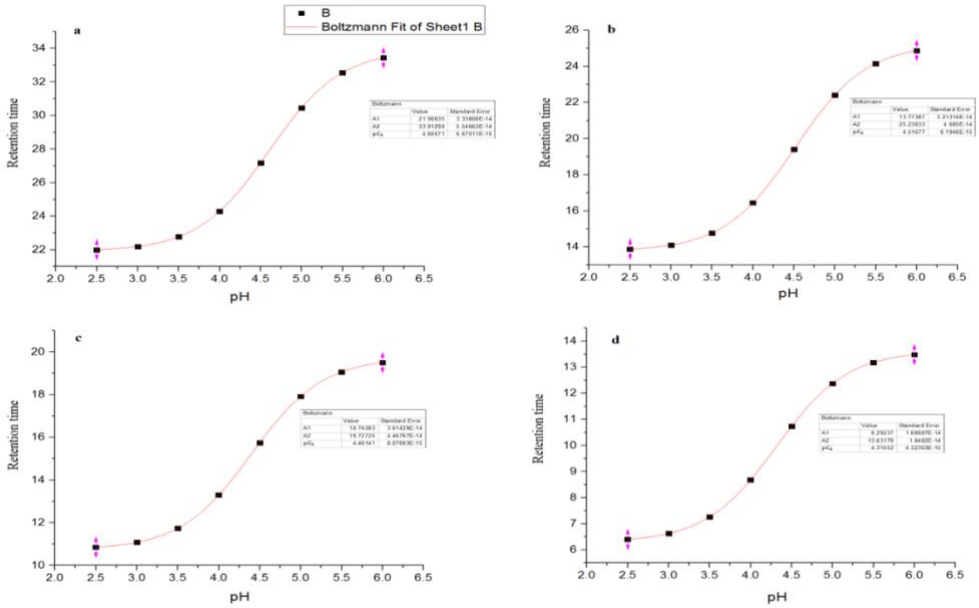

Figure 3. Graphs showing the nonlinear regression behavior of the compound. **a** 15%(v/v), **b** 20% (v/v) **c** 25%(v/v) **d** 30%(v/v) ethanol.

Table 2. Experimental and calculated theoretical retention time values for thiabendazole.

	15% (v/v)				30% (v/v)			
pH	$t_{Rexperimental}$	$t_{Rtheoretical}$	$t_{Rexperimental} - t_{Rtheoretical}$	$(t_{Rexperimental} - t_{Rtheoretical})^2$	$t_{Rexperimental}$	$t_{Rtheoretical}$	$t_{Rexperimental} - t_{Rtheoretical}$	$(t_{Rexperimental} - t_{Rtheoretical})^2$
2.5	22.000	21.999	$4.20.10^{-4}$	$1.77.10^{-7}$	6.403	6.403	$2.25.10^{-4}$	$5.07.10^{-8}$
3.0	22.197	22.196	$5.72.10^{-4}$	$3.28.10^{-7}$	6.631	6.631	$-6.05.10^{-5}$	$3.66.10^{-9}$
3.5	22.779	22.778	$9.91.10^{-4}$	$9.82.10^{-7}$	7.266	7.267	$-7.63.10^{-4}$	$5.82.10^{-7}$
4.0	24.292	24.290	$1.86.10^{-3}$	$3.46.10^{-6}$	8.685	8.687	$-1.84.10^{-3}$	$3.37.10^{-6}$
4.5	27.183	27.180	$2.64.10^{-3}$	$6.98.10^{-6}$	10.730	10.732	$-2.18.10^{-3}$	$4.74.10^{-6}$
5.0	30.462	30.460	$2.13.10^{-3}$	$4.55.10^{-6}$	12.374	12.376	$-1.42.10^{-3}$	$2.01.10^{-6}$
5.5	32.555	32.554	$1.03.10^{-3}$	$1.06.10^{-6}$	13.181	13.182	$-7.11.10^{-4}$	$5.06.10^{-7}$
6.0	33.447	33.447	$3.76.10^{-4}$	$1.42.10^{-7}$	13.483	13.483	$-3.90.10^{-4}$	$1.52.10^{-7}$
			RSS	**$1.77.10^{-5}$**			**RSS**	**$1.14.10^{-5}$**

When Table 2 is examined, it is seen that the obtained RSS values are quite small, and the experimental and theoretical values are compatible with each other. In addition, the slope and intercept values of the linear function obtained when $K_a/[H^+]$ is plotted against the $t_{Rtheoretical}(1 + K_a/[H^+])$ data give, t_{RB} and t_{RBH^+} values, respectively. Graphs showing this linear relationship are given in Figure 4. Obtained slope and intercept values are in perfect agreement with NLREG results.

Retention modeling was performed using the linear relationship between the initial t_R values calculated from the NLREG program and the solvatochromic parameter E_T^N values calculated against each ethanol percentage studied (Equations (2), (3)) [13]. e_B, e_{BH^+}, C_B and C_{BH+} values were calculated from the slope and intercept values of this linear relationship. Theoretical values were calculated using the pK_a values determined in the percentage of ethanol studied and the initial t_R values (Eq. (4)). The parameters found to explain the retention behavior of these analytes by applying this equation are demonstrated in Table 3, together with the relative root mean squared differences (RRMSD). These results show the quality of the description of retention time values. In conclusion, the RPLC method is suitable for the pK_a determination of compound with very low water solubility.

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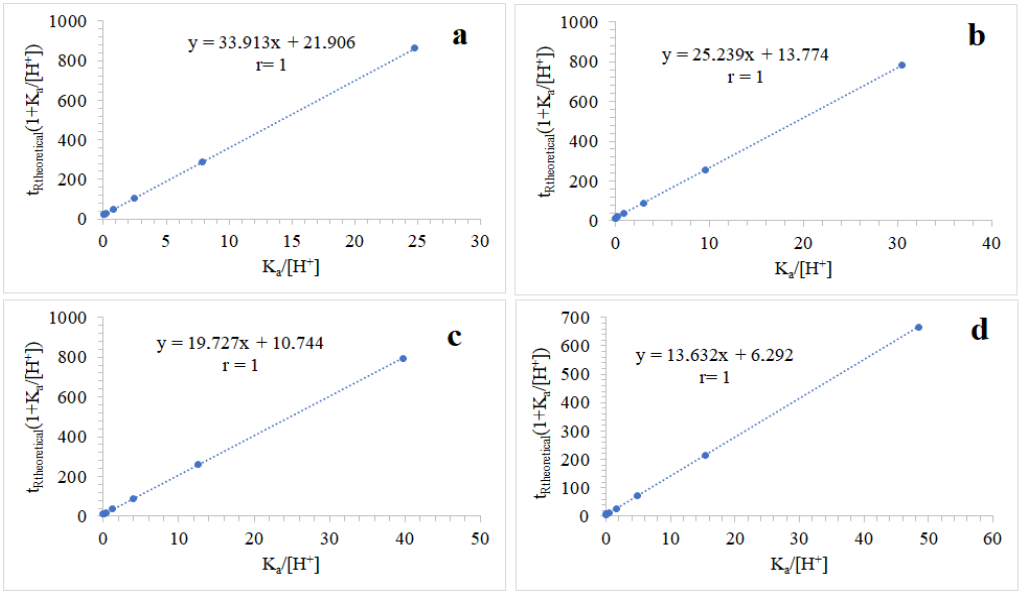

Figure 4. Graphs showing the linear relationship $K_a/[H^+]$ - $t_{Rtheoretical}(1 + K_a/[H^+])$ **a** 15%(v/v), **b** 20% (v/v) **c** 25%(v/v) **d** 30%(v/v) ethanol.

Table 3. Results of the application of the retention model proposed to all the experimental retention data available for studied compound.

Data	Thiabendazole
N[a]	32
C_{BH+}	-4.327
e_{BH^+}	6.084
C_B	-2.659
e_B	4.544
RRMSD[b]	3.379

[a] N, Number of all available experimental retention data for thiabendazole

[b] RRMSD, relative root mean squared differences = $100\sqrt{\sum (k_{obs} - k_{pred})^2} / \sum (k_{obs})^2$.

2.2. Calculation of pK_a value of compound in the aqueous medium

The aqueous solubility (logS) of thiabendazole is -4.33 [26]. Since the compound has moderate solubility, it was aimed to determine the chromatographic behavior of water-ethanol binary mixtures containing 15, 20, 25, and 30% (v/v) ethanol, and the ${}^{s}_{s}pK_a$ value of the compound was calculated with the NLREG program. The pK$_a$ value (${}^{w}_{w}pK_a$) of the thiabendazole in the water medium could also be calculated by using these ${}^{s}_{s}pK_a$ values obtained in the water-ethanol mixture and some solvent constants of ethanol. For this, firstly, the mole fraction ($X_{ethanol}$) values corresponding to the studied volume percent of ethanol were used [27]. When the ${}^{s}_{s}pK_a$ values calculated with the NLREG program are plotted against the $X_{ethanol}$ values, the intercept value of the linear functions obtained gives the ${}^{w}_{w}pK_a$ value of the compound in water. (Figure 5).

The dielectric constant (ε) is an important property of hydro-organic mixtures. This constant is a necessary parameter for determining the solubility and ionization constants of active pharmaceutical ingredients. In the second approach, a linear relationship was obtained by plotting the ${}^{s}_{s}pK_a$ values against 1/ε

[28]. The ε value was used as 79.72 for the water medium. The results of the calculations using these linear functions are shown in Table 4.

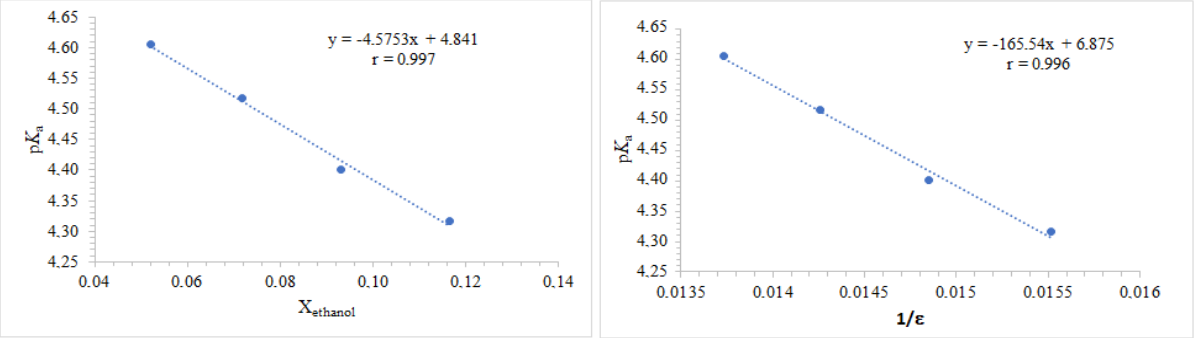

Figure 5. Calculation of ${}^{w}_{w}pK_a$ values with $X_{ethanol}$- ${}^{s}_{s}pK_a$ and $1/\varepsilon$-${}^{s}_{s}pK_a$ approaches

Table 4. Results of the aqueous pK_a value

Compound	${}^{w}_{w}pK_a$
$X_{ethanol}$-${}^{s}_{s}pK_a$	4.841
$1/\varepsilon$ -${}^{s}_{s}pK_a$	4.799
d	+0.042

d, the difference between paired values

In the study performed by Demiralay and Poturcu, the ${}^{s}_{s}pK_a$ values of thiabendazole were determined by the RPLC method in acetonitrile-water binary mixtures containing 15%, 20%, and 25% (v/v) acetonitrile [20]. Since the elution strength (ε°) of ethanol (3.6) is higher than that of methanol (3.0) and acetonitrile (3.1), ethanol can be used less than these solvents in the analyzes made with the RPLC method [29]. For this reason, thiabendazole could be analyzed with approximately the same ethanol amounts in the study. In addition, since the viscosity of ethanol is higher than that of acetonitrile, the flow rate of the mobile phase is kept lower. In the study conducted by Demiralay and Poturcu, ${}^{w}_{w}pK_a$ values were calculated as 4.597 for thiabendazole [20]. The results of the studies using these two different hydro-organic mixtures are compatible with each other. In the study by Chamberlain et al., thiabendazole was determined in methanol-water binary mixtures by potentiometric method. Using the pK_a values calculated in these environments, the pK_a value of thiabendazole in the water environment was calculated by the Yasuda-Shedlovsky method. The pK_a value calculated according to this approach is 4.7. This value is consistent with the pK_a value calculated from this study [30]. pK_a of thiabendazole (pK_a 4.7) was also estimated by the commercial Pallas software package [31].

By using the ${}^{w}_{w}pK_a$ values calculated at the experimentally selected temperature (37°C) and the Abraham solute parameters determined for the basic groups (Table 5), the value of the compound at 25 °C was calculated without an experimental study [32]. When Abraham solute parameters (Table 5) and ${}^{w}_{w}pK_a$ values at 37 °C were substituted in Eq. (6), the ΔpK_a value was calculated. When this value is replaced in Eq. (7), ${}^{w}_{w}pK_a$ values at 25 °C were calculated [32].

$$\Delta pK_a = k_0 pK_a{}^{37} + c_0 + c_1 \sum \alpha_2{}^{H} + c_2 \sum \beta_2{}^{H} + c_3\pi_2 + c_4 R_2 + c_5 V_x \qquad \text{[Eq. 6]}$$

$$\Delta pK_a = pK_a{}^{37} - pK_a{}^{25} \qquad \text{[Eq. 7]}$$

Table 5. Abraham solute parameters

	k_0	c_0	$c_1\sum\alpha_2^H$	$c_2\sum\beta_2^H$	$c_3\pi_2$	c_4R_2	c_5V_x
Base	-0.026	-0.136	0.008	0.018	0.035	-0.032	0.020

Using the ${}^{w}_{w}pK_a$ values calculated using the ${}^{s}_{s}pK_a$ - 1/ε relationship at 37°C, the ${}^{w}_{w}pK_a$ values at 25°C were calculated as 5.011 for thiabendazole. When the study by Sun and Avdeef was examined, the pK_a values of compounds containing basic functional groups at 25 °C were higher than the pK_a values calculated at 37 °C. The results obtained with this approach support the accuracy of the calculation.

2.3. Calculation of the percentage of ionization of the basic group at different pHs

pK_a is the pH value of the environment in which an ionizing compound is 50% ionized. In general, in a healthy person, stomach pH varies between 1 ~ 3, small intestine pH 4 ~ 7, and blood pH 7.3 ~ 7.45. Depending on the pH values in these tissues, the drug shows different ionization behavior in different parts of the body [33].

In this study, the ${}^{s}_{s}pK_a$ and ${}^{w}_{w}pK_a$ values of N-3 in the benzimidazole ring in the structure of thiabendazole were determined. Calculation of the degree of ionization is very important since a drug can pass through the cell membrane in its unionized (uncharged) form. The % ionization values of this compound, which have low solubility in the aqueous medium, were calculated by the Henderson-Hasselbach equation [34]. Using the Henderson-Hasselbalch equation, the ionization percentage of the basic functional group was calculated at different pHs (1-12) values with the calculated ${}^{w}_{w}pK_a$ value for each compound (Figure 6).

At a pH value of 2 pH units below the pK_a value, the basic form of the compound is in the non-ionized form. These findings highlight the potential of the physicochemical properties of the drugs to provide an understanding of different biological systems, such as cell permeability, human oral absorption, and plasma protein binding.

3. CONCLUSION

In this study, the retention behavior of thiabendazole was determined by the RPLC method depending on the changing mobile phase pH and ethanol amounts. Using the mobile phase pH values determined by the retention time and pH standardization, the ${}^{s}_{s}pK_a$ and intrinsic t_R values of the compound were determined by the NLREG program. These ${}^{s}_{s}pK_a$ data obtained are the first data obtained in the ethanol-water binary mixture in the literature. The linear relationship between the intrinsic retention times of the compound and the solvatochromic parameter E_T^N and the theoretical retention times were calculated as a function of different ethanol percentages (15%, 20%, 25%, 30%, *v/v*). The RRMSD values determined according to these data showed that the theoretical and experimental data were compatible with each other. The ${}^{w}_{w}pK_a$ values of the compound was calculated by using linear functions obtained by using ${}^{s}_{s}pK_a$ values against the macroscopic solvent constants ($X_{ethanol}$, ε) of ethanol in the hydro-organic mixture.

Apart from this, the pK_a value of the compound in the water environment at 25°C was also estimated using the Abraham solute parameters without any experimental work. The ionization percentages of the thiabendazole at different pH values at 37°C were also calculated in this study. As a result, the study is an environmentally friendly analysis. The determined data are predictive for method optimization studies in liquid chromatographic analyses without the need for any trial and error.

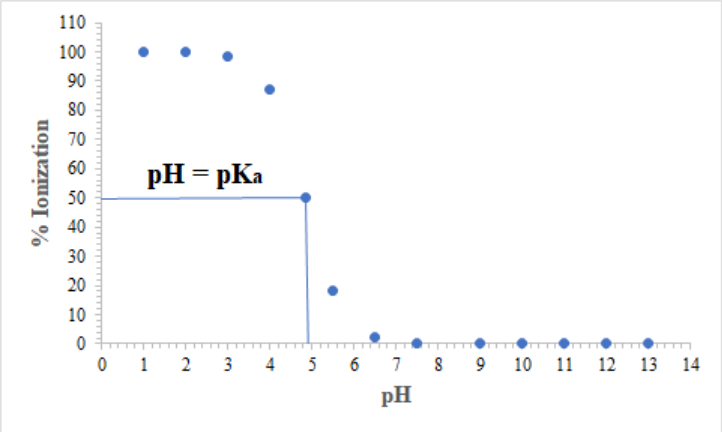

Figure 6. % ionization values of thiabendazole at different pH values

4. MATERIALS AND METHODS

4.1. Instrumentation and apparatus

Shimadzu HPLC device (Kyoto, Japan) was used for chromatographic analysis of thiabendazole. The system used consists of a UV detector (SPD-20A), column oven (CTO-20A), pump (LC20AD), degasser (DGU-20A3), and manual injection system. Mettler Toledo brand pH analyzer and glass electrode In Lab 412 (Schwerzenbach, Switzerland) was used for mobile phase pH analysis.

The pH was measured in the mixed mobile phase, considering the reference pH values of primary standard buffer solutions (pHs) for the standardization of potentiometric sensors in ethanol-water mixtures. For the standardization of potentiometric systems according to the IUPAC rules, standardization of pH measurements was carried out using the primary standard reference solution (KHP, 0.05 mol/kg) used in ethanol-water mixtures [35]. The ultrapure water used throughout the study was obtained from the Direct-Q3® UV water purification system (Millipore, Bedford, MA, USA).

4.2. Chemicals and reagents

The chemicals used in this study are of analytical purity. Thiabendazole was acquired from Sigma-Aldrich (St. Louis, USA). Ethanol, sodium hydroxide (NaOH), o-phosphoric acid (o-H_3PO_4), potassium hydrogen phthalate (KHP) were obtained from Merck (Darmstadt, Germany). The analyzed compound was prepared by dissolving in the working mobile phase at a concentration of 50 µg/mL.

4.3. Chromatographic analysis conditions

For the chromatographic analysis of the compound, a Pinnacle DB cyano (5 µm, 250 x 4.6 mm, Restek) column with a carbon content of 11% and a pH working range of 2.5-6.0 was preferred. 40 mM o-H_3PO_4 was added to the mobile phase prepared in water-ethanol binary mixtures containing 15, 20, 25, and 30% (v/v) ethanol. The desired pH value was adjusted using 1 M NaOH solution. By keeping the mobile phase pH between 2.5 and 6.0, the chromatographic retention behavior of the compound in the cyano column and the ${}_{s}^{s}pK_a$ value were determined. Analysis of the compound was performed at a constant flow rate of 0.3 mL/min with HPLC pump pressure. The compound was analyzed with a UV detector at 205 nm and determined at 37°C column temperature. Compound injected with 20 µL into the HPLC system were analyzed in triplicate and the relative standard deviation value of these analyses with high precision was calculated below 1%.

4.4. Calculation of theoretical retention time and ${}^{s}_{s}pK_a$ data

Based on the pH changes of the mobile phase containing the studied hydro-organic binary mixtures of thiabendazole, using the retention time (t_R) values determined by the RPLC method, using the nonlinear regression program (NLREG) ${}^{s}_{s}pK_a$ values and the intrinsic t_R of the ionized and non-ionized forms (BH^+, B) values could be calculated [36]. Using these calculated data, the theoretical t_R values were calculated and the agreement between the theoretical and experimental data was given with the residual sum of squares (RSS).

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