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A Green RPLC Approach to Some Tetracyclines Analysis in Human Urine

İnsan İdrarında Bazı Tetrasiklinlerin Analizine Yönelik Yeşil RPLC Yaklaşımı

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ABSTRACT

Tetracyclines are the main antibiotic group used for many years in the treatment of infections in both humans and animals due to their broad spectrum of action. Therefore, analytical methods developed for analysis of drugs attract the interest of many researchers. Studies conducted for the analysis of tetracycline group compounds in literature are generally focused on the reverse phase liquid chromatographic method. Toxic organic solvents such as acetonitrile and methanol, which have negative effects on environment, are used in the analyses. Moreover, optimum analysis conditions in these studies were determined by the trial-error principle without using a systematic approach. A green reverse phase liquid chromatographic method was developed for determination of tetracycline group compounds 4-epitetracycline, 4-epioxytetracycline and tetracycline in spike human urine. Nontoxic ethanol was used as mobile phase used in method. The liquid chromatographic behaviors of the compounds in ethanol-water binary mixture determined in the liquid chromatographic method development were systematically investigated in pH 2.5-7.0. The optimum separation condition was selected as a binary mixture containing 10% (v/v) ethanol with pH 3.0 and the analysis was carried out at 37 ºC. The developed method was validated according to International Council for Harmonization guidelines. The recovery results of studied compounds from spike human urine were found to be close to 100%. In addition, the greenness of method developed in study was evaluated using the Analytical Greenness Calculator and Green Analytical Procedure Index. The obtained results determined that developed method is suitable for use in routine analyses and is environmentally friendly.

Keywords: *Tetracyclines, Green RPLC, Method gevelopment, pK_a*

ÖZ

Tetrasiklinler, geniş etki spektrumları sayesinde hem insan hem de hayvanlarda görülen enfeksiyonların tedavisinde uzun yıllardır kullanılan temel antibiyotik grubudur. Bundan dolayı bu grup ilaçların analizi için geliştirilen analitik yöntemler birçok araştırmacının ilgisini çekmektedir. Literatürde tetrasiklin grubu bileşiklerin analizi için gerçekleştirilen çalışmalar genellikle ters faz sıvı kromatografik yöntem üzerinde yoğunlaşmıştır. Yapılan analizlerde çevreye olumsuz etkileri olan asetonitril ve metanol gibi toksik organik çözücüler kullanılmaktadır. Üstelik bu çalışmalarda optimum analiz koşulları sistematik bir yaklaşım kullanılmadan deneme yanılma prensibiyle belirlenmiştir. Gerçekleştirilen çalışmada tetrasiklin grubu bileşiklerden 4-epitetrasiklin, 4-epioksitetrasiklin ve tetrasiklinin katkılandırılmış insan idrarında tayini için yeşil ters faz sıvı kromatografik yöntem geliştirilmiştir. Yöntemde kullanılan mobil fazda toksik olmayan etanol organik çözücü olarak kullanılmıştır. Sıvı kromatografik yöntem geliştirmede belirlenen etanol ve su ikili karışımında, mobil faz pH 2,5 ile 7,0 aralığında bileşiklerin sıvı kromatografik davranışları sistematik olarak incelenmiştir. Optimum ayırma koşulu pH'sı 3,0 olan %10 (h/h) etanol içeren etanol-su ikili karışımı seçilmiştir ve 37 ºC'de analiz gerçekleştirilmiştir. Geliştirilen yöntem Uluslararası Uyum Konseyi yönergesine göre valide edilmiştir. Çalışılan bileşiklerin katkılandırılmış insan idrarından geri kazanım sonuçları %100'e yakın olarak bulunmuştur. Ayrıca bu çalışmada geliştirilen yöntemin yeşilliği Analitik Yeşillik Hesaplayıcı ve Yeşil Analitik Prosedür İndeksi kullanılarak değerlendirilmiştir. Elde edilen sonuçlar geliştirilen yöntemin rutin analizlerinde kullanımı için uygun ve çevre dostu olduğu belirlenmiştir.

Anahtar Kelimeler: *Tetrasiklinler, Yeşil RPLC, Metot geliştirme, pK_a*

INTRODUCTION

Tetracyclines belong to the basic antibiotic drug groups that have been used for many years to treat bacterial infections in humans and animals thanks to their broad-spectrum anti-inflammatory and antibacterial effects (1-3). Tetracyclines are structurally very similar and are derived from the hydronaphthacene skeleton, which consists of four linearly linked 6-rings. Oxytetracycline, tetracycline, chlortetracycline, minocycline, metacycline, demeclocycline, and doxycycline are representatives of this class of antibiotics. These compounds have 5 or 6 chiral centers in their structures (4). Tetracyclines are structurally unstable and polar compounds. Moreover, they transform themselves in a slightly acidic or basic environment into 4-epitetracycline, an epimer of tetracycline, 4-epioxytetracycline, an epimer of oxytetracycline, and 4-epichlortetracycline, an epimer of chlortetracycline (5,6).

Since drugs of the tetracycline group are widely used today, there are many studies on their determination. The literature shows that the experimental studies carried out for this group are mostly studies on their determination in drug preparations or biological fluids using reversed-phase liquid chromatography (RPLC) (7-11). These studies in the literature are similar and were carried out by trial and error without a systematic approach. In the trial-and-error method, the parameters that affect the liquid chromatographic retention (t_R) values of the compounds are tried one after the other to determine the best operating condition. However, it is expected that the large number of experimental combinations randomly generated by varying the individual parameters often searches for the best operating condition complex and time-consuming. The probability that the best operating condition cannot be determined is often high (12-15). In addition, the hydrophobic nature of tetracycline compounds requires working in binary mixtures of organic solvents and water. Liquid chromatography studies often use toxic solvents such as acetonitrile and methanol, which are harmful to the environment. Therefore, liquid chromatography studies performed with the trial-and-error method for compounds of the tetracycline group result in the release of large amounts of waste of high concentrations of toxic solvents into the environment. For these reasons, in this study, an RPLC method was developed for the determination of tetracycline, 4-epitetracycline, and 4-epioxytetracycline, which are compounds of the tetracycline group, in spiked human urine using ethanol, an environmentally friendly organic solvent, with a systematic approach that significantly reduces the number of experiments and is based on the pH-retention time relationship of the compounds. In this way, an environmentally friendly RPLC method using eco-friendly ethanol as an alternative to toxic solvents in the liquid chromatographic determination of the investigated tetracyclines was introduced in the literature for the routine analysis of these compounds.

In this study, the developed RPLC method was validated to test the applicability of the method developed for the investigated tetracyclines in routine analyses. The criteria of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use - ICH were used to validate the developed method (16).

MATERIAL and METHOD

Chemicals and Reagents

Tetracycline, 4-epitetracycline, 4-epioxytetracycline and fluconazole used in the study were supplied by Sigma-Aldrich (St. Louis, MO, USA). Ethanol (EtOH), sodium hydroxide (NaOH), and o-phosphoric acid (o-H_3PO4) used in the preparation of the mobile phase were from Merck (Darmstadt, Germany). The pure water used for the preparation of the mobile phase was prepared using the Millipore Direct-Q®3 UV device. Potassium hydrogen phthalate was selected based on the IUPAC criteria for electrode calibration as the primary standard substance to adjust the pH of the mobile phase consisting of the binary EtOH-water mixture.

Apparatus

The study was performed with a Shimadzu high-performance liquid chromatography unit consisting

of a pump (LC-20AD), a UV detector (SPD-M20A), a column oven (CTO-10AS VP), and degassing sections. The mobile phase was adjusted using a combined Ag/AgCl glass electrode (Mettler Toledo InLab 413) and a Mettler Toledo MA 235 pH/ion analyzer.

Chromatographic Method

Although there are many analytical methods for the qualitative and quantitative determination of compounds, the RPLC method is the basic method used in drug analysis due to the high precision, accuracy, and repeatability of the data obtained (17). In this method, the retention of the compound in a stationary phase is determined by the descriptor retention time (t_R). Depending on the type of analysis performed, other descriptors such as the capacity factor (k), the selectivity (α), and the resolution (R_s) are calculated using the t_R values obtained from the compounds. When developing a liquid chromatographic method, an attempt is made to control the t_R values of the compounds according to certain rules by using parameters that are known to influence them. To control the t_R values of the compounds, it is necessary to systematically change some chromatographic parameters. The parameters that change the retention of the compound in a column are generally the concentration of the organic solvent in the mobile phase, the pH of the mobile phase, and the column temperature (12-15). However, due to the large number of experimental combinations that can be obtained by changing these parameters one after another, this situation must be based on a systematic approach (15).

In the development of the green RPLC method for tetracycline, 4-epitetracycline, and 4-epioxytetracycline, binary EtOH-water mixtures with 30 mM o-phosphoric acid and 10% EtOH (v/v) were studied. The pH range of the studied mobile phases was set at 2.5-7.0. Tetracycline, 4-epitetracycline, 4-epioxytetracycline, and fluconazole, which were used as internal standard (I.S.), were prepared daily at a concentration of 200 $\mu g.mL^{-1}$ throughout the study. The calibration solutions of the investigated tetracyclines and I.S. were prepared by dilution with the mobile phase according to the concentrations determined on the calibration curve.

All liquid chromatographic measurements were performed on a YMC Triart C18 (150x 4.6 mm I.D, 3µm) column. Throughout the study, the column temperature was kept constant at 37 ^oC and the mobile phase flow rate was maintained at 1 $mL.min^{-1}$. For the determination of tetracycline, 4-epitetracycline and 4-epioxytetracycline the UV detector was set to 270 nm, and for fluconazole, the UV detector was set to 255 nm. The wavelength values for the investigated compounds were taken from our previous work (18).

Preparation of Human Urine Samples

The use of the developed method in real sample analysis was investigated through direct analysis of compounds determined in human urine samples. Urine sample preparation was done by the spike method and a certain amount of tetracycline, 4-epitetracycline and 4-epioxytetracycline was added to the urine sample according to the following method. Urine samples from a healthy person not taking medication were first diluted at 1:20. Then 2 mL of each sample was taken and 3 mL of EtOH was added to precipitate the proteins in the samples. The precipitated proteins were filtered through a 0.45 µm filter and removed from the solution. The solution samples were completed to 10 mL by adding the I.S. and studied tetracyclines to these solutions at a constant concentration of 0.5 $\mu g.mL^{-1}$ for I.S. The samples were then analyzed using the green RPLC method (19).

Evaluation of Greenness of The Developed RPLC Method

The environmental impact, in other words, the "greenness" of the method developed for the determination of tetracyclines investigated in spiked urine using the RPLC technique, was determined using the Analytical Greenness Calculator (AGREE) and the Green Analytical Procedure Index (GAPI), which are programs developed for this purpose (20,21).

RESULTS

The compounds of the tetracycline group are highly polar compounds with a partially conjugated four-ring structure with a carbonamide function. Moreover, due to their precarious structure, these compounds easily transform into their epimers in an acidic or basic environment. The transformation of compounds of the tetracycline group into epimers only occurs through the exchange of ammonium and hydrogen at carbon number 4 in their structure (Figure 1). The similar and polar chemical structures of tetracycline compounds and their epimers make their simultaneous liquid chromatographic determination difficult. Although an attempt was made in this study to develop a liquid chromatographic method for the simultaneous determination of tetracycline, oxytetracycline, chlortetracycline and their epimers, which belong to the tetracycline group compounds, the RPLC method was able to provide sufficient conditions for the simultaneous determination of tetracycline, 4-epitetracycline and 4-epioxytetracycline (Figure 1). However, the method developed for these compounds can also be easily used to simultaneously determine single or binary combinations of the other tetracyclines, as these have retention values similar to those of the tetracyclines under investigation.

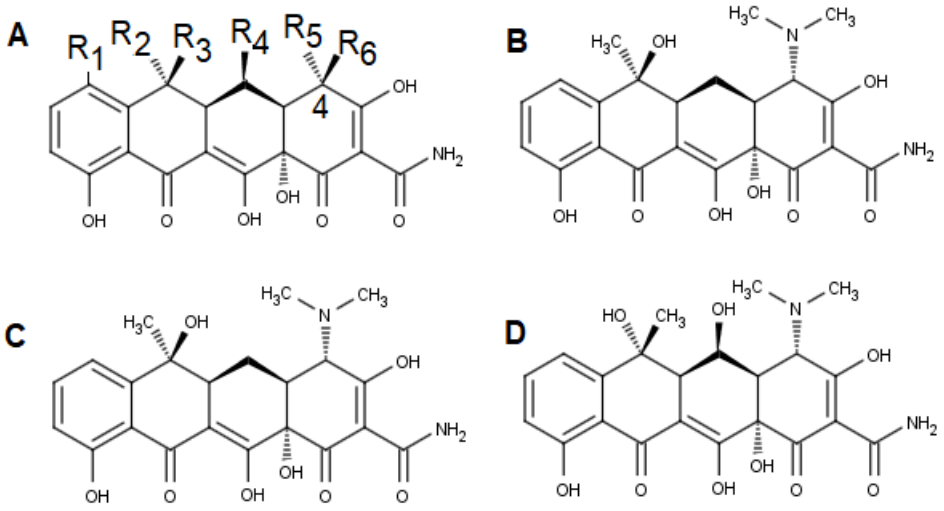

Figure 1: Chemical Structure of The Studied Compounds A) General Structure Of Tetracyclines, B) Tetracycline C) 4-Epitetracycline D) 4-Epioxytetracycline

In this study, the retention behavior of studied compounds at different pH values was systematically investigated to determine the optimum condition for their simultaneous determination in spiked human urine. To this purpose, the pH values of their neutral and ionic forms were first determined using the pK_a values of the tetracyclines studied (approximately 4.278 for tetracycline, 4.805 for 4-epitetracycline and 4.706 for 4-epioxytetracycline) (18). For the binary mixture of water and organic solvent studied, the compounds are present in their neutral and ionic forms at pH values 1.5 pH units below (approximately pH 3.0) and 1.5 pH units above their pK_a values, and the t_R values obtained for the compounds in this pH range are the highest and lowest values that can be obtained, respectively. The binary EtOH-water mixtures to be used for the tetracyclines studied were determined based on the t_R values obtained at these pH values with a small number of preliminary experiments. As a result of this investigation, binary EtOH-water mixtures containing 10% (v/v) EtOH were established as the working condition. Subsequently, the t_R values of tetracyclines studied were determined in the mobile phases prepared at systematically increasing pH values in the pH range of 2.5-7.0 and the capacity factor (k) of the compound was determined from these values (18). When the determined k values were plotted against the pH values, the liquid chromatographic retention behavior of tetracycline, 4-epitetracycline and 4-epioxytetracycline was obtained (Figure 2).

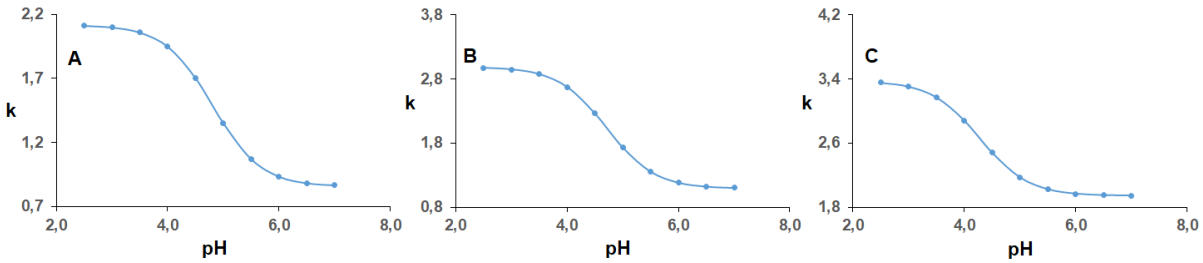

Figure 2: pH-k Relationship of The Studied Compounds Obtained in 10% EtOH-Water (v/v) Environment A) 4-Epitetracycline B) 4-Epioxytetracycline C) Tetracycline

Tetracyclines and their epimers are compounds with amphoteric properties due to the acidic substituents and basic dimethylamino groups in their structures. In this study, the liquid chromatographic behavior of the investigated tetracyclines was examined in the pH range of 2.5-7.0 (Figure 2). The liquid chromatographic behavior of tetracycline, 4-epitetracycline and 4-epioxytetracycline in this pH range is due to the ionization of the tricarbonyl group in their structures (pK_{a_1}) (18). Figure 3 shows the ionization behavior of tetracycline, 4-epitetracycline and 4-epioxytetracycline in the studied pH range.

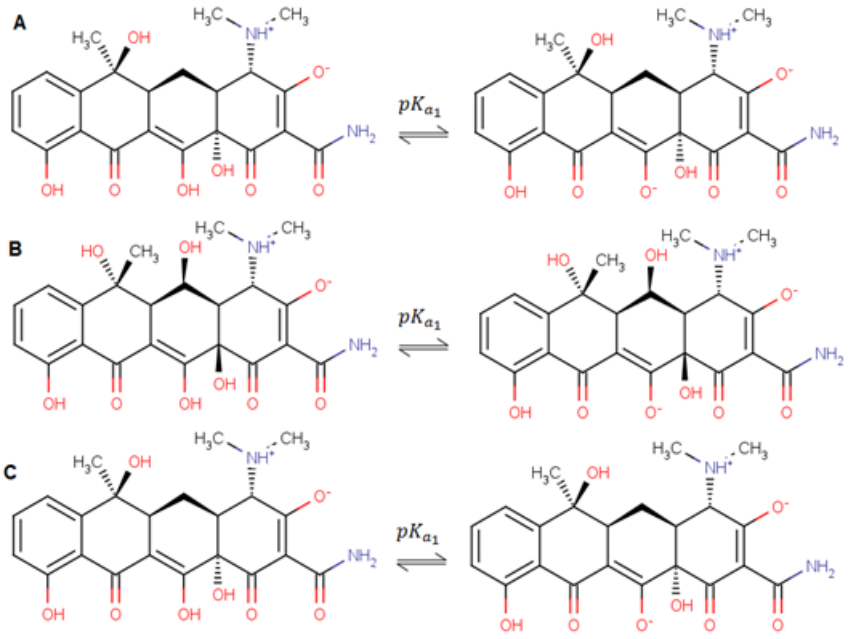

Figure 3: Dissociation Equilibrium of The Investigated Compounds A) 4-Epitetracycline B) 4-Epioxytetracycline C) Tetracycline

When determining the optimum working condition for the compounds under study, the k value in the liquid chromatographic behavior of the compound should be in the range of $1 \leq k < 10$, the value of the selectivity factor (α) between the consecutive peaks of the tetracyclines under study and the compound used as an internal standard should be at least 1.15, and the resolution (R_s) values are at least 2.0 (13,14). For this study, the condition that was closest to these criteria was determined as the optimum working condition for the binary EtOH-water mixture with 10% (v/v) EtOH and pH of 3.0 at a column temperature of 37°C. The chromatogram obtained under the optimum conditions determined is shown in Figure 4, and the chromatographic data obtained are listed in Table 1.

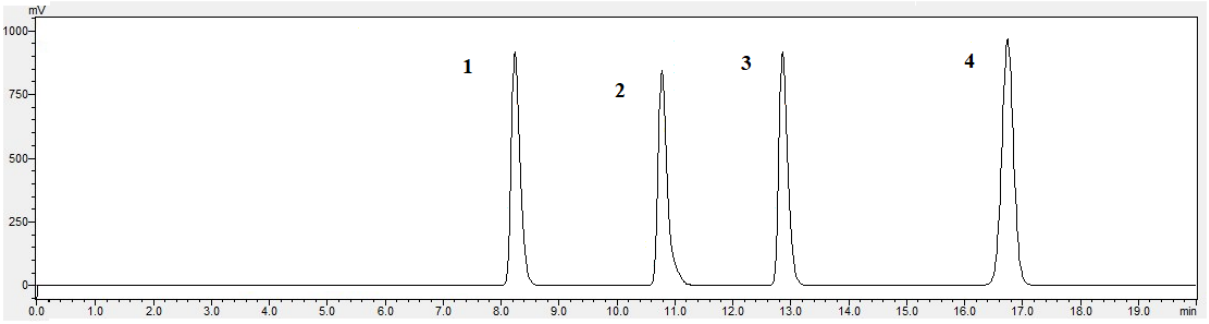

Figure 4: Standard Chromatogram of The Analyzed Compounds, Obtained Under Optimal Conditions: 1) 4-Epitetracycline (120 µg.mL^{-1}) 2) 4-Epioxytetracycline (80 µg. mL^{-1}) 3) Fluconazole (I.S.) (160 µg. mL^{-1}) 4) Tetracycline (160 µg. mL^{-1})

Table 1: Liquid Chromatographic Data Obtained Under Optimum Separation Condition

Compounds	t_R	k	α	$\frac{(a-1)}{a}$	$\frac{k_2}{k_2+1}$	$(\frac{1}{4})\sqrt{N}$	R_s
4-epitetracycline	8.796	2.008					
4-epioxytetracycline	10.760	2.680	1.334	0.728	0.251	24.533	4.478
Fluconazole (I.S.)	12.912	3.416	1.275	0.774	0.215	26.273	4.379
Tetracycline	16.786	4.741	1.388	0.826	0.279	23.731	5.477
Urasil	2.924						

To determine the suitability of the optimum working conditions established in the determination of the studied tetracyclines for quantitative determination, the method was evaluated using validation tests consisting of criteria of repeatability, precision, accuracy, linearity, and robustness (16). The criteria established by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) were used as a reference for method validation (16). Since the confounding effects in the urine samples affected the measurement of the analyte with the UV detector, the internal standard method (I.S.) was used for the calibration process in the study. The substance selected as the I.S. substance must have similar chemical properties to the substances to be analyzed. Therefore, fluconazole, which contains similar functional groups to the compounds selected for analysis in this study, was selected as the internal standard substance (16).

The repeatability and stability of the developed method were examined with system suitability tests. The parameters that make up the system suitability tests, the value ranges that must be provided for these parameters, and the results obtained are listed in Table 2. Table 2 shows that the values obtained are within the recommended limits. This shows that the method developed for the determination of tetracyclines investigated has good reproducibility and is suitable for the validation process.

Table 2: System Suitability Values of The Investigated ~~İnvestigated~~ Compounds

Parameters	**4-epitetra cycline**	**4-epioxytetra cycline**	**Fluconazole (I.S.)**	**Tetra cycline**	**Recommended value**
Retention time (t_R)	8.797	10.760	12.912	16.786	
Asymmetry factor (A)	1.135	1.297	1.078	1.208	0.90-2.0
Retention factor (k)	2.009	2.680	3.951	4.741	>1
Resolution factor (R_s)		4.413	4.317	5.441	>2
Theoretical plates (N)	4741	9467	10803	8892	>2000
Selectivity factor (α)		1.334	1.274	1.389	>1
RSD% (for retention time)	0.111	0.038	0.046	0.024	≤1
RSD% (for peak area)	0.275	0.059	0.098	0.258	≤1

A calibration curve was created to determine the linearity of the developed method. The calibration curve created is linear between 0.5 μg.mL^{-1} and 3.0 μg.mL^{-1}. The calibration curve was created by plotting the value obtained by dividing the area of the tetracyclines studied against the concentration with the area of fluconazole used as I.S. The results of the linear regression are shown in Table 3. The values for the detection limit (LOD) and quantitation limit (LOQ) given in Table 3 were calculated based on signal-to-noise ratios of 3.3:1 and 10:1, respectively (16).

Table 3: Calibration Curve Data of Studied Tetracyclines

Sample	Linearity range (μg.mL^{-1})	m*	b*	S.E. of m*	S.E. of b*	r	LOD (μg.mL^{-1})	LOQ (μg.mL^{-1})
4-epitetra cycline	0.5-3.0	2.140	-0.007	0.003	0.006	0.999	0.010	0.031
4-epioxy tetra cycline	0.5-3.0	2.719	0.002	0.004	0.008	0.999	0.011	0.032
Tetra cycline	0.5-3.0	1.952	0.007	0.008	0.015	0.999	0.027	0.081

*m=slope, b= intercept

The accuracy and precision of the developed method were evaluated with intra-day and inter-day studies. Intra-day studies were evaluated on the same day and for two different concentrations. Inter-day studies were performed on three different days from the analysis day and for two different concentrations (15). Both analyses are performed with three independent solutions prepared for the studied tetracyclines and I.S. The results obtained from this evaluation are shown in Table 4. The fact that the relative standard deviation (RSD%) values from the intraday studies in Table 4 are below 1 % and the RSD% values from the inter-day studies are below 2% indicates that the method developed has high sensitivity, accuracy, and precision.

Table 4: Results of The Intra-Day And Inter-Day Precision Data

Compounds	Theoretical Concentration (μg.mL^{-1})	Intra-day measured concentration mean	RSD %	Inter-day measured concentration mean	RSD %
4-epitetra cycline	1.00	1.002	0.284	0.989	0.824
	2.50	2.503	0.158	2.493	0.918
4-epioxy tetracycline	1.00	1.017	0.164	0.992	0.449
	2.50	2.502	0.220	2.485	0.521
Tetracycline	1.00	1.001	0.182	0.895	0.480
	2.50	2.501	0.158	2.475	0.407

Urine consists of certain components such as water, urea, ions, creatine, drugs, and metabolites produced in the liver and kidney. Urine sampling is non-invasive. It can be used during drug evaluation studies as it does not require complex sample preparation steps. This method was carried out to evaluate the efficiency of the proposed method for the simultaneous determination of analyzed compounds in a complex matrix. For this purpose, recovery tests are applied to the developed method. In this study, the accuracy of the developed method was determined by recovery testing of the investigated tetracyclines from spiked human urine samples. Urine is preferred as a biological sample because it is easier to obtain than other biological fluids and the sample volume is relatively large. Normally, there are no compounds that are analyzed in the urine of a healthy person unless they are taken into the body from outside.

For the recovery test, solutions containing the investigated tetracyclines in two different concentrations and I.S. in a fixed concentration (0.5 µg.mL^{-1}) were added to the urine samples, the preparation of which was indicated in the method section. This analysis was performed in triplicate. The chromatograms of the recovery studies performed in three replicates are shown in Figure 5 and the results obtained are listed in Table 5. Figure 5-A shows the chromatogram of the urine samples that were not spiked with the studied compounds, Figure 5-B shows the chromatogram of the urine samples that were spiked with the studied tetracyclines at a concentration of 1.0 µg.mL^{-1} and fluconazole at a concentration of 0.5 µg.mL^{-1}, and Figure 5-C shows the chromatogram of the urine samples that were spiked with the studied tetracyclines at a concentration of 2.0 µg.mL^{-1} and fluconazole at a concentration of 0.5 µg.mL^{-1}. Table 5 shows that a result close to 100% recovery was obtained. This shows that the accuracy of the developed method is high, and the matrix effect caused by urine samples does not negatively influence the method (15).

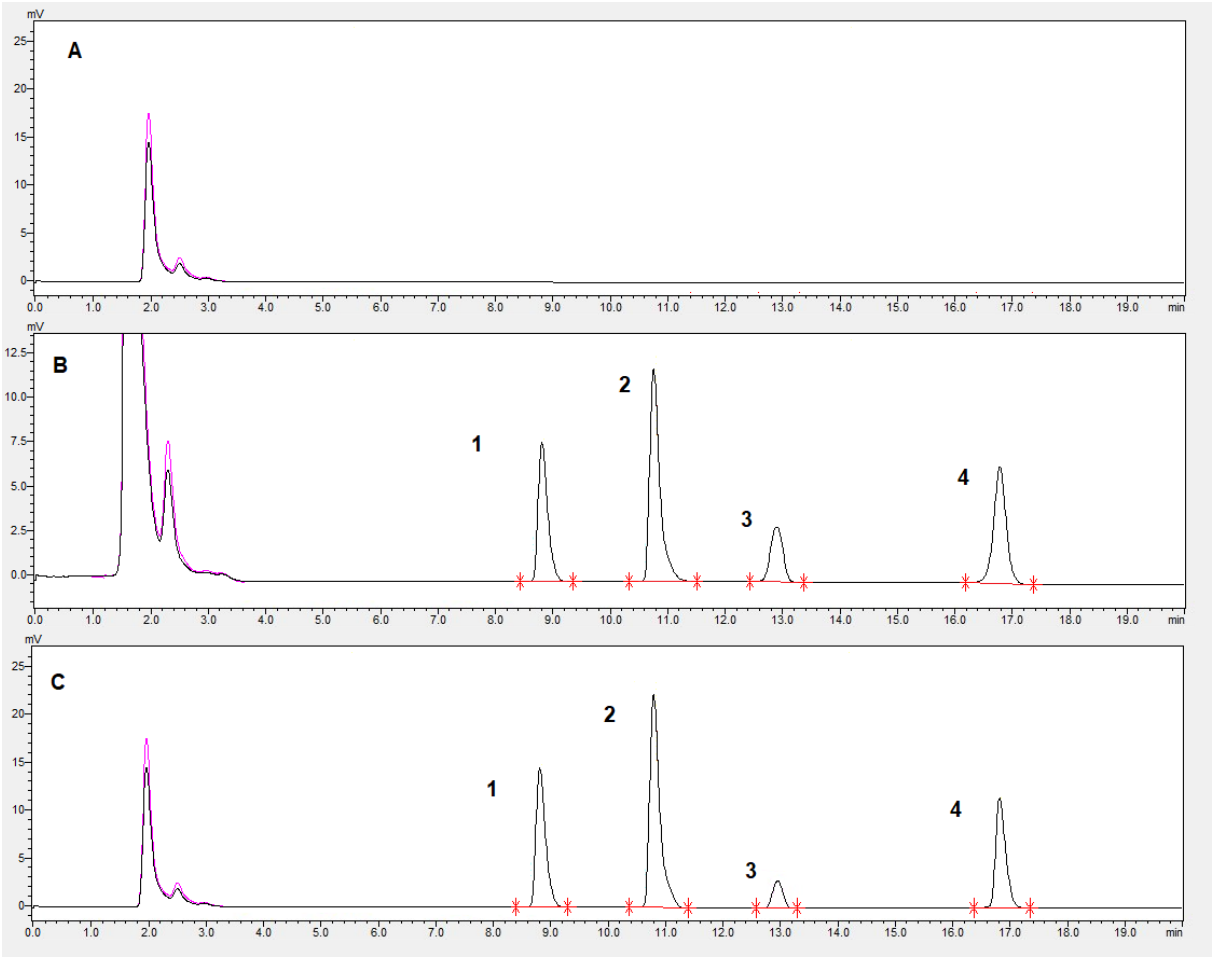

Figure 5: Sample Chromatograms: 1) 4-Epitetracycline 2) 4-Epioxytetracycline 3) Fluconazole (I.S.) 4) Tetracycline

Table 5: Results of Accuracy Data

Compounds	N	Theoretical Concentration (µg.mL^{-1})	Average concentration found (µg.mL^{-1})	Recovery (%)	Standard Deviation	RSD (%)
4-epitetracycline	3	1.0	1.001±0.006	100.031±0.570	0.002	0.232
		2.0	2.002±0.001	100.083±0.057	0.001	0.023
4-epioxytetra cycline	3	1.0	1.007±0.005	100.721±0.594	0.002	0.238
		2.0	2.013±0.002	100.642±0.031	0.001	0.013
Tetracycline	3	1.0	1.002±0.004	100.063±0.370	0.001	0.149
		2.0	2.009±0.001	100.435±0.070	0.001	0.028

The robustness of the developed method was evaluated with robustness tests. This test examines whether small but significant changes in the components that create the optimum operating condition change the area and tailing factor values of the method. This means that the fewer changes in the components that make up the working condition change the area and tailing factor values determined in the method, the more robust the method is (22). The results of the robustness test of the method developed for the investigated tetracyclines are shown in Table 6. From these data, it can be seen that minor changes in the analytical method do not cause any changes in the results obtained with the method.

Table 6: Results of The Robustness Test

Conditions	4-epitetracycline		4-epioxytetracycline		Tetracycline	
	Peak area	Asymmetry factor (A)	Peak area	Asymmetry factor (A)	Peak area	Asymmetry factor (A)
%10 EtOH (v/v), pH 3.0, 37 °C, flow rate 1.0 ml/min. (Optimum Condition)	122562	1.134	158478	1.295	854361	1.207
%10 EtOH (v/v), pH 3.0, 37°C, flow rate 0.5 ml/ min.	122082	1.138	158768	1.294	854950	1.132
%10 EtOH (v/v), pH 3.5, 37°C, flow rate 1.0 ml/ min.	122455	1.139	158444	1.295	854481	1.097
%10 EtOH (v/v), pH 2.5, 37°C, flow rate 1,0 ml/ min.	122671	1.138	158486	1.297	854588	1.075
%8 (v/v) EtOH, pH 3.0, 37°C, flow rate 1.0 ml/ min.	122919	1.137	158732	1.297	854326	1.029
%12 (v/v) EtOH, pH 3.0, 37°C, flow rate 1.0 ml/ min.	122108	1.139	158316	1.296	854341	1.059
%10 (v/v) EtOH, pH 3.0, 25°C, flow rate 1.0 ml/ min.	122526	1.138	158007	1.296	854342	1.052
%10 EtOH (v/v), pH 3.0, 42°C, flow rate 1.0 ml/ min.	122526	1.133	158007	1.294	854372	1.052

The environmental impact (i.e. the greenness) of the RPLC method developed was evaluated using the AGREE and GAPI programs. In both methods, the responses to the test criteria are expressed on a scale with different colors, from red to green. The closer to the green color, the closer to the desired environmental outcome, while the red colored areas indicate the opposite (20,21). In addition, the AGREE program evaluates the results obtained using these criteria and expresses the greenness of the developed method on a scale between 0 and 1.0. The closer the result is to 1.0, the greener the method is (21). The results obtained with these programs for the RPLC determination of studied tetracycline in spiked human urine are shown in Figure 6. Accordingly, both calculation methods show that the developed RPLC method is an environmentally friendly analytical procedure.

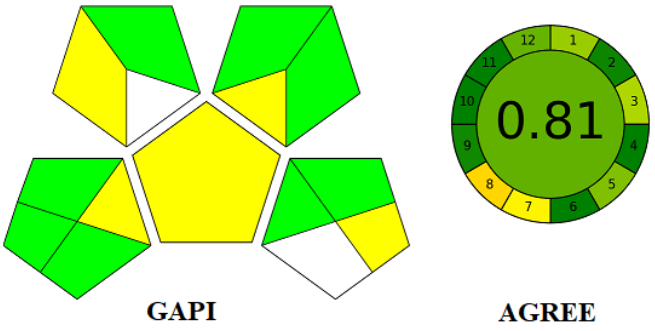

Figure 6: Evaluation of The Greenness of The Developed Method with The AGREE and GAPI Programs

DISCUSSION and CONCLUSION

The antibiotics of the tetracycline group are compounds that are used very frequently in animal husbandry as they have a very broad spectrum of activity. For this reason, there are many studies in the literature on their liquid chromatographic determination in biological fluids, tablet formulations, and animal tissues (7-11). Some of the current studies are summarized below.

Butovskaya et al. (2024) in their study, developed a liquid chromatographic method for the quantitative determination of oxytetracycline, chlortetracycline, and doxycycline in animal feed. The method developed by the researchers works in gradient mode, and a binary mixture of 10%-30% (v/v) acetonitrile and water is reported as the optimal separation condition. The developed method shows linearity in the range of 40-1000 $mg.kg^{-1}$ (8) Uddin et al. (2024) in their study developed a liquid chromatographic method for the determination of tetracycline, oxytetracycline and ciprofloxacin in tablet formulations and bovine milk. The liquid chromatographic study is performed in gradient mode and the researchers used a condition consisting of different concentrations of acetonitrile (16-30%, v/v), methanol (2-12 v/v %), and 0.05 M oxalic acid for the optimum separation condition. The method is linear in the range of 0.5-8 $\mu g.mL^{-1}$ and was validated according to ICH guidelines (11). In their study, Nakhonchai et al. (2024) developed a liquid chromatographic method for the determination of oxytetracycline, tetracycline, and doxycycline in bovine milk. In this study, a solid phase extraction of the investigated tetracyclines with a biological material was performed before the liquid chromatographic determination. In the study, mobile phases containing 20%-30% (v/v) acetonitrile were used. The researchers found that the developed method was linear in a range of 15.0-500 $\mu g.L^{-1}$ (10).

When examining the current studies presented above and older studies in the literature, it is noticeable that these studies were conducted in binary mixtures of organic solvents and water, and even in the current studies, toxic organic solvents such as acetonitrile and methanol were used. In addition, these studies were conducted using the trial-and-error method, which did not use a systematic approach to determine the optimum separation condition. Therefore, in the current study, an environmentally friendly RPLC method was developed for the determination of 4-epitetracycline, 4-epioxytetracycline, and tetracycline in spiked human urine. In this study, the environmental impact of the developed method was evaluated using the GAPI and AGREE programs, and the results obtained proved that the method is an environmentally friendly analytical procedure. Since the method developed for the determination of the investigated tetracyclines is a systematic study that is not based on trial and error, this is an important step in the development of an analytical method. The developed method was validated according to the ICH criteria and the results show that the obtained green RPLC method can be used as an alternative to traditional RPLC techniques in routine analyses. According to the results obtained in this study, the developed method can be used for the RPLC determination of tetracyclines in biological fluids as well as in drug formulations containing these compounds.

Declaration of Ethical Code: In this study. we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with. and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

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