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ORIGINAL ARTICLE / ÖZGÜN MAKALE

GREEN HPLC DETERMINATION OF PHENYTOIN AND METHOD VALIDATION

FENİTOİNİN YEŞİL HPLC TAYİNİ VE METOT VALİDASYONU

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ABSTRACT

Objective: *In this study, the chromatographic behavior of the antiepileptic drug phenytoin was determined by the green HPLC method. The optimization of the developed method was based on the capacity factor values of phenytoin in varying water-ethanol binary mixtures and the ethanol concentration in the mobile phase where the compound was analyzed.*
Material and Method: *Ethanol-water binary mixtures containing 35%, 40%, and 45% (v/v) ethanol were used in the optimization for the determination performed by the RPLC method. Retention times of the compound were determined with the Zorbax SB-CN (150x4.6 mm, 3.5 µm ID) column. Analyzes were performed at a constant flow rate (0.3 ml/min) and column temperature (37°C). The optimum condition for quantitative analysis was determined as an ethanol-water binary mixture containing 40% (v/v) ethanol with a pH of 6.5.*
Result and Discussion: *In this study, the hydrophobicity of phenytoin was calculated using the logk-φ relationship. The optimum condition was determined using the obtained chromatographic data, and the quantitative determination of phenytoin in the commercial tablet formulation was made by the internal standard method. Under these conditions, excellent linearity (r>0.99) was obtained in the concentration range of 0.8-2.8 µg/ml. The detection limit of the developed method is 0.021 µg/ml; the limit of quantitation was calculated as 0.064 µg/ml. The recovery value of the method was determined as 99.61%. It was concluded that the parameters of precision, accuracy, and method robustness were appropriate for the validation procedures.*
Keywords: *Antiepileptic drugs, binary mixture, green chemistry, method optimization, RPLC*

ÖZ

Amaç: *Bu çalışmada antiepileptik ilaç fenitoinin kromatografik davranışı yeşil HPLC yöntemi ile belirlenmiştir. Geliştirilen yöntemin optimizasyonu, değişen su-etanol ikili karışımlarındaki fenitoinin kapasite faktörü değerlerine ve bileşiğin analiz edildiği mobil fazdaki etanol derişimine dayandırılmıştır.*
Gereç ve Yöntem: *RPLC yöntemiyle geliştirilen metot optimizasyonunda %35, %40 ve %45 (h/h) etanol içeren etanol-su ikili karışımları kullanılmıştır. Bileşiğin alıkonma süreleri Zorbax SB-CN*

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(150x4.6 mm, 3.5 µm ID) kolonu ile belirlenmiştir. Analizler sabit akış hızı (0.3 ml/dakika) ve kolon sıcaklığında (37°C) gerçekleştirilmiştir. Kantitatif analiz için optimum koşul, pH'ı 6.5 olan %40 (h/h) etanol içeren bir etanol-su ikili karışımı olarak belirlenmiştir.

Sonuç ve Tartışma: *Bu çalışmada logk-φ ilişkisini kullanılarak fenitoinin hidrofobisitesi hesaplanmıştır. Elde edilen kromatografik veriler kullanılarak optimum koşul belirlenmiş, ticari tablet formülasyonunda fenitoinin kantitatif tayini dahili standart yöntemiyle yapılmıştır. Bu koşullar altında 0.8-2.8 µg/ml derişim aralığında mükemmel doğrusallık (r>0.99) elde edilmiştir. Geliştirilen yöntemin tespit limiti 0.021 µg/ml; miktar belirleme sınırı 0.064 µg/ml olarak hesaplanmıştır. Yöntemin geri kazanım değeri %99.61 olarak belirlenmiştir. Kesinlik, doğruluk ve metot sağlamlığı parametrelerinin validasyon prosedürleri için uygun olduğu sonucuna varılmıştır.*

Anahtar Kelimeler: *Antiepileptik ilaçlar, ikili karışım, RPLC, yeşil kimya, yöntem optimizasyonu*

INTRODUCTION

Epilepsy is one of the neurological disorders that affects approximately 1% of the world's population. Antiepileptic (Anticonvulsants) drugs are a wide variety of pharmacological agents used in the treatment of epileptic seizures. Phenytoin has been used as an anticonvulsant drug since the late 1930s. It limits the spread of seizure discharges by blocking voltage-dependent sodium channels. A usage mediates this response- and voltage-dependent slowdown in the recovery rate of voltage-activated sodium channels from inactivation [1,2]. Phenytoin is a broadly effective ion channel blocker because it inhibits several sodium and calcium channels [3].

Phenytoin is a weakly basic compound with a dissociation constant (pK_a) value between 8.06 and 9.2. Slightly soluble in water, phenytoin is soluble in alkaline and most organic solvents. The poor solubility of phenytoin in water is due to the hydrophobic nature of the diphenyl structure at the C-5 position of hydantoin. (Figure 1) [4].

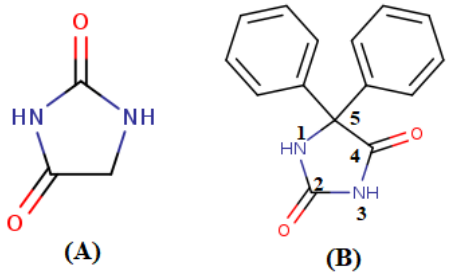

Figure 1. Structure of hydantoin (A) and phenytoin (B)

Analyzes performed by reversed-phase liquid chromatography (RPLC) method for the qualitative and quantitative analysis of the antiepileptic drug phenytoin are available in the literature [5-8]. RPLC is considered the most common technique used in many fields, including developing and analyzing drugs in quality control laboratories and analyzing active substances in biological fluids [9,10]. In most studies carried out with the RPLC method, significant amounts of organic solvents that can hurt the environment, produce large quantities of waste to be disposed of, and cause problems related to ecological impact continue to be used. In this method, methanol and acetonitrile are most commonly used. These solvents with high elution power have some problems in terms of safety and environment. This study aims to introduce a new chromatographic method using the less hazardous solvent ethanol, which is considered an alternative to methanol and acetonitrile. The most suitable green organic solvent for the green RPLC method is ethanol. Due to its lower vapor pressure, ethanol has less toxicity than methanol and acetonitrile, which have higher vapor pressures. In terms of selectivity, ethanol is in the same group as methanol, as in the classification of organic solvents. The main disadvantage of ethanol is that increasing column temperature creates high pressure in the HPLC system due to its higher viscosity compared to acetonitrile and methanol [11,12].

In RPLC optimization, the best separation conditions must be provided. For this purpose, various approaches have been developed to predict the retention behavior of the analyte. Thus, mobile phase polarity, pH, and column temperature values are used in the analysis of ionizable and neutral compounds without the need for trial and error [13-15]. Among these three parameters, the change in the organic solvent content in the mobile phase also causes changes in the degree of ionization and retention of the compound. Achieving the desired separation is very important and is often possible at certain pH values. For this reason, mobile phase pH is important in the determination of ionizable compounds [16,17].

According to the solvophobic theory, the retention will be a function of the chromatographic behavior of the analyte determined in the mobile phase [16]. Using the equations for estimation of retention in chromatography ensures the performance of the experiment in a shorter time and the selectivity of the compounds [14,18]. The relationship between the percentage of the organic solvent in the mobile phase and the capacity factor value is given in Equation 1.

$$\log k = log k_w - S\varphi \qquad (1)$$

φ is the volume fraction of the organic modifier in the binary mixture. The S value is a factor associated with the solvent strength of the organic modifier. As seen in Equation 1, the k value of the compounds shows a linear relationship depending on the volume percentage of organic solvent in the mobile phase. In the determined linear function, S can be calculated with the slope value, and capacity factor (k_w) values of the compounds in pure water can be calculated with the intercept value. The $logk_w$ value calculated in the RPLC method is a measure of hydrophobicity [19]. Although the S value depends on the solvent power of the pure organic modifier, it is not constant for analytes with different chemical structures. S values can range from -3.0 to -6.0. This value indicates that a given increase in organic modifier concentration causes large differences in retention [20]. Equation 1, also referred to as the linear solvent strength (LSS) model, is used to develop methods in RPLC [21]. It is also used to predict chromatographic separations in both isocratic and gradient studies [21,22].

This study focused on the ease of replacing conventional mobile phases with less toxic and greener solvents without altering the performance of the developed method. A green RPLC method was developed for the qualitative and quantitative analysis of phenytoin, an antiepileptic drug. With this developed method, the chromatographic analysis of phenytoin, a hydrophobic compound, was performed using less ethanol than the analyses made with toxic solvents. This study aimed to determine the effect of ethanol content and pH change in the mobile phase on the retention of phenytoin. The pH value of the mobile phase to be studied was chosen in the range of $pK_a \pm 1.5$ of the compound. For the quantitative determination of phenytoin, the k value should be in the range of 1-5 [9,19]. Selectivity factor (α) and separation factor (R_s) values were calculated to be able to separate with the selected internal standard dofetilide in the quantitative determination. In addition, the method was validated according to the International Conference on Harmonization (ICH) and Association of Official Analytical Chemists (AOAC) parameters [23,24] and then the quantitative determination in the pharmaceutical formulation was performed.

MATERIAL AND METHOD

Chemical Substances

The chemicals used for the developed method are of analytical purity and have not been subjected to any purification process. Phenytoin (5,5-diphenylhydantoin), internal standard dofetilide, and uracil were obtained from Sigma Aldrich (St. Louis, USA). Ethanol, ortho-phosphoric acid, sodium hydroxide, and potassium hydrogen phthalate (KHP) were purchased from Merck (Darmstadt, Germany).

Apparatus

The qualitative determination of phenytoin and its quantitative determination in drug formulation was made in an HPLC device. Shimadzu brand HPLC device consists of a UV detector (SPD-20A), pump (LC-20AD), column oven (CTO-20A), and degasser unit (DGU-20A3). The pH values of the prepared mobile phases were measured with a pH meter (Mettler Toledo, Switzerland) using an Ag/AgCl combined glass electrode. According to the International Union of Fundamental and Applied

Chemistry (IUPAC), potassium hydrogen phthalate was chosen as the reference standard for the calibration of the electrode in the ethanol-water mixture [25]. The ultrapure water used in the preparation of the mobile phase was obtained from the Direct Q3 (Millipore, Bedford, MA, USA) device.

Liquid Chromatographic Conditions

Analysis of phenytoin and selected internal standard dofetilide was performed on the Zorbax SB-CN (150x4.6mm, 3.5µm ID) column. The column temperature is 37°C and the flow rate is 0.3 ml/min. Compounds injected into the manual injection system in a volume of 20 µl were analyzed in triplicate and the relative standard deviation of the analysis was calculated below 1%. The wavelengths of the compounds analyzed in the UV detector were 230 nm for phenytoin, 215 nm for dofetilide, and 210 nm for uracil, respectively.

pH Measurement in the Mobile Phase

In a water-organic solvent mixture, pH was measured by IUPAC guidelines [25] considering the determined reference pH of the National Institute of Standards and Technology (NIST) buffer solutions in the hydroorganic mixture studied [26]. According to IUPAC rules, pH standardization was carried out with the primary standard reference solution (KHP, 0.05 mol/kg) used in ethanol-water binary mixtures [25].

Solutions Used and Preparation of Mobile Phase

Stock solution concentrations of phenytoin and selected internal standard dofetilide were prepared as 50 µg/ml. Compounds and mobile phases were stored in the refrigerator at +4°C unless used. Uracil solution prepared by dissolving it in a mobile phase medium was used to determine the capacity factors. The primary standard was prepared using the reference Potassium hydrogen phthalate compound in the mobile phase medium, where the electrode calibration solution was 0.05 mol/kg. For phenytoin analysis, ethanol-water binary mixtures containing 35%, 40%, and 45% (v/v) ethanol with pH adjusted to 5.0 and 6.5 were prepared as mobile phases. For the robustness test, the ethanol-water mixture containing 35% (v/v), 45% (v/v) ethanol, and mobile phases at different pH values (5.0 and 8.0) were prepared in the same way.

Preparation of Tablet Solution

To determine the active ingredient of phenytoin in tablet formulation, 10 tablets were crushed in a porcelain mortar, and tablet powder equivalent to 1 tablet was weighed and taken into a 100 ml balloon jug. Some mobile phase was added to it and it was dissolved in the ultrasonic bath and its volume was completed with the mobile phase. This solution was filtered through a blue band filter paper and diluted at different concentrations so that phenytoin was within the determined linear calibration range.

Method Robustness Test

The robustness of the developed method was evaluated by examining the changes in the amount of ethanol in the mobile phase (±5%, v/v), mobile phase pH (±1.5), and column temperature (25°C, 40°C) in the analysis of phenytoin.

RESULT AND DISCUSSION

In this study, the simultaneous effect of mobile phase pH and ethanol concentration was used for the retention of phenytoin. The analysis of this acidic and basic functional group-containing compound using the RPLC method is highly dependent on the degree of ionization of this compound and thus the pH of the mobile phase.

Since the analyzed phenytoin has moderate solubility and dofetilide has poor solubility [27], water-organic solvent binary mixtures are preferred in liquid chromatographic analyses with these compounds. In the study, water-soluble and polar organic solvent ethanol was preferred. Solvent polarity in the mobile phase affects chromatographic separation. The selection of more polar solvents increases the retention in the column in RPLC [9]. In these analyses made with polar solvent ethanol, classical

alkyl chain columns such as C18, and C8 were not preferred so that the interaction of the compounds with the column is not too much. For the analysis of compounds, a cyano column with a stable bond structure, which provides less interaction of analytes with the column, was preferred. Since the Zorbax SB-CN column (150x4.6 mm, 3.5 µm; Agilent) is nonpolar, the water-ethanol binary mixture was chosen as the mobile phase components.

The t_R values of phenytoin in mobile phases containing ethanol-water hydro-organic mixtures containing 35%, 40%, and 45% (v/v) ethanol at a constant column temperature of 37°C and a flow rate of 0.3 ml/min were determined by averaging the results of three replications. In addition, dead time (t_o) values were determined by using the type of uracil that was not retained in the column under each condition. The capacity factor values of the compound were calculated for each condition using the data obtained as a result of the qualitative analysis. The pH value of the mobile phase to be studied was chosen in the pK_a±1.5 range of phenytoin. Since there is no experimental data on the pK_a value of the compound, the Chemicalize program [28], which makes an estimation, was used to determine this physicochemical parameter. The pK_a value of the acidic nitrogen (Number 3 nitrogen) atom in the hydantoin ring in the chemical structure of the compound is around 8.0 [28]. For this, the pH values to be analyzed were chosen as pH 6.5 and pH 5.0. Since the limit pH value of the analyzed column is 8.0, the working pH value did not exceed 6.5. Since the peak symmetry and reproducibility of phenytoin will not be good at pH 8.0, no study has been carried out at pH where the pK_a value of the compound [9,19].

According to the LSS model, the linear relationship obtained when the logarithmic capacity factor (logk) values of phenytoin are plotted with the volume percentage of ethanol (φ, 35%, 40%, and 45%, v/v) in the mobile phase is given in Figure 2 for pH 5.0 and pH 6.5.

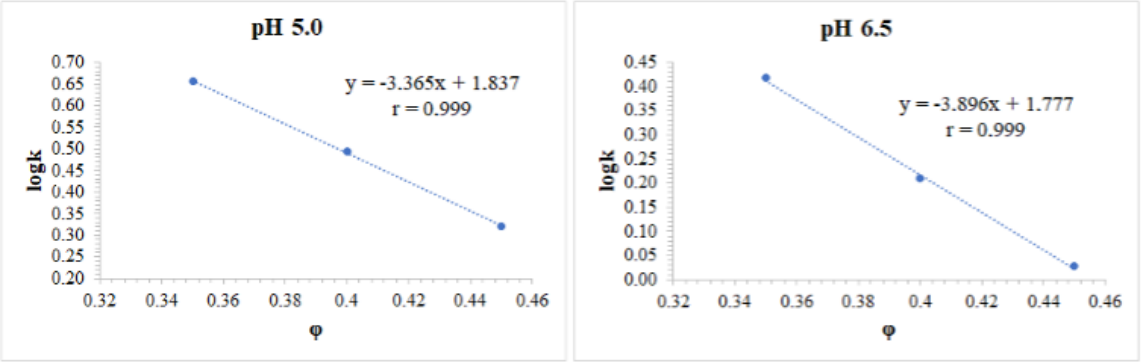

Figure 2. Graphs showing the φ-logk relationship at pH 5.0 and pH 6.5

The intercept value of the linear functions in these graphs gives the $logk_w$ value. According to this data, the k_w value of phenytoin at the studied pH values was calculated without any experiment. Accordingly, while the k_w value was 68.77 at pH 5.0, the k_w value was calculated as 59.83 at pH 6.5. The desired k value in chromatographic analyses is between 1-5. According to these calculated data, the calculated k values in the aqueous medium are very large. According to these values, it was concluded that the t_R values would also be very high, and under this condition, this compound could not be analyzed in a 100% water environment. In addition, the compound with an ionizable acidic functional group depends on the pH change, and the retention time in the column decreases as the pH value increases [16]. The k and k_w values obtained at two different pH values support this situation.

In the linear functions of the graphs given in Figure 2 (Equation 1), k values for phenytoin can be estimated when any percentage by volume value of ethanol outside the experimental study is substituted for the φ value. For ethanol-water binary mixtures containing 5%, 20%, and 70% (v/v) ethanol outside the experimental working range, the k values of the compound could also be calculated without any experiment (Table 1).

Table 1. k values calculated for phenytoin

Compound	pH 5.0			pH 6.5		
	5% (v/v)	20% (v/v)	70% (v/v)	5% (v/v)	20% (v/v)	70% (v/v)
Phenytoin	46.68	14.60	0.30	38.20	4.06	0.11

According to the data in Table 1, since the k value must be between 1-5, it is not possible to analyze phenytoin in studied binary mixtures containing 5%, 20%, and 70% (v/v) ethanol at pH 5.0. At pH 6.5, it is possible to determine phenytoin in binary mixtures containing only 20% (v/v) ethanol. In addition, since the ionized functional group in the structure of the compound is acidic, the k value of the compound will decrease as the pH value of the mobile phase increases. In addition, as the amount of ethanol in the environment increases, the interaction of the compound with the HPLC column will decrease and the k value will also decrease. The data in Table 1 supports these situations [9,19].

Hydrophobicity or lipophilicity is defined as a measure of a compound's tendency to "prefer" non-aqueous media over aqueous media. The logarithm of the partition coefficient, logP, and its logarithmic form, log k_w, are used as the hydrophobicity index in the RPLC method [22]. The relationships between logP values and RPLC retention parameters can be used to predict the hydrophobicity of the compound by estimating the log k_w values from the linear model using the k values in ethanol-water binary mixtures.

In this study, the logk_w (1.77-1.84) values calculated according to pH values, which differ slightly, are very close to the estimated logP (1.89) value of phenytoin [27]. logP > 0 indicates hydrophobic substances soluble in the lipid phase, and log P < 0 indicates polar compounds soluble in the water phase [22]. In this case, the determined and predicted hydrophobicity values show that the phenytoin compound is hydrophobic. In this study, the k values that can be estimated using the logk-φ relationship and the low solubility of phenytoin in water (logS -5.62) also support that the compound is hydrophobic [27].

Optimization of Chromatographic Analysis

For the qualitative and quantitative determination of phenytoin, which will be determined by the RPLC method, the capacity factor (k) value should be 1 and above. The determination of the compound with low solubility by the green RPLC method was carried out in a water-ethanol binary mixture. For this, three mobile phases containing 35%, 40%, and 45% (v/v) ethanol were prepared. Since phenytoin is an ionizable compound, it is affected by changing mobile phase pH values. In determining the pH value to be studied, the pH value of the compound in the range of $pK_a \pm 1.5$ is selected. The pK_a value of phenytoin is 8.33 [28]. Accordingly, the mobile phase pH value of the compound to be studied was chosen as 6.5. This is because the HPLC column Zorbax SB-CN (150x4,6mm, 3.5µm ID) where the determination will take place has a working pH range of 1-8, so the pH value 1.5 units beyond the pK_a value of the compound could not be reached. In a chromatographic analysis, it is desired to analyze a compound as quickly as possible. For this reason, it is important to determine the k value. In the ethanol-water binary mixture containing 40% ethanol adjusted to pH 6.5, a very symmetrical peak with a k value above 1 was obtained at 37 °C for phenytoin. At this pH, the compound is in molecular form.

In this study, the internal standard method was used for the quantitative determination of phenytoin in commercial tablet formulation. For this, different standards with UV properties and chromatographic separation from phenytoin were tried. In the separation for quantitative determination, to separate two compounds from each other, the k value must be 1 and above 1, the selectivity factor (α) value must be 1.15 and above, and the resolution factor (R_s) value must be 2 and above. As a result, dofetilide, an antiarrhythmic drug that meets these conditions, was chosen as the internal standard (IS). Dofetilide has poor solubility in water (logS -6.61) and is less hydrophobic than phenytoin (logP 0.79) [27]. Chromatographic parameter values calculated with the Purnell equation [19] under specified conditions are given in Table 2.

In this assay performed in triplicate, the first dofetilide (IS) was eluted from the HPLC column in 9.108 minutes. Phenytoin eluted from the column at 11.803 minutes. To calculate the k values of the compounds, the uracil solution given to the column was taken from the column in 4.502 minutes.

According to these data, it is seen that the k value calculated for both compounds is above 1. In addition, the calculated α and R_s values show that the compounds are separated from each other.

Table 2. Calculated chromatographic parameter values

Compounds	k_2	α	$k_2/(k_2+1)$	$\alpha - 1/\alpha$	$(1/4)\sqrt{N}$	R_s
Phenytoin/ Dofetilide (IS)	1.622	1.585	0.619	0.369	12.544	2.864

System Suitability Test

The tests performed to determine the suitability and effectiveness of the chromatographic system before the quantitative determination in a chromatographic separation is called the system suitability test (SST). The results obtained in these analyses performed according to the United States Pharmacopeia (USP) [29] are given in Table 3.

Table 3. SST results

Chromatographic parameter	Dofetilide (IS)	Phenytoin	Recommended values
Retention time	9.030	11.696	
Tailing factor	1.410	1.400	<2
Theoretical plate number	2464	2517	>2000
Capacity factor	1.045	1.649	>1
Selectivity factor		1.578	>1
Resolution factor		2.859	>2
RSD (%) of peak retention time	0.188	0.058	≤1
RSD (%) of peak area	0.059	0.057	≤1

Results from SST meet United States Pharmacopoeia requirements. These values seem to be suitable for the method developed for the quantitative determination of phenytoin. SST according to the AOAC guideline, RSD% of the t_R and the peak area of the phenytoin are below 2%. This indicates that the change in repeatable injections is small [24]. Spectral data versus retention time were taken from the HPLC device. Using these data, chromatograms were drawn in the Origin LabPro 2017 program. The standard mixture chromatogram showing the separation of the compounds is given in Figure 3. The two compounds were separated with good peak sharpness and symmetry in a total time of 13 min.

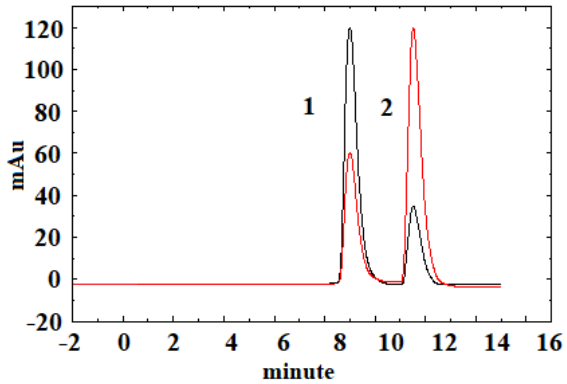

Figure 3. Standard mixture chromatogram showing separation of compounds.1) Dofetilide (IS) 2) Phenytoin – :215 nm; –:230 nm

Validation of the Green RPLC Method

Determination of Linear Range

The purpose of chemical analysis is quantitative analysis in which the amount of a substance in the sample is determined. Careful preparation of the sample is very important to accurately calculate the concentration of an unknown substance from the sample. Some of the samples may be lost with each preparation. However, there are some strategies to minimize sample loss. The internal standard (IS) method is widely used to deal with sample loss and still make accurate concentration measurements. The concentration of selected IS in this study was kept constant at 0.5 µg/ml throughout the study. The working range of the developed method was determined to be in the range of 0.8-2.8 µg/ml. A calibration graph was drawn using the peak area (mAu) ratio values obtained as a result of the analysis of solutions containing phenytoin at six concentration levels and IS at a fixed concentration, against varying phenytoin concentrations (µg/ml). According to the results, there is an excellent correlation (correlation coefficient (r)>0.999) between phenytoin concentration and peak area ratio. The regression equation is as follows:

$$y = 2.601x + 0.012 \qquad (2)$$

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated for phenytoin. LOD and LOQ values are 0.021 µg/ml and 0.064 µg/ml, respectively. The results obtained meet the acceptance criteria according to ICH and AOAC guidelines [23,24].

Precision Data of the Method

To determine the precision of the developed Green RPLC method, intraday (repeatability) and interday (reproducibility) studies were carried out. For this, phenytoin solutions containing a constant concentration of IS were prepared at two different concentrations within the linear working range determined in the calibration. Independent solutions prepared at 1.2 µg/ml and 2.4 µg/ml concentrations were analyzed three times a day. These prepared solutions were kept in a refrigerator at +4°C by cutting off contact with air. After the intraday analysis, the retention times and peak area values of the compound were recorded in the analysis performed on the 3rd day. Measured concentration and RSD% values calculated using the calibration function according to these data are given in Table 4.

Table 4. Intraday-interday data

Compounds	Theoretical concentration (µg/ml)	Intraday measured concentration mean (µg/ml)	RSD%	HorRat value	Interday measured concentration mean (µg/ml)	RSD %	HorRat value
Phenytoin	1.2	1.201	0.137	0.009	1.197	0.326	0.021
	2.4	2.401	0.178	0.013	2.396	0.413	0.039

According to ICH guidelines, the RSD% value should be 1% and below in intraday repeatability data, and the RSD% value should be 2% and below in interday repeatability data. The RSD% values calculated in these data are presented in Table IV. show that the precision of the developed method is high. Additionally, precision values are calculated from the Horwitz (HorRat) equation [30], which represents the empirical relationship between acceptable precision and the corresponding analyte concentration in the analyzed sample. HorRat value (Equation 3) was calculated by AOAC guidelines [24].

$$\text{HorRat} = \frac{RSD_R}{PRSD_R} \qquad (3)$$

$PRSD_R$ is the RSD value estimated from the Horwitz equation. This value is calculated with Equation 4.

$$PRSD_R = 2^{(1-0.5\log C)} \qquad (4)$$

RSD_R is the relative standard deviation under reproducibility conditions. C is the mass concentration expressed in the power of 10, i.e. 1 µg/g=10^{-6}. The precision is better than expected if the ratio is less than 1, and poorer if greater than 1 [30]. Reproducibility and reproducibility results for phenytoin also demonstrated compliance with the AOAC guidelines (Table 4).

Accuracy Data of the Method

For quantitative analysis, tablets containing phenytoin active ingredient (Eptandoin, Exeltis®, 100 mg Phenytoin) were prepared as stated in the material and method section. As a result of the HPLC analysis, when the peak area ratio values of the compounds were substituted in the calibration function, the amounts in the tablet samples for phenytoin were calculated. The data are presented in Table 5.

Table 5. The amount of phenytoin in the tablet sample and recovery results ([1]Five experiments)

Labeled claim (mg)	100
Amount found (mg)[1]	100.50
RSD %	0.88
Bias%	0.50
Recovery%	99.61
RSD %	0.65
Bias%	0.39

According to the data obtained from the table, an average value very close to the amount of 100 mg of phenytoin active ingredient in the tablet sample was obtained. The calculated RSD% value shows that the precision of the results is good, and the accuracy is high since the bias percentage value is below 1%.

A recovery study was carried out to express the accuracy of the developed method. For this purpose, the tablet sample prepared from the standard solution of phenytoin, not exceeding the calibration working range, was added. As a result of the HPLC analysis, when the peak area ratio values of the compounds in the additive sample were substituted in the calibration function, their amounts in the sample were calculated. Using these results, the recovery values of the method could be calculated (Table 5). According to the ICH guideline, the average % recovery should be between 95-105% [23]. This result shows that the accuracy of the method is high.

In this quantitative analysis study, excipients commonly used in tablets did not hurt the analysis results. Chromatograms showing the tablet sample and the tablet sample containing phenytoin spiked at a certain concentration for the recovery study are given in Figure 4. No interfering peaks were found in the chromatograms.

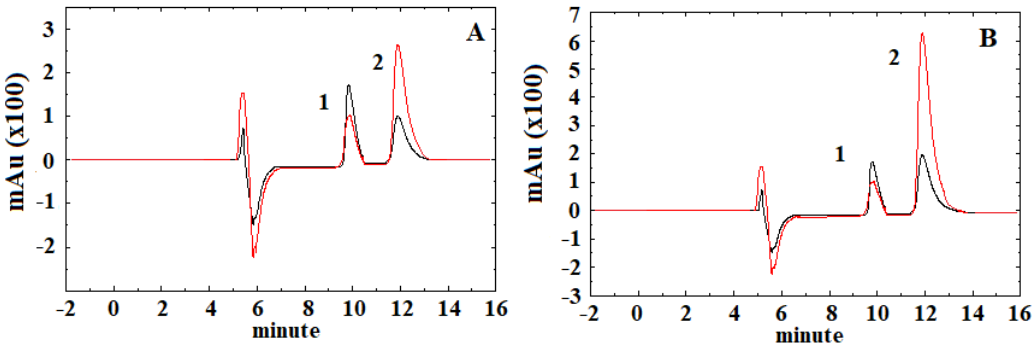

Figure 4. A) Tablet sample analysis (1-Dofetilide 0.5 µg/ml; 2-Phenytoin 1.2 µg/ml) B) Spiked sample analysis (1-Dofetilide 0.5 µg/ml; 2-Phenytoin 2.4 µg/ml)

Robustness Test

The robustness test shows the degree to which the method is affected when a small but significant change in the study parameters of the method is made. The smaller the effect of small changes in method parameters during routine processing on the analysis result, the more robust the method. In the optimized chromatographic condition, the changes in the amount of ethanol in the mobile phase, the pH change and the column temperature change, the tailing factor, and the peak area of phenytoin are given in Table 6.

Table 6. Robustness test results with different conditions

Parameter	Optimized condition	Used	Retention time (min)	Tailing factor	Peak area (mAu)
Mobile phase	**40:60 (v/v%) ethanol-water binary mixture**	45:60 (v/v%)	9.301	1.392	6725621
		35:65 (v/v%)	16.272	1.419	6649433
pH	**6.5**	8.0	9.322	1.407	6537047
		5.0	12.717	1.411	6419294
Column temperature	**37 ºC**	40 ºC	11.569	1.402	6581862
		25 ºC	13.243	1.410	6696332

According to these results, minor changes made in the repeatability of the results proved that the developed method was effective. There are many HPLC studies on the separation of phenytoin alone or simultaneously with different compounds in different samples. In these studies, qualitative and quantitative analysis of the compound was carried out using classical trial and error methods. The stationary phases used in the studies are generally C18 and the organic solvents used are common solvents in RPLC [31,35]. In the study conducted by Ayman et al., the micellar liquid chromatography method, known as green chromatography, was used. In this study, 10% (v/v) acetonitrile was used, although it was low compared to the amount of solvent used in RPLC [36].

Conclusion

In this study, the green RPLC method, an environmentally friendly method for the qualitative and quantitative analysis of the antiepileptic drug phenytoin, which is widely used in the treatment of epilepsy, one of the neurological disorders, was developed. This study, in which the optimum condition is determined depending on the pK_a value of phenytoin, is far from trial and error. First of all, the pH value measured in the ethanol-water binary mixtures prepared to determine the retention time of phenytoin was measured by pH standardization. This type of study is the first for this hydro-organic mixture. According to the LSS method, the $logk_w$ value, known as the hydrophobicity index of the compound, was calculated using the $logk\text{-}\varphi_{ethanol}$ linear relationship. Chromatographic conditions were determined for the analysis of phenytoin according to the suitability of the chromatographic parameters. This developed method is the first study in the literature with this optimization and suitability for green chemistry. In this study, an analytical procedure suitable for routine use was developed, and the method was validated for the determination of the amount of phenytoin in a single-active ingredient pharmaceutical dosage form. Method validation showed excellent results for linearity, precision, accuracy, limit of quantitation and limit of detection, and robustness parameters. This environmentally friendly analysis is a pioneering study for method optimization and validation without the need for any trial and error.

AUTHOR CONTRIBUTIONS

Concept: E.Ç.D., Y.D.D.; Design: E.Ç.D., Y.D.D.; Control: E.Ç.D., Y.D.D., İ.K.; Sources: E.Ç.D.; Materials: E.Ç.D., Y.D.D., E.F.Ö., İ.K.; Data Collection and/or Processing: E.Ç.D., E.F.Ö., İ.K.;

Analysis and/or Interpretation: E.Ç.D., Y.D.D., E.F.Ö.; Literature Review: E.Ç.D., Y.D.D., E.F.Ö., İ.K.; Manuscript Writing: E.Ç.D.; Critical Review: E.Ç.D., Y.D.D.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

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