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Chiral Separation of Econazole by High Performance Liquid Chromatography Method using Cyclodextrin as Chiral Column

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ABSTRACT. The chiral separation of econazole, an antifungal drug with one chiral center has been successfully carried out using the high-performance liquid chromatography (HPLC) method. Enantioresolution of econazole (*Rs* = 2.29) was achieved using cyclodextrin-based chiral column (Astec Cyclobond, 25 cm × 4.6 mm × 5 µm), mobile phase composition of acetonitrile : water (0.2% HCOOH) (20:80, v/v), and UV detection of 220 nm. The optimized HPLC method has been applied for the quantitative determination of econazole in the pharmaceutical (liquid) sample with percentage recovery of 100.75 % (RSD = 0,95%; *n* = 3). The effect of several HPLC parameters on the chiral separation of econazole was also evaluated and the method was successfully validated in terms of linearity, accuracy, precision, and selectivity. The present HPLC method was simple, short analysis time, and high resolution.

Keywords: Chiral separation, econazole, high-performance liquid chromatography.

INTRODUCTION

The enantiomeric separation is one of the most important fields in modern analytical chemistry, mainly in pharmaceutical and agrochemical products since a stereochemistry has a crucial role on biological activity. Enantiomers have the same chemical properties except to the reactivity of the enantiomers to optically active reagents. The physical properties of enantiomers are identical, but their direction e.g. rotation is different (Yardimci, 2020).

Many biological and pharmacological compounds are asymmetrical showing optical activity (Sahu et al., 2018; Zhang et al., 2020). Administration of pure, pharmacologically, and pharmacokinetically active enantiomers are important. Enantioselective synthesis is the most ideal method for obtaining pure enantiomers. However, this method is not recommended because it is impractical and expensive. On the other hand, the demand for enantiomeric separation methods on an analytical scale to control synthesis, check the racemization process, control the enantiomeric purity, and pharmacokinetic studies are very urgent at this time (Zhang et al., 2020; Dubey et al., 2012).

The physical and chemical properties of the enantiomers are identical in the symmetrical environment but differ in the pharmacological properties of the receptor. Enantiomeric separation is carried out to determine one of the enantiomers of a stereoisomer that has active drug properties. Most of the synthetic drugs still contain chiral compounds that are still marketed in racemic conditions and only a small portion are pure enantiomers. This racemic condition means that a drug in addition to having active enantiomeric properties also has enantiomeric properties that are inactive with some unwanted side effects or that it is toxic (Valimana-Traverso et al., 2019).

The antifungal drug imidazole has two nitrogen atoms in the azole ring. Butoconazole, ketoconazole, bifonazole, isoconazole, clotrimazole, oxiconazole, fenticonazole, miconazole, and econazole are some of the examples of this azole class (Yardimci, 2020). Econazole has a chemical name 1-[2-(2,4-dichlorophenyl) -2-(4-chlorobenziloxy) -ethyl]-imidazole with the molecular formula $C_{18}H_{15}C_{13}N_{20}$ (Salido-Fortuna et al., 2020). Currently, imidazole antifungals are widely used in the treatment of

vulvovaginal candidiasis, superficial fungal infections, keratitis, and water fleas. Econazole is generally administered in conventional topical formulations (solutions, ointments, gels, creams, and tablets). Econazole is an antimycotic drug with a structure similar to miconazole. Antimycotics are drugs that have the property to remove or kill fungi that live on the surface of the skin (Thomson et al., 2017; Azhari et al., 2020). This substance can be used in candidiasis with a night dose of 1 ovule for 3 days. Skin infections can be treated using ointments containing 1% econazole (Yardimci, 2020).

Econazole is an imidazole group that has antifungal activity. A popular and widely used method in the separation of enantiomers is the chromatographic technique. So far, the analytical method used for the separation of econazole enantiomers includes high-performance liquid chromatography (HPLC) using chiral column used was an AmyCoat RP and combinations of water, acetonitrile, diethylamine and acetic acid as mobile phase (Ali et al., 2009). Ultra high-performance thin layer chromatography (HPTLC) using an ethyl acetate–tetrahydrofuran–ammonia mobile phase (10.0 + 7.0 + 0.1, v/v/v) and silica gel HPTLC plates (Abbas et al., 2018).

Enantiomeric separations of econazole drugs are used chromatographic methods, where chiral stationary phase or chiral columns are often utilized to achieve optical recognitions. Several chiral additives to mobile phases are also used instead of chiral stationary phases in HPLC method (Ali et al., 2009; Taschwer et al., 2014). The advantages of the HPLC method are versatile, high resolution, high sensitivity, and rapid analysis (Dubey et al., 2012). This method is also capable of separating a large number of mixtures with similar substances. The resulting chromatogram provides both qualitative and quantitative information which is directly related to the elution time of each compound in the mixture which is represented by the chromatogram peaks that appear on the screen and the number of substances that correspond to the area and height of the peaks of each chromatogram.

Econazole is a chiral drug that has chiral carbon and contains two stereoisomers. The structure of econazole is shown in **Figure 1**. This compound is an anti-fungal with low solubility in water solvents and high solubility in methanol solvents (Abbas et al., 2018; Hermawan et al., 2010). Econazole can be used for tropical fungal infections as well as used as a nitrate salt in various medicinal formulations. S-econazole produced higher antifungal activity against several microorganisms especially against Aspergillus than R-econazole and racemic mixtures (Baker et al., 2016; Zhu et al., 2018).

HPLC is a method that has been used for chiral separation from econazole analysis. It interacts with the cytochrome P450 enzyme, 14 α-demethylase by converting lanosterol into ergosterol which is an important component in inhibiting the synthesis process in fungal cell membranes (Hall, 2011). In this research, chiral separation of econazole has been successfully achieved by cyclodextrin-based HPLC chiral column (Astec Cyclobond column (I 2000 HP-RSP, 5 µm) size 25 cm x 4.6 mm). Several parameters were also observed including the effect of the acetonitrile mobile phase, flow rate, injection volume, and administration of formic acid on the resolution value of the enantiomeric separation of econazole. The quantitative determination of econazole in the pharmaceutical sample was also investigated by the optimized HPLC system.

EXPERIMENTAL SECTION

Materials and Instrumentation

Materials used in this study include econazole standard (Sigma-Aldrich), methanol (Merck), dichloromethane (Merck), formic acid (Merck), acetonitrile (Merck), water (twice destilation), pharmaceutical samples (Epi Pevaryl p.v 1% econazole). Experiments were carried out using the HPLC Hitachi L (UV-Vis detector L-2420, pump L-2130, L-2200 autosampler, D-2000 Elite software, Astec CYCLOBOND column (I 2000 HP-RSP, 5 µm) size 25 cm x 4.6 mm. The detector was set to operate at 220 nm wavelength.

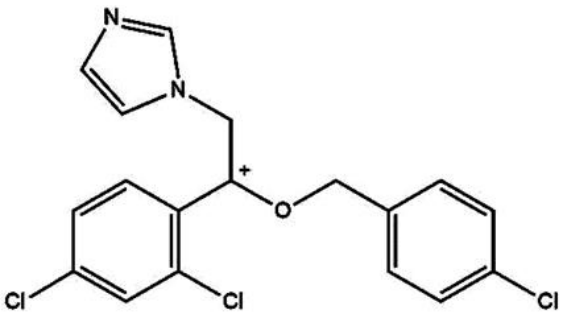

Figure 1. Chemical structure of econazole (note: The sign (*) indicates a chiral carbon atom)

Preparation of Standards

An accurate weight of 10 mg econazole standard was dissolved in 10 mL of methanol (1000 mg/L). The standard solution is diluted to 25, 50, 75, and 100 mg/L using methanol. standard solutions are labeled and covered with aluminum foil to avoid evaporation and stored in the refrigerator before use.

Preparation of Pharmaceutical Sample (1% Econazole)

Pharmaceutical samples (1% econazole) equivalent to about 2 g were dissolved in 5 mL of methanol, sonicated for 5 minutes, and the volume was adjusted to 25 mL with the same solvent. The solution was re-sonicated for 5 minutes and filtered. The filtrate (2 mL) was diluted in 10 mL of methanol. Then, the resulting solution was used for HPLC analysis.

Chiral Separation Conditions

In this research the HPLC method using a cyclodextrin-based (Astec Cyclobond I 2000 HP-RSP) is developed. Isocratic mode is used for the mobile phase at a flow rate of 1.0 mL/min. The wavelength for UV detection was fixed at 220 nm. Standard solution of econazole was injected into the injection valve (5 µL) by the autosampler. The column temperature was controlled at 25 °C. Chromatographic experiments were done using acetonitrile-water (0.1 % HCOOH) (20:80 v/v) and then degassed before application.

The following equation is the separation performance monitored in terms of retention factor (k), resolution (Rs) and selectivity (α).

$$K = \frac{t_R - t_0}{t_0} \quad (1)$$

$$Rs = \frac{t_2 - t_1}{w_2 + w_1} \quad (2)$$

$$\alpha = \frac{K_2}{K_1} \quad (3)$$

Where t_R and t_0 are on behalf of the analyte and unretained solute, respectively (Liu et al., 2020). t_1 and t_2 are the retention time, w_1 and w_2 are the peak widths at the baseline, k_1 and k_2 are the retention factors for the first and second eluting enantiomer, respectively (Wang et al., 2018).

Optimization of The HPLC Method

Enantiomeric separation of econazole was performed using a cyclodextrin-based Astec Cyclobond column (I 2000 HP-RSP, 5 μm) of 25 cm x 4.6 mm with a wavelength of 220 nm. The composition of the mobile phase, e.g. acetonitrile: water (0.1% HCOOH) was varied at the ratio of 10 : 90; 20 : 80; 30 : 70. Flow rate variations were done at 0.8; 0.9; and 1.0 mL/min. The variations in the injection volume were 1; 3; and 5 μL. The percentage addition of HCOOH to the mobile phase was also varied in the range of 0.1; 0.2; and 0.3 %. All samples were analyzed at room temperature. The optimum condition was determined based on the resolution value (Rs) between the two enantiomeric peaks of econazole.

Analytical Performance of The HPLC Method

The standard econazole solutions (25-100 mg/L) for linearity were prepared by dilution using methanol. All standard solutions were injected in triplicate. The peak area versus concentration will be plotted to create a calibration curve. The least squares method was used to determine the equation of the regression line to evaluate the slope, intercept, and correlation coefficient (Zanwar et al., 2020). Theoretical concentration with the concentration obtained is then compared for precision and accuracy testing. Calibration range is used to determine the value of the coefficient of variation (CV 5 SD/mean) and standard deviation (Rane & Shinde, 2008). The precision was determined in three replicates at a concentration of 50 mg/L in a short time interval. The recoveries of each econazole in the pharmaceutical samples were determined to analyze quality control samples (Mskhiladze et al., 2013). The sample was subjected to a sonication process before being analyzed. Detection and quantification limits were estimated to analyze econazole standard solutions at low concentrations. The limit of detection (LOD) and limit of quantification (LOQ) were defined on a ratio of 3 and 10, respectively (Wang et al., 2018).

Determination of Econazole Levels in Pharmaceutical (Liquid) Sample by HPLC

Econazole in pharmaceutical preparations is prepared by dissolving 2 grams of econazole sample in 5 mL of methanol. Furthermore, the substance is sonicated for 5 minutes before adding methanol to a volume of 25 mL. The solution was re-sonicated for 15 minutes. The solution is filtered and then 1 mL of the solution is taken. The filtrate is then diluted using methanol in a 10 mL volumetric flask until the limit mark. Furthermore, the sample solution was analyzed using HPLC with optimum conditions. Analysis of econazole levels was carried out by three repetitions (Romich, 2012).

RESULTS AND DISCUSSION

Huang et al., (2012), have succeeded in separating enantiomers from econazole compounds using a chiralpak AGP column. However, in this method there is a strong interaction between the AGP column as a stationary phase with the enantiomer of econazole, resulting in a long retention time of up to 22 min, making it less efficient. Zhang et al. (2020), have succeeded in separating the econazole enantiomers by using the chiralpak IC column using the acetonitrile-ammonium acetate buffer (5 mM) mobile phase (85: 15, v/v), but it still has weaknesses in terms of analysis time because it requires 16 min to complete the analysis, therefore it is less efficient. Moreover, Gaona-Galdos et al., (2008), have been able to separate the econazole enantiomer by using a C18 bondclone column. However, the interaction

between the stationary phase of the column and the enantiomer of econazole is still quite strong, resulting in a relatively long retention time of ± 13 min.

Hence, it is necessary to develop an analytical method that has a high-resolution value with a short analytical time of less than 10 min. The developed method will save analytical time so that it will be more efficient compared to the previously available methods. The need for short analytical methods has always been a priority in the development of analytical methods because these methods will be used for routine analysis purposes with relatively big sample sizes of various pharmaceutical formulations (Kenari et al., 2021).

Acetonitrile-water solution (0.2% HCOOH) (20:80 v/v) as the mobile phase was used in this HPLC method. Avoid long analysis time and better resolution can be obtained by using acetonitrile solution (Wang et al., 2018). To obtain the best enantioresolution of econazole, cyclodextrin-based chiral column (Astec Cyclobond I 2000 HP-RSP) was used in this study. Cyclodextrin-based columns have been popularly used for the separation of the enantiomers of azole compounds with excellent separation results (Scriba, 2019). Azhari et al., (2020) were able to separate ketoconazole and miconazole compounds using a cyclodextrin as chiral selector by capillary electrophoresis method, with good resolution. The best results were obtained in this HPLC method with analysis time of less than 10 minutes for chiral separation of econazole.

Optimization of HPLC

Parameters of mobile phase composition, flow rate, injection volume, and % formic acid (% HCOOH) were carried out for optimization using cyclodextrin-based chiral column (Astec Cyclobond I 2000 HP-RSP). The standard concentration of econazole and ketoconazole used was 50 mg/L and the peak was monitored at a wavelength of 220 nm. In research conducted by Valimana-Traverso et al., (2019), wavelength 220 nm is the optimum condition of econazole.

The Effect of Mobile Phase Composition

An important step to improve the first separation of the econazole enantiomer is by changing the percentage of acetonitrile in the mobile phase. The acetonitrile mobile phase was chosen because it provides a better resolution value among other polar mobile phases such as methanol, as described by Zhang et al., (2020). At the 10% of acetonitrile, the mobile phase gives a poor resolution value (Rs = 1.01) with a retention time of peak 1 of 8.02 min and peak 2 of 8.45 min. Increasing the percentage of acetonitrile from 10% to 20% results in the increase of resolution value of the econazole peak (Rs = 1.66) with a shorter retention time, i.e. 7.61 min for peak 1 and 8.39 min for peak 2. However, further increase in the acetonitrile percentage up to 30%, gives rise to a significant decrease in the resolution value of the econazole peak (Rs = 0.44). Therefore, the 20% of acetonitrile in the mobile phase was selected because it gives the best resolution (Rs = 1.66) with the retention time of peak 1 = 7.61 min and peak 2 = 8.39 min. These results are in good agreement with the reported results by Ali & Aboul-Enein (2001) which states that acetonitrile provides the best resolution value in the separation of enantiomers from econazole compounds. The results obtained in this study clearly show that the use of the acetonitrile mobile phase provides better resolution (Rs = 1.66) and shorter retention time (± 8 min).

The Effect of Flow Rate

The mobile phase flow rate may influence the separation value of the second econazole enantiomer. The flow rate of 0.8 mL/min has been found to give a fairly good resolution value (Rs = 1.59), but a stronger interaction between cyclodextrin in the stationary phase and the enantiomer of econazole results in a longer retention time of peak 1 (10.50 min) and peak 2 (11.81 min). In fact, increasing the flow rate to 0.9 mL/min has decreased the retention time of peak 1 and peak 2 into 7.97 min and 8.83 min, respectively. However, the resolution value of econazole decreases significantly (Rs = 1.45). To obtain the best resolution and shorter analysis time, the flow rate has been increased again up to 1 mL/min. The results show that the resolution value of the econazole peak improves (Rs = 1.66), and its retention times are shorter, 7.61 min and 8.39 min for peak 1 and peak 2, respectively. These results are due to the efficiency of the column which produces better values so that a symmetrical chromatogram peak is obtained. Based on the results of this investigation, the mobile phase flow rate of 1 mL/min was selected due to the best resolution value (Rs = 1.66) and a shorter analysis time (within 8 min).

The Effect of Injection Volume

The sample size represented by injection volume can influence the quality of the econazole compound separation. In this study, we have found that the injection volume of 5 μL gives a fairly good resolution value (Rs = 1.59) with the retention time of 7.61 min and 8.39 min for peak 1 and peak 2, respectively. A better resolution value (Rs = 1.60) has been obtained after reducing the injection volume to 3 μL. The shorter retention time is also obtained using this injection volume, e.g. at 7.03 min and 7.77 for peak 1 and peak 2, respectively. Further decrease in the injection volume down to 1 μL gives rise to a significant increase in the resolution value (Rs = 1.82) and their retention time is not much different with that of 3 μL. Based on these results, the injection volume of 1 μL has been selected because it gives the best resolution value (Rs = 1.82) and shorter retention time. It was very beneficial because utilizing the smaller volume of injection would reduce the waste which supports the separation method of green chemistry concept.

The Effect of Formic Acid Addition

The concentration of formic acid in water has a significant effect on the resolution value of econazole enantiomers. The concentration of 0.1 % formic acid resulted in a fairly good resolution value (Rs = 1.82). Increasing the concentration of formic acid to 0.2% results in a very significant increase in the resolution value of econazole (Rs = 2.29). Further increasing the concentration of formic acid to 0.3% decreases the resolution value of econazole (Rs = 2.05). Therefore, it is understood that the optimum concentration of formic acid addition is achieved at 0.2%, exhibiting the best separation. The parameters of the optimization results of the enantiomeric separation of econazole are shown in **Table 1**.

In the reverse phase HPLC method, the formation of hydrophobic inclusion complexes between the cyclodextrin chiral selector and the econazole enantiomer occurs. The variation of the organic phase can affect the resolution value, retention time, and level of selectivity. There is a competition between the organic formic acid solvent and the econazole enantiomer in obtaining the non-polar cavity of the cyclodextrin, as a result, the econazole retention time is longer when the concentration of the organic phase is increased (Szabó et al., 2016). In this case, increasing the concentration of formic acid from 0.2% to 0.3% decreases the resolution of econazole enantiomers.

Method Validation

The validation of the analytical method is a procedure of assessing certain parameters based on experimental works carried out in the laboratory. Method validation has the objective of ensuring and confirming that the analytical methods used in a certain analysis have met the requirements set and are following the desired objectives. Method validation conducted in this study has applied the optimum conditions obtained from the HPLC optimization results.

Linearity

Linearity is one of the tests in method validation that shows a linear relationship between variables (Baker et al., 2016). This test has been carried out by constructing a standard calibration curve of econazole standards with concentration variations of 25-100 mg/L analyzed by HPLC under optimum conditions. Measurements were performed in three replicates and plotted peak area vs concentration to determine linear regression, using the least squares method. Determination coefficient test is used to measure how

Table 1. Optimization with some parameters includes variations in the mobile phase, flow rate, injection volume, and percentage of formic acid (HCOOH)

Optimation	Retention time of solvent (min)	Retention time (min)		Rs
		Peak 1	Peak 2	
Acetonitrile: water (0.1 % HCOOH)				
10:90	5.21	8.02	8.45	1.01
20:80	**3.19**	**7.61**	**8.39**	**1.66**
30:70	3.11	4.51	4.69	0.44
Flow rate (mL/min)				
0.8	3.99	10.50	11.81	1.59
0.9	3.52	7.97	8.83	1.45
1.0	**3.19**	**7.61**	**8.39**	**1.66**
Injection volume (μL)				
5	3.19	7.61	8.39	1.59
3	3.17	7.03	7.77	1.60
1	**3.19**	**7.03**	**7.78**	**1.82**
% HCOOH				
0.1	3.19	7.03	9.56	1.82
0.2	**3.21**	**7.54**	**8.61**	**2.29**
0.3	3.23	8.69	7.78	2.05

well the regression line is. Based on the experiment results, the standard calibration curve can be obtained with the linear equation of y = 1561.5x-7118.2 for the first peak of enantiomer and the determination coefficient value r^2 of 0.9992. The second peak of the enantiomer has the linear equation of y = 1557.2x-10713 with r^2 value of 0.9998. Based on the determination coefficient value of both enantiomer peaks, this method can be included in the meticulous criteria because their determination coefficient value is greater than 0.997.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ of this proposed method has been determined to know the lowest concentration of analyte that can still be detected and can be measured quantitatively to calculate the value of accuracy and precision of the method. The detection limits and the quantification limits are calculated from a linear line of the calibration curve (Pesek et al., 2007). According to the experimental data, the detection limit values for the first and second enantiomeric peaks are 3.309 and 1.798 mg/L, respectively. The quantification limit test results of the first and second enantiomeric peaks are 11.029 and 5.995 mg/L, respectively. This value is in accordance with research by Kenari et al., (2021) which separated the econazole enantiomer with a beta-cyclodextrin-based column with LOD values of 1.6 mg/L and LOQ 5.3 mg/L, respectively combined with ionic liquids based on L-glutamic acid and L-lysine. The smaller the detection limit and the quantification limit, the better and more sensitive the method will be in detecting an analyte.

Precision

The precision test expressed as relative standard deviation (RSD) has been evaluated, measuring in six repetitions using the standard solution of econazole 50 mg/L. This test uses the principle of repeatability in which the test is carried out on the same day at the same equipment condition, solvent, and analysis (Hermawan et al., 2020). Based on the test results, the first and second peak enantiomeric standard deviation values were 0.42 and 0.22, respectively. The relative standard deviation (RSD) of the first and second enantiomeric peaks were 0.84 and 0.45%, respectively. The RSD value did not exceed 2%, indicating that this method had a very precise level of accuracy (Harmita, 2004).

Accuracy

The accuracy is determined using the standard addition method using the following procedure. The pharmaceutical sample is first analyzed, then the standard solution of econazole is added to the econazole sample and the sample solution is then analyzed again. Accuracy is expressed in percent recovery of the standard amount found in the sample by HPLC analysis after the addition of the standard to the sample solution. This accuracy was determined by analyzing the standard solution of econazole 25 mg/L added to the sample solution using HPLC under the optimum conditions. Based on the experimental results, the value of percentage recovery is 102.25 %. This shows that the proposed method has a high level of accuracy so that it can be recommended for routine analysis purposes. The accepted range of recovery percentage for analytes with the concentration of 25 mg/L is 90-107% (Harmita, 2004).

Selectivity

Selectivity is the ability to produce accurate and specific measurements in the presence of other components contained in the sample matrix. selectivity is indicated by the separation between two compounds that are still of the same group or kind. Ketoconazole compounds have been used in this study to test the econazole selectivity. The selection of ketoconazole as a compound for the selectivity test is based on fact that it is an antifungal compound with a structure similar to econazole. The standard solution of econazole 100 mg/L was added to the standard solution of ketoconazole 100 mg/L (1:1). Then the solution mixture was analyzed using HPLC under optimum conditions and the results are presented in **Table 2.**

Based on the calculations, it is found that the selectivity factor of econazole in the presence of ketoconazole at the same concentration is 1.29. This value indicates that the analytical method can accurately and specifically measure econazole compounds in the presence of other compounds, namely ketoconazole. This method can also be used to separate econazole and ketoconazole well which is characterized by an alpha value of more than 1.

Table 2. Selectivity (alpha) and retention time (tR) of Econozole and Ketoconazole Enantiomers

Repetition	tR of solvent (min)	tR of Econazole (min)		tR of Ketoconazole (min)	
		Peak 1	Peak 2	Peak 1	Peak 2
1	3.22	8.15	9.13	10.86	11.74
2	3.22	8.12	9.09	10.79	11.75
3	3.22	8.10	9.05	10.75	11.59
			α = 1.29		

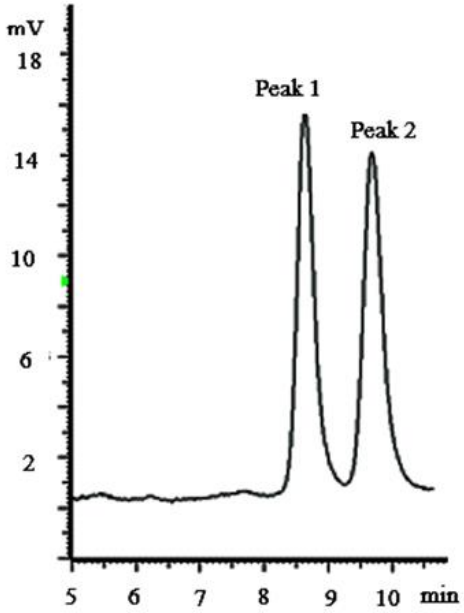

Figure 2. Chromatogram of econazole enantiomers in the pharmaceutical (liquid) sample. Peaks 1 and 2 are econazole enantiomers. HPLC conditions: mobile phase of acetonitrile-water (0.2 % HCOOH) (20:80, v/v), cyclodextrin-based Astec Cyclobond (25 cm × 4.6 mm × 5 µm) as stationary phase, UV detection of 220 nm, flow rate of 1.0 mL/min, and injection sample volume of 1 μL.

Determination of Econazole in The Pharmaceutical (Liquid) Sample

The proposed method has been applied in the determination of econazole enantiomers in the pharmaceutical (liquid) sample. Chromatogram of econazole enantiomers is depicted in **Figure 2**. The two peaks on the chromatogram indicate that econazole is a chiral compound. Enantiomers can be separated using chiral columns, such as cyclodextrin-based (Arranz et al., 2000; Mskhiladze et al., 2013). Based on the results, the econazole content in the cream sample obtained was 100.75%. This result clearly shows that this method is very accurate and can be used for routine analysis purposes.

CONCLUSIONS

The HPLC method using cyclodextrin-based (Cyclobond) as a chiral column has been successfully developed for chiral separation of econazole with *Rs* = 2.29 and analysis time within 9 min. The calibration curve of econazole is linear with r^2 = 0.9992. LOD and LOQ of econazole are 3.31 and 11.03 mg/L, respectively. Quantitative determination of econazole in the pharmaceutical (liquid) sample can be obtained with percentage recovery of 100.75% (*RSD* = 0. 95% ; *n* = 3). The present HPLC method is simple, short analysis time, and high resolution.

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