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Molecular Docking Approach and Enantioseparation of Hydroxychloroquine by High-Performance Liquid Chromatography using Amylose Tris (3,5-dimethyl phenyl carbamate) as the Chiral Selector

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Abstract

The chiral separation of hydroxychloroquine, an antimalarial drug with one chiral center, has been predicted using molecular docking and was proven using the HPLC method. Docking utilized the PM3 semi-empirical method with specific grid coordinates (X = 19.977, Y = 20.069, and Z = 25.901) and a grid size of (X = 20, Y = 20, and Z = 60), employing a grid spacing of 1,000 Å, an exhaustiveness value of 8, and num_modes of 10. The analysis revealed the enhanced stability of R-hydroxychloroquine within the tris amylose complex, resulting in slower retention and elution rates compared to S-hydroxychloroquine. The HPLC experimental validation demonstrates resolution (Rs = 2.23), successfully achieved by employing amylose tris-based chiral columns. The mobile phase composition employed in this study consisted of acetonitrile:aquabidest: dimethylamine (47:52:1, v/v). Detection was performed at 343 nm, and the optimized HPLC method successfully quantitatively determined hydroxychloroquine in a liquid pharmaceutical sample with a percentage recovery of 98.47%.

1. Introduction

The pharmaceutical industry produces various classes of drugs, including the aminoquinoline. One common aminoquinoline drugs is 4-aminoquinoline [1]. Drugs in the 4-aminoquinoline group have derivatives, one of which is hydroxychloroquine. Hydroxychloroquine is an immunomodulatory drug used to treat malaria and autoimmune diseases such as systemic lupus erythematosus and arthritis by preventing and suppressing the activation of its receptors [2]. Hydroxychloroquine has also been recommended by the Food and Drug Administration (FDA) for emergency treatment in hospitalized COVID-19 patients [3].

Chiral compounds, such as hydroxychloroquine, are used as drugs. Certain chiral drug compounds, like hydroxychloroquine, are typically crafted as a blend of both enantiomers during the pharmaceutical formulation process. Nevertheless, it is imperative to emphasize that not all pharmaceuticals adhere to this strategy. Some drugs employ one of the two enantiomers. Only one of the enantiomers in the racemic can provide a significant pharmacological effect when consumed for treatment, while the other enantiomers have no effect or even have a toxic effect [4]. The hydroxychloroquine toxicity of the S and R isomers is unknown, but the S isomer outperforms the R isomer in treating malaria [5]. This is evidenced by a study conducted by Ni *et al.* [6] that found the R-hydroxychloroquine configuration showed higher antiviral activity than the S configuration and ransemic hydroxychloroquine. Separation of chiral compounds in medicinal preparations is a process that needs to be

carried out. This is because it determines the purity of the active substance and the levels of chiral compounds in drugs [7].

The difference in retention time of the two enantiomers depends on their interaction with the chiral column. The differences in molecular interactions between the two enantiomers can be analyzed using the molecular docking approach. Molecular docking is a computational method for analyzing the recognition and interaction of ligands with their receptors [8]. These interactions include hydrogen bonds, electrostatic interactions, van der Waals forces, and hydrophobic interactions. The process of interaction of the ligand to the receptor causes constant structural changes so that the most stable bond is obtained [9]. The uses of this method include analyzing binding and affinity strength, creating structure-based drug designs, predicting the protein structure of ligand complexes, and drug screening [8].

The necessary methods that allow to prove the results of separation of the S and R using molecular docking include liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE) [7]. Another method that can be used to separate S and R enantiomers is high-performance liquid chromatography (HPLC) [10]. The HPLC method was selected in this study because it has several advantages, including good sensitivity, specificity, and automation; many detection techniques; and various chiral columns available for separation [11].

The method of detecting hydroxychloroquine compounds using HPLC was carried out by Harahap *et al.* [12] using a C18 column with a mobile phase of 1% acetonitrile: diethylamine in water (65: 35, v/v), and the retention time was 5.29 minutes. However, two enantiomers have not yet been separated successfully. The separation of chiral compounds on hydroxychloroquine was conducted by Xiong *et al.* [13]. In their research, they utilized HPLC with a Chiralpak AD-H column with n-hexane and isopropanol as mobile phases with the addition of 0.50% diethylamine in n-hexane. The results showed the successful separation of the hydroxychloroquine enantiomers. Specifically, the R- hydroxychloroquine enantiomer was identified at 26 minutes, while the S-hydroxychloroquine enantiomer was detected at 29 minutes, achieving a resolution of 2.08.

Research results conducted by Xiong *et al.* [13] were quite good from the point of view of its resolution; however, the retention time of >25 minutes was relatively prolonged. Therefore, developing a separation method that can reduce the retention time of the two enantiomers is urgently needed. Based on these circumstances, this research endeavors to devise an analytical method to achieve shorter retention times, thereby enhancing the efficiency of enantioseparation analysis for the drug. Several method enhancements have been explored, including utilizing a modified mobile phase composition, namely acetonitrile:aquabidest: dimethylamine [12]. This was employed in conjunction with an amylose tris (3,5-dimethyl phenyl carbamate)-based chiral column [14] operating in the reverse phase mode [15]. The modification was undertaken to achieve improved separation outcomes and reduced retention times.

The development of analytical methods for chiral drug separation is very important because it is required to control drug quality, pharmacological and pharmacokinetic studies, and the development of single enantiomer formulations [4]. Molecular docking to predict the interaction between the enantiomers and the column is a beneficial approach to help reduce the experimental optimization that usually requires a lot of reagents and time. This method modification must be validated to prove that the developed procedures have met the requirements. The development of chiral drug analysis methods has been validated based on the 1992 FDA statement regarding chiral drugs [16]. Validation must be done on racemic drug compounds to determine the S-isomer and R-isomer [17]. Validation of the analytical method uses several parameters, including linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, accuracy, and selectivity [18].

2. Experimental

2.1. Materials and Instrumentation

Molecular docking simulations were conducted using a personal computer with a Windows 10 x64 Operating System (OS) and an Intel® CoreTM i5-6500 CPU @ 3.20 GHz. The software employed for this study included Gaussian 09W, Gaussian 6.0, Open Babel, AutoDock Tools, AutoDock Vina, and PyMOL. The receptor structure utilized in the simulations was tris amylose, and the ligands examined were in the form of R-hydroxychloroquine and S-hydroxychloroquine, available for download on PubChem with PubChem CID: 178395 and 178396, respectively.

The enantioseparation results predicted by molecular docking were then proved experimentally using HPLC Hitachi L such as UV-Vis detector L-2420, pump L-2130, D-2000 Elite software, and L-2200 autosampler (PT. Indotech Scientific) and amylose tris (3,5-dimethyl phenyl carbamate)-based chiral column (Lux® 5 Amylose-1) of 250 x 4.6 mm. The analytes were operated at a wavelength of 343 nm. The materials used include acetonitrile (Merck, 99.90%), dimethylamine (Merck, 99.90%), aquabidest (twice distillation), hydroxychloroquine standard (Sigma-Aldrich), and pharmaceutical samples (PT. Tempo Scan Pacific Tbk., 200 mg hydroxychloroquine).

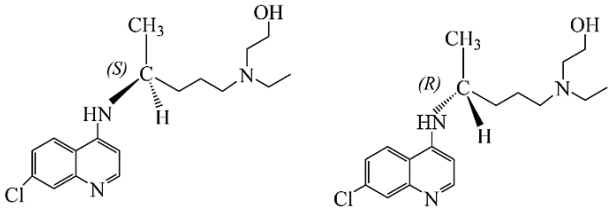

Figure 1. Chemical structures of (R and S) hydroxychloroquine

2.2. Molecular Docking Analysis

2.2.1. Computational Methods Optimization

A suitable processing methodology was essential to achieve optimal conformation of the ligand structure. The HyperChem program was employed to select the most suitable calculation methods from a range of semi-empirical methods, including PM3, PM6, PM7, AM1, and others. In the context of this research, PM3 was identified as the best method applied to this research [19].

2.2.2. Molecular Docking

The introduction of macromolecular structures for MD simulations can be facilitated through the Protein Data Bank (PDB), accessible at rcsb.org. After accessing the website, the 'Tris Amylose' macromolecule was located and downloaded from the UniProt page in its beta version. Subsequently, structure visualization was performed using PyMol, and macromolecular preparation involved water removal, ligand preparation, and atom adjustments to ensure the integrity of the macromolecules and their associated ligands [20].

The process of ligand docking was conducted employing specific grid coordinates, namely X = 19.977, Y = 20.069, and Z = 25.901, facilitating the precise positioning of the ligand within the binding site. The grid dimensions encompassed X = 20, Y = 20, and Z = 60, providing an encompassing search space for potential interactions. Moreover, a grid spacing of 1,000 Å was employed to ensure sufficient granularity for accurate calculations. To ensure a thorough exploration of conformational space, an exhaustiveness value of 8 was selected, ensuring comprehensive sampling, with a num_modes value of 10 to capture multiple potential binding modes.

2.3. Development of Analytical Methods

2.3.1. Preparation of Standards

The accurate weight of 10 mg of hydroxychloroquine standard was dissolved in 10 mL of water. The hydroxychloroquine standard solution was diluted to 50, 100, 150, and 200 mg/L using water. Standard solutions were covered and stored refrigerated until they were ready for use.

2.3.2. Optimization of the HPLC Method

The ratios of acetonitrile:aquabidest: dimethylamine were tested at 46:53:1, 47:52:1, 48:51:1, 49:50:1, 47:52:1, 45:54:1, 47:53:0, 47:52:1, and 47:51:2. Flow influence ratios of 0.6, 0.8, and 1 mL/min were investigated. The injection volume was examined at 5, 3, and 1 µL. The Rs value was calculated using Equation (1) [21].

$$Rs = 2 \times \frac{t_2 - t_1}{w_2 + w_1} \quad (1)$$

where, w_1 and w_2 are the peak widths at the baseline [22]. Retention times are denoted by t_1 and t_2 [23].

2.3.3. Preparation of Hydroxychloroquine Standard Calibration Curve

Standard solutions of hydroxychloroquine 50, 100, 150, and 200 mg/L were injected as much as 1 µL using a syringe into the column and carried out twice a row. The obtained data were then utilized to form the calibration curve for the hydroxychloroquine standard solution.

2.3.4. Preparation of Pharmaceutical Sample (Hydroxychloroquine)

Hydroxychloroquine pharmaceutical samples (200 mg) were crushed, dissolved in 25 mL of methanol, sonicated for 30 minutes, and filtered. The filtrate (0.9375 mL) was diluted in 50 mL water to make a 150 mg/L hydroxychloroquine pharmaceutical sample.

2.3.5. Determination of Hydroxychloroquine Levels in Pharmaceutical (Liquid) Samples by HPLC

The hydroxychloroquine 150 mg/L sample solutions were analyzed using HPLC under optimum conditions. Analysis of hydroxychloroquine levels was carried out by three repetitions [21].

2.4. HPLC Method Validation

The validation of the developed method is essential to ensure its reliability in yielding accurate results that closely approximate the true values [24]. The development of this method was validated using several parameters, including linearity, the limit of detection (LOD) and limit of quantification (LOQ), precision, accuracy, method range, and selectivity [18]. Standard solutions of hydroxychloroquine at a concentration of 50, 100, 150, and 200 mg/L were analyzed under optimum conditions to calculate LOD and LOQ, and the injection was performed three times to calculate linearity. The LOD and LOQ were calculated using Equation (2) and (3), respectively.

$$\text{LOD} = \frac{3(\frac{sy}{x})}{b} \quad (2)$$

$$\text{LOQ} = \frac{10(\frac{sy}{x})}{b} \quad (3)$$

where sy/x is the standard deviation of y to x, b is the slope [25].

Precision and accuracy were determined by injecting a 150 mg/L hydroxychloroquine standard solution repeatedly in a short period for a precision of six repetitions and an accuracy of three repetitions. The results of precision were shown in the value of standard deviation (SD), relative standard deviation (RSD), the coefficient of variation (CV), and Horwith Ratio (HORRAT), while the accuracy results were shown in the percentage of the recovery. The selectivity was determined by mixing 0.5 mL of 150 mg/L hydroxychloroquine standard solution with 0.5 mL of 150 mg/L chloroquine standard. Then, the retention time of the mixture was compared with the respective standard solutions (Equations 4 and 5).

$$K = \frac{t_R - t_0}{t_0} \quad (4)$$

$$\alpha = \frac{K_2}{K_1} \quad (5)$$

where, t_R and t_o are on the sake of the analyte and unretained solute [26]. The k_1 and k_2 are the retention factors [23].

3. Results and Discussion

3.1. Molecular Docking

Enantiomers with different retention times were identified using molecular docking. The preparation process involved both receptors and ligands. The tris amylose receptor was performed using AutoDock Tools with files stored in the .pdbqt extension. This preparation was carried out by adding polar hydrogen to tris amylose. Meanwhile, the ligands were prepared using the PM3 semi-empirical method using the HyperChem program. This preparation is intended to optimize the geometry, ensuring the stabilization of the R-hydroxychloroquine and S-hydroxychloroquine structures at their lowest energy states.

Molecular docking in this study was carried out using the Command Prompt (CMD) to facilitate the docking process. The procedure involved the removal of the H_2O molecule from the receptor, as its presence was found to impede the docking process. Subsequently, polar hydrogen atoms were added to the receptor to conduct further docking. The grid boxes employed in this docking process had grid centers specified as X = 19.977, Y = 20.069, and Z = 25.901, with corresponding grid sizes of X = 20, Y = 20, and Z = 60, and a grid spacing of 1,000 Å. The exhaustiveness value used is 8 with num_modes of 10.

The docking results obtained were the ΔG values of the tris amylose/R-hydroxychloroquine inclusion complex in the range of -5.6 to -4.9 kcal/mol (Table 1). In another inclusion complex, tris amylose/S-hydroxychloroquine, the ΔG value was obtained from -5.5 to -5.1. This ΔG value indicates the conformational stability of the formed inclusion complexes. A more negative ΔG value indicates that the complex conformation is stable, while a less negative ΔG value indicates that the complex conformation is less stable [27]. In this case, R-hydroxychloroquine has a more stable conformation than S-hydroxychloroquine. Therefore, S-hydroxychloroquine was expected to be eluted first, followed by R-hydroxychloroquine during the HPLC separation process.

3.2. Optimization of the HPLC Method

Some HPLC parameters, including mobile phase composition, flow rate, injection volume, and wavelength, were optimized to obtain the best enantioresolution (Table 2).

3.2.1. Effect of mobile phase composition

In this study, the mobile phase composition of acetonitrile:aquabidest:dimethylamine (47:52:1, v/v) demonstrated the optimal resolution (Rs = 2.23). The retention times for peak 1 (S-hydroxychloroquine) and peak 2 (R-hydroxychloroquine) were observed to be 14.77 minutes and 16.3 minutes, respectively (Table 2).

3.2.2. Effect of Flow Rate

The investigation revealed that a 0.6 mL/min flow rate yielded the optimal resolution of enantiomers, with a Rs value of 2.23. Further increasing the flow rate up to 0.8 mL/min and 1.0 mL/min results in the lower resolution of the two enantiomers (Table 2).

3.2.3. Effect of Injection Volume

The best resolution of the enantiomers is achieved at the injection volume of 1 µL (Table 2). Increasing the injection volume to 3 µL leads to a lower separation resolution.

Table 1. Affinity and RMSD values for R- and S-hydroxychloroquine

Mode	R-hidroxychloroquine			S-hidroxychloroquine		
	Affinity	RMSD L.B.	RMSD U.B.	Affinity	RMSD L.B.	RMSD U.B.
1	-5.6	0.000	0.000	-5.5	0.000	0.000
2	-5.5	9.224	10.790	-5.4	14.374	16.505
3	-5.2	13.787	16.689	-5.4	12.937	16.011
4	-5.2	10.245	12.257	-5.3	10.148	11.423
5	-5.1	13.136	17.099	-5.3	13.847	17.388
6	-5.1	14.528	16.573	-5.3	9.851	11.147
7	-5.1	9.696	10.791	-5.2	9.780	11.026
8	-5.0	3.359	6.762	-5.2	13.813	17.097
9	-4.9	14.472	16.389	-5.2	2.137	3.693
10	-4.9	2.233	3.575	-5.1	9.553	10.814

3.2.4. Effect of Detector Wavelength

The detector wavelength was optimized at 333, 343, and 353 nm. The best resolution of enantiomer separation (Rs = 2.23) was obtained at a detector wavelength of 343 nm (Table 2).

There are several interactions in chiral recognition between the analyte and the stationary phase, including electrostatic forces, steric effects, hydrophobicity, and hydrogen-bonding interaction [1]. According to Matarashvili *et al.* [28], the interaction that occurs between the chiral selector, namely amylose tris (3,5-dimethylphenylcarbamate) stationary phase with this mobile phase is a hydrophobic interaction because the water content used is more than 20%. Increasing the content of water in the mobile phase results in the increase of the hydrophobic interaction. In contrast, increasing the acetonitrile content in the mobile phase gives rise to a decrease in the hydrophobic interaction [28]. The hydrophobic interaction occurs because of the presence of hydrophobic groups on or near the surface of molecules that can interact with the hydrophobic column matrix [29].

The polar group in the hydroxychloroquine enantiomer interacts more strongly with the polar mobile phase; the enantiomer with more polar properties will be eluted earlier from the column [30]. The more or stronger the polarity of the group attached, the more or stronger bonds are formed. Consequently, the higher interaction affinity between the polar groups of the compound and the mobile phase is formed. This causes enantiomers with more polar groups to have a shorter retention time because they elute faster than those with weaker polar groups.

3.3. Method Validation

3.3.1. Linearity

The calibration curve constructed based on peak 1 has the regression equation y = 0.0092x + 0.0733 with a correlation coefficient (r) of 0.9999 and coefficient of determination (r^2) of 0.9997. The regression equation of the calibration curve for peak 2 is y = 0.007x+0.0117 with r of 0.9993 and r^2 of 0.9986. The two peaks produce the determination and correlation coefficients that meet the standard requirements. The analytical method is considered to be satisfactorily fulfilled with a high degree of accuracy when the correlation coefficient (r) (linearity) of the calibration curve exceeds 0.995 and its coefficient of determination (r^2) is greater than 0.997 [31]. Based on this reference, the value of the determination and the correlation coefficients obtained from this study meet the standard requirements. Therefore, the developed method of hydroxychloroquine chiral compounds in this study has good accuracy and can be used for routine analysis.

Table 2. Optimization of the HPLC method

HPLC parameter	Retention time (min)		Rs
	Peak 1	Peak 2	
acetonitrile: aquabidest: dimethylamine (%)			
45:54:1	16.95	18.33	0.76
46:53:1	17.71	18.29	1.04
47:52:1	14.77	16.30	2.23
48:51:1	14.33	15.52	0.38
49:50:1	13.78	15.01	1.28
47:53:0	16.25	18.09	1.38
47:51:2	14.72	16.19	0.76
flow rate (mL/min)			
1.0	8.67	9.55	0.94
0.8	10.97	12.09	1.05
0.6	14.77	16.25	1.23
injection volume (µL)			
5	14.81	16.27	0.19
3	14.95	16.38	0.23
1	14.77	16.30	2.23
Wavelength (nm)			
333	15.65	16.76	1.10
343	14.77	16.30	2.23
353	21.17	22.64	0.65

3.3.2. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The determined LOD values for peak 1 and peak 2 are 13.70 mg/L and 8.99 mg/L, respectively. These values signify the minimum concentration of the hydroxychloroquine standard solution that the HPLC method can detect. If the analysis involves concentrations below these respective values, the instrument may not be able to detect the compound reliably. Moreover, the LOQ values are determined to be 45.67 mg/L for peak 1 and 29.98 mg/L for peak 2. These values of LOQ indicate the lowest concentrations of hydroxychloroquine standard solution that can be used for precision and accuracy testing. The smaller the value of LOD and LOQ obtained, the more sensitive the method is [32].

3.3.3. Precision

The calculation of the standard deviation for the method, as derived from peak 1, results in a value of 1.80, whereas for peak 2, it is 1.51. Furthermore, the coefficient of variation is determined to be 1.43 at peak 1 and 0.96 at peak 2. These values of the relative standard deviation, which is less than 2, suggest that the method has high precision and meets the requirements of good precision [33]. The HORRAT value generated at peak 1 is 0.13, and at peak 2 is 0.09. The HORRAT value obtained in this study is less than 1, indicating that the method can be classified as valid and meets the requirements of good acceptance [22].

3.3.4. Accuracy

The average percentage of recovery determined at the two peaks is 92.43%. These results indicate that the analytical method used has met the minimum standard of acceptability for an analyte concentration of > 100 mg/L, specifically falling within the range of 90–107% [34].

3.3.5. Selectivity

The selectivity test was determined by mixing 0.5 mL of 150 ppm hydroxychloroquine standard solution with 0.5 mL of 150 ppm chloroquine standard solution into the vial. In the mixed solution of hydroxychloroquine and chloroquine, the retention time of the standard hydroxychloroquine solution is 15.52 minutes, while the standard chloroquine solution is 34.53 minutes. The results obtained from the mixed solution were then compared to those of two standard solutions determined separately, yielding a selectivity value of 2.68. A selectivity test is considered good if the selectivity value obtained is greater than 1. Hence, the separation method of chiral hydroxychloroquine compounds is very selective against chloroquine in this study [35].

3.4. Determination of Hydroxychloroquine in the Pharmaceutical (Tablet) Sample

For the determination of real pharmaceutical drugs, the sample of hydroxychloroquine drug obtained from PT Tempo Scan Pacific has been used. The analysis was done by injecting 1 µL of 150 mg/L hydroxychloroquine sample solution into HPLC with three replications under optimum analysis conditions. The concentration of hydroxychloroquine in the drug preparation sample at peak 1 is 147.91 mg/L, and at peak 2 is 147.49 mg/L. According to the Kementerian Kesehatan Republik Indonesia [36], it is stated that the level of hydroxychloroquine in the tablet should not less than 93.00% and not exceed 107.00%. The standard peaks appear at 14.52 min and 16.26 min. Furthermore, the percentage recovery of hydroxychloroquine in the pharmaceutical sample with a concentration of 150 mg/L is 98.47%. The HPLC chromatogram of hydroxychloroquine enantiomers for the standard and sample are given in Figure 2, showing the very similar peaks between the standard and sample and the good resolution of each enantiomer.

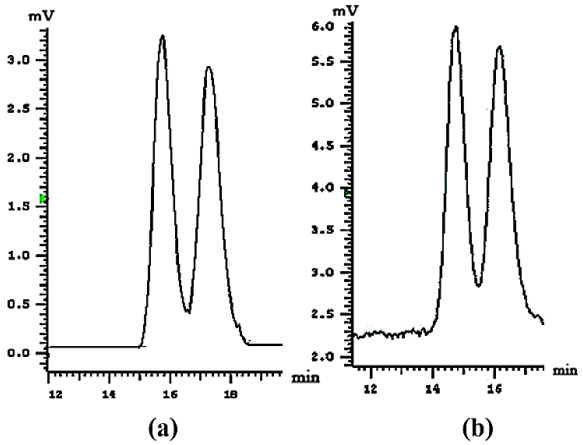

Figure 2. Chromatogram of hydroxychloroquine enantiomers (a) standard (150 mg/L), (b) tablet sample. HPLC conditions: acetonitrile:aquabidest: dimethylamine (47:52:1, v/v) as mobile phase, amylose tris (3,5-dimethyl phenyl carbamate) as stationary phase (Lux® 5 Amylose-1, 250 x 4.6 mm), UV detection at 343 nm, flow rate of 0.6 mL/min, and injection sample volume of 1 µL

4. Conclusion

The chiral separation of hydroxychloroquine was predicted based on a molecular docking approach using amylose tris (3,5-dimethyl phenyl carbamate) as a chiral selector. Subsequent analysis indicated the higher stability of R-hydroxychloroquine when forming a complex with tris amylose, leading to a decelerated rate of retention and elution in comparison to S-hydroxychloroquine. Based on molecular docking approach, chiral separation of hydroxychloroquine was successfully achieved by the HPLC method using acetonitrile:aquabidest: dimethylamine (47:52:1, v/v) as mobile phase, amylose tris (3,5-dimethyl phenyl carbamate) as stationary phase (250 × 4.6 mm), with resolution (Rs) = 2.23 and retention time less than 18 min. Determination of hydroxychloroquine in the pharmaceutical (tablet) sample gives a percentage of recovery of 98.47%, indicating the accuracy of the proposed method. The developed HPLC method is simple, gives high resolution, and has a shorter analysis time. This study also confirms that molecular docking is a suitable approach to predict the enantioseparation of chiral drugs before real analysis using analytical methods such as HPLC.

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