5 th International Conference on Multidisciplinary Approaches for Sustainable Rural Development (ICMA - SURE)

LETTER OF ACCEPTANCE AND INVITATION

Date : November 5, 2022
No. : 066/LoA/ICMA-SURE-2022

Dear **Dadan Hermawan, Salsabil Rahmadina, Irmanto Irmanto, Mudasir Mudasir, Hassan Y Aboul-Enein**

Thank you for submitting your abstract for presentation at the 5th International Conference on Multidisciplinary Approaches for Sustainable Rural Development (ICMA-SURE) 2022. After reviewing your abstract, we are pleased to inform you that your abstract entitled:

Chiral Separation of Hydroxychloroquine by High-Performance Liquid Chromatography Method using Amylose Tris (3,5-dimethyl phenyl carbamate) as Chiral Column

ID Paper: 3413

meets preliminary acceptance requirements set forth by our Scientific Committee to be presented as Oral Presentation at the conference. The conference will be held online on 8-9 November 2022 using the Zoom application.

The Oral/Poster Presentation Guidelines can be found at the following link:
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If you require any further information, please do not hesitate to contact us or visit our website.We look forward to seeing you at the conference.

Yours Sincerely,

Amin Fatoni, S.Si., M.Si., Ph.D.
Chairman of ICMA-SURE 2022

ICMA SURE 2022
INTERNATIONAL CONFERENCE ON MULTIDISCIPLINARY APPROACHES FOR SUSTAINABLE RURAL DEVELOPMENT

CERTIFICATE

THIS CERTIFICATE IS PRESENTED TO

Dadan Hermawan, Salsabil Rahmadina, Irmanto Irmanto, Mudasir Mudasir, Hassan Y Aboul-Enein

WITH THE TITLE

Chiral Separation of Hydroxychloroquine by High-Performance Liquid Chromatography Method using Amylose Tris (3,5-dimethyl phenyl carbamate) as Chiral Column

IN RECOGNITION OF THE OUTSTANDING CONTRIBUTION AS

Presenter(s)

ON INTERNATIONAL CONFERENCE

5 th International Conference on Multidisciplinary Approaches for Sustainable Rural Development 2022

" ICMA-SURE 2022 "

" The Advanced Strategies To The Development of Rural Resources For A Smart Society "

Purwokerto - Indonesia, November 8-9, 2022

Prof. Dr. Rifda Naufalin, S.P., M.Si.
Head of LPPM Jenderal Soedirman University

Chiral Separation of Hydroxychloroquine by High-Performance Liquid Chromatography and Method using Amylose Tris (3,5-dimethyl phenyl carbamate) as Chiral Column

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ABSTRACT. The chiral separation of hydroxychloroquine, an antimalarial drug with one chiral center, has been successfully carried out using the high-performance liquid chromatography (HPLC) method. Enantioresolution of hydroxychloroquine (Rs = 2.23) was accomplished using an amylose tris (3,5-dimethyl phenyl carbamate)-based chiral column (Lux® 5 Amylose-1, 250 4,6 mm), acetonitrile:aquabidest:dimethylamine (47:52:1, v/v) mobile phase, and 343 nm UV detection.The optimized HPLC method has been applied to quantitatively determine hydroxychloroquine in the pharmaceutical (liquid) sample with percentage recovery of 98.47%. The effect of several HPLC parameters on the chiral separation of hydroxychloroquine was also evaluated, and the method was successfully validated in terms of linearity, accuracy, precision, and selectivity. The HPLC method generated in this study was simple, had a short analysis time, and had a high resolution.

Keywords: Chiral separation; Hydroxychloroquine; High-performance liquid chromatography; Molecular docking

1. Introduction

The pharmaceutical industry produces various classes of drugs, one of which is the aminoquinoline class of drugs. One of the common aminoquinoline drugs is 4-aminoquinoline (Wang et al., 2020). Drugs of 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 (Shippey et al., 2018). Hydroxychloroquine has also been recommended by the Food and Drug Administration (FDA) for emergency treatment in hospitalized COVID-19 patients (Mahase, 2020).

Chiral compounds such as hydroxychloroquine are used as drugs. Chiral drugs are used as racemic mixtures in drug formulations. Only one of the enantiomers in the racemic can provide a pharmacological effect when consumed for treatment, while the other enantiomers have no effect or even have a toxic effect (Zhu et al., 2019). The hydroxychloroquine toxicity of the S and R isomers is unknown, but the S isomer outperforms the R isomer in the treatment of malaria (George and Mathew, 2021). This is evidenced by a study conducted by Ni et al. (2022) 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 is to determine the purity of the active substance and to determine the levels of chiral compounds in drugs (Yu and Quirino, 2019).

The need for a method capable of separating the S and R enantiomers is very important so that the two enantiomers can be separated for further analysis. The compound analysis methods

commonly used to determine the levels of active chiral compounds in column chromatography-based drugs include Liquid Chromatography (LC), Gas Chromatography (GC), and Capillary Electrophoresis (CE) (Yu & Quirino, 2019). Another method that can be used for the separation of S and R enantiomers is High-Performance Liquid Chromatography (HPLC) (Naghdi & Fakhari, 2018). The method used in this study is HPLC because it has several advantages, including good sensitivity, specificity, and automation; it has many detection techniques; many modes; and is available in various chiral columns (Chankvetadze, 2021).

The method of detecting hydroxychloroquine compounds using HPLC has been carried out by Harahap et al. (2021) using a C18 column with a mobile phase of 1% acetonitrile:diethylamine in water (65: 35, v/v) and the retention time is 5.29 minutes. However, the results of research by Harahap et al. (2021) are in racemic form, so the two enantiomers have not been separated. The separation of chiral compounds on hydroxychloroquine has been carried out by Xisheng Xiong et al. (2021). This study used HPLC with a Chiralpak AD-H column with n-hexane and isopropanol as mobile phases with the addition of 0.5% diethylamine in n-hexane. The results obtained in this study showed that the enantiomer of hydroxychloroquine was separated; namely, the R-hydroxychloroquine enantiomer was detected at 26 minutes and the S-hydroxychloroquine enantiomer was detected at 29 minutes with a resolution of 2.08. However, research conducted by Xisheng Xiong et al. still has a relatively long retention time, so method development is needed. The development of analytical methods for chiral drug separation is very important because it is used to control drug quality, pharmacological and pharmacokinetic studies, and the development of single enantiomer formulations (Zhu et al., 2019).

The development of the analytical method from the research of Xisheng Xiong et al. (2021) which will be carried out in this study is by modifying the column used in the form of Lux® 5 Amylose-1 (250 x 4.6 mm). The mobile phase modification in this study was acetonitrile:aquabidest:dimethylamine. This modification was carried out to obtain better separation results and shorter retention times. This modification of the method carried out needs to be validated to prove that the procedures used have met the requirements to be used. The validation of the development of chiral drug analysis methods has been based on the 1992 FDA statement regarding chiral drugs (Calcaterra and D'Acquarica, 2018). Validation needs to be done on racemic drug compounds to determine the S-isomer and R-isomer (Ragab and Eman, 2017). Validation of the analytical method uses several parameters, including linearity, the limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and selectivity (Rao, 2018).

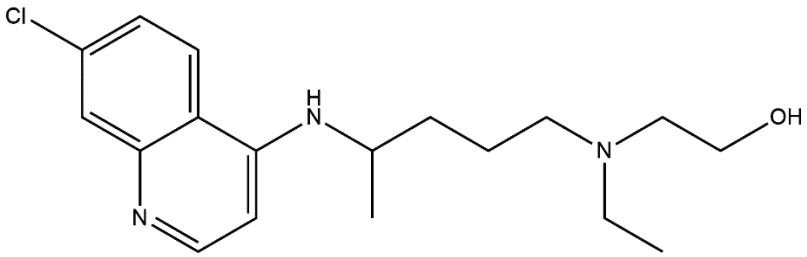
Identification of the types of R and S enantiomers that have different retention times can be done using molecular docking. The difference in retention time of the two enantiomers depends on their interaction with the chiral column. The differences in molecular interactions between the two enantiomers 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 (Chen et al, 2020). 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 the most stable bond is obtained (Tao et al, 2020). 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 (Chen et al, 2020).

2. Methodology

3.1. Materials and Instrumentation

Hydroxychloroquine standard (Sigma-Aldrich), acetonitrile (Merck), dimethylamine, water (twice distillation), pharmaceutical samples (PT. Tempo Scan Pacific Tbk., 200 mg HCQ). Experiments were carried out by using HPLC Hitachi L (UV-Vis detector L-2420, pump L-2130, D-2000 Elite software, L-2200 autosampler, and amylose tris (3,5-dimethyl phenyl carbamate)-based chiral column (Lux® 5 Amylose-1) of 250 4,6 mm. The analytes were operated at a wavelength of 343 nm. The instrument used for molecular docking is a personal computer with a

Windows 10 x 64 Operating System (OS) with an Intel® CoreTM i5-6500 CPU @ 3.20 GHz. The software used in this study are Gaussian 09W, Gaussian 6.0, Open Babel, AutoDock Tools, AutoDock Vina, and PyMOL.The structure used in this study as the receptor is tris amylose and its ligands in the form of R-hydroxychloroquine and S-hydroxychloroquine which can be downloaded on PubChem with PubChem CID: 178395 and 178396.



2-[4-[(7-chloroquinolin-4-yl)amino]pentyl-ethylamino]ethanol

Figure 1. Chemical structure of hydroxychloroquine

3.2. Development of Analytical Methods

2.2.1. Preparation of standards

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

2.2.2. 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.

$$\mathrm{Rs} = \frac{t_2 - t_1}{w_2 + w_1}$$

w1 and w2 are the peak widths at the baseline (Younes et al., 2018). Retention times are denoted by t1 and t2 (Patel et al., 2021).

2.2.3. Preparation of Hydroxychloroquine Standard Calibration Curve

Standard solutions of hydroxychloroquine 50, 100, 150, and 200 ppm were injected as much as 1 L using a syringe into the column and carried out two times in a row. The information is utilized to form the calibration curve for the hydroxychloroquine standard solution.

2.2.4. Preparation of pharmaceutical sample (hydroxychloroquine)

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

2.2.5. Determination of hydroxychloroquine levels in pharmaceutical (liquid) samples by HPLC

The HCQ 150 ppm sample solutions were analyzed using HPLC under optimum conditions. Analysis of hydroxychloroquine levels was carried out by three repetitions (Jire, J. and Doua, 2022).

2.3. HPLC method validation

The development of the obtained method needs to be validated to ensure that the method can be trusted to provide reliable results and is close enough to the true value (Raposo & Ibelli-Bianco, 2020). 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 (Rao, 2018). Standard solutions of hydroxychloroquine at a concentration of 50; 100; 150; and 200 ppm were analyzed under optimum conditions able to calculate LOD and LOQ, and the injection was performed 3 times to calculate linearity.

$$\mathrm{LOD} = \frac{3(\frac{sy}{x})}{b}$$

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

where sy/x = standard deviation of y to x; b = slope (Elkhazein et al., 2022).

Precision and accuracy are determined by injecting a 150 ppm hydroxychloroquine standard solution repeatedly in a short period for a precision of 6 repetitions and an accuracy of 3 repetitions. The result of precision can be shown in the value of standard deviation (SD), relative standard deviation (RSD), or the coefficient of variation (CV), and Horwith Ratio (HORRAT), while the accuracy results are shown in the percentage of the recovery. The selectivity was determined by mixing 0.5 mL of 150 ppm hydroxychloroquine standard solution with 0.5 mL of 150 ppm chloroquine standard, and then the retention time of the mixture was compared with the respective standard solutions.

$$K = \frac{t_R - t_0}{t_0}$$

$$\alpha = \frac{K_2}{K_1}$$

The t_R and t_0 are on the sake of the analyte and unretained solute (Liu et al., 2020). The k_1 and k_2 are the retention factors (Patel et al., 2021).

3. Results and Discussion

3.1. Optimization of HPLC

For optimization, parameters of mobile phase composition, flow rate, injection volume, and wavelength were carried out. In research conducted by Xisheng Xiong et al., a wavelength of 343 nm is the optimum condition to separate the enantiomer of hydroxychloroquine.

3.2.1. Effect of mobile phase composition

Based on the table above, the best variation of acetonitrile composition was obtained with a percentage mobile phase acetonitrile:aquabidest:dimethylamine of 47:52:1, which resulted in the best resolution (Rs=2.23) with a retention time of peak 1 at a minute to 14.77 and peak 2 at minute 16.3.

3.2.2. Effect of flow rate

The flow rate varies with the best results (Rs = 2.23) was 0.6 mL/minute, with a retention time of peak 1 at minute 14.77 and peak 2 at minute 16.25. Varying the flow rate at a flow rate of 0.8 mL/min obtained resolution at 1.05 with a retention time of peak 1 at minute 10.97 and peak 2 at minute 12.09. Meanwhile, at a flow rate of 1.0 mL/minute, the resolution obtained at 0.94.

3.2.3. Effect of injection volume

The injection volume variation with the best results (Rs = 2.23) was the injection volume of 1 µL with a retention time of peak 1 at a minute to 14.77 and peak 2 at minute 16.3. Variation of the flow rate by increasing it at a flow rate of 3 µL resulted in a decreased resolution (Rs = 0.23) and a longer retention time, namely peak 1 at 14.95 minutes and peak 2 at 16.38 minutes. If the injection volume was continuously increased at 5 µL, the resolution obtained was decreased (Rs = 0.19) and the retention time was at peak 1 at 14.81 minutes and peak 2 at 16.27 minutes.

3.2.4. Effect of wavelength

The optimization of the wavelength was carried out at 333, 343, and 353 nm. The best resolution (Rs = 2.23) was obtained at a wavelength of 343 with a retention time of peak 1 at minute 14.77 and peak 2 at minute 16.3. Wavelength was reduced to 333 then obtained 1.1 resolution. On the other hand, if the wavelength is increased to 353, the resolution obtained decreases (Rs = 0.65) and the retention time is longer at peak 1 at minute 21.17 and peak 2 at minute 22.64.

Table 1. Optimization of HPLC method; mobile phase, flow rate, injection volume, wavelength, and concentration

Optimation	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	16.3	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	16.3	2.23
wavelength			
333	15.65	16.76	1.1
343	14.77	16.3	2.23
353	21.17	22.64	0.65

There are several interactions in chiral recognition between the analyte and the stationary phase, including electrostatic forces, steric effects, hydrophobicity and hydrogen-bonding interaction (Wan Ibrahim et al, 2010). According to Matarashvili et al. (2017), 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, the hydrophobic interaction can increase. Increasing the content of acetonitrile in mobile phase, the hydrophobic interaction can decrease (Matarashvili et al, 2020). The hydrophobic interaction is based on the presence of hydrophobic groups on or near the surface of molecules that can interact with the hydrophobic column matrix (Reuhs, 2017).

The polar group contained in the hydroxychloroquine enantiomer more strongly interacts with the polar mobile phase, the more polar enantiomer will be eluted early from the column. The more or stronger the polarity of the group owned, the more or stronger the bonds formed so that the affinity between the polar groups of the compound and the mobile phase becomes greater. This causes enantiomers with more polar groups to have a shorter retention time because they elute faster than those with weaker polar groups.

3.2. Method Validation

3.2.1. Linearity

The peak of 1 has the regression equation $y = 0.0092x + 0.0733$ with a correlation coefficient (r) of 0.9999 and a coefficient of determination (r^2) of 0.9997. The regression equation for peak 2 is $y = 0.007x+0.0117$ and is obtained with a correlation coefficient (r) of 0.9993 and a coefficient of determination (r^2) of 0.9986. The two peaks produce the coefficient of determination and correlation coefficient that meet the requirements. Tiris (2022) states that the linearity requirements can be met with good accuracy if the value of the correlation coefficient (r) is more than 0.995 and the value of the coefficient of determination (r^2) > 0.997. Based on this reference, the value of the coefficient of determination and the correlation coefficient produced in this study have met the requirements, so this method of analysis of hydroxychloroquine chiral compounds has good accuracy and can be used for routine analysis.

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

The LOD value at peak 1 was 13.7 ppm and at peak 2 was 8.99 ppm. These two values indicate the lowest concentration of hydroxychloroquine standard solution that can be detected by HPLC. If the analysis is carried out with a concentration below this value, it cannot be detected by the tool. The LOQ value obtained at peak 1 was 45.67 ppm and at peak 2 was 29.98 ppm. These two values indicate the lowest concentration of hydroxychloroquine standard solution that can be used for precision and accuracy testing. The smaller the value of LOD and LOQ obtained, the better the method has the good ability (Miller and Miller, 1988). These two values indicate the lowest concentration of hydroxychloroquine standard solution that can be detected by HPLC and . If the analysis is carried out with a concentration below this value, it cannot be detected by the tool. The smaller the value of LOD and LOQ obtained, the better the method has the good ability (Miller and Miller, 1988).

3.2.3. Precision

The standard deviation obtained at peak 1 is 1.8 at peak 2 is 1.51. The value of the coefficient of variation produced at peak 1 is 1.43 and at peak 2 is 0.96. The value of relative standard deviation obtained in this study is less than 2, it indicates that the method meets the requirements of good precision so that it has high accuracy (Bose, 2014). 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 (Turner et al., 2020).

3.2.4. Accuracy

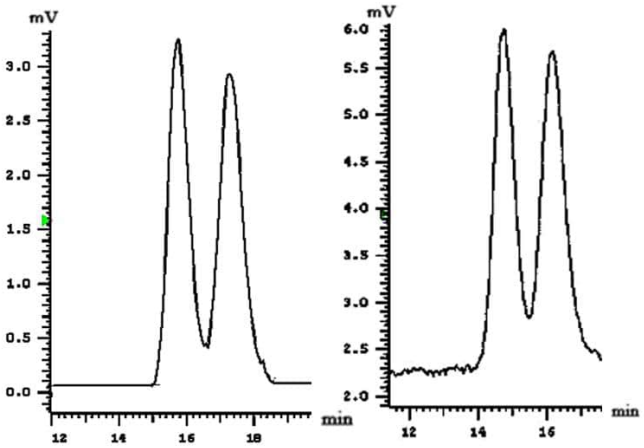
The average percentage recovery at both peaks is 92.43%. These results indicate that the analytical method used has met the acceptability requirements at an analyte concentration of > 100 mg/L, which is in the range of 90–107% (Jesenkovi-Habul, 2019).

3.2.5. Selectivity

The selectivity 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 a mixed solution of hydroxychloroquine and chloroquine, the retention time of the standard hydroxychloroquine solution was 15.52 minutes, while the standard solution of chloroquine was 34.53 minutes. The results obtained were then compared with the two standard solutions separately. The results of this analysis obtained a selectivity value of 2.68. The selectivity test is said to be good if the value obtained is greater than 1, so in this study, the separation of chiral hydroxychloroquine compounds with chloroquine was selective (Ali et al, 2009).

3.3. Determination of hydroxychloroquine in the pharmaceutical (tablet) sample

The sample was used in the form of hydroxychloroquine drug preparation produced by PT Tempo Scan Pacific. The analysis was carried out by injecting 150 ppm hydroxychloroquine sample solution using HPLC for 3 repetitions at optimum conditions. The concentration of hydroxychloroquine in the drug preparation at peak 1 was 147.91 ppm and at peak 2 was 147.49 ppm. According to the Indonesian Pharmacopoeia VI edition of 2022, it is stated that the level of hydroxychloroquine is not less than 93.0% and not more than 107.0% (Directorate General of POM, 2022). The percentage recovery of hydroxychloroquine in the pharmaceutical sample with a concentration of 150 ppm is 98.47%. The following is an image of a hydroxychloroquine chromatogram on a standard and a drug sample.



(a) (b)

Figure 2. Chromatogram of hydroxychloroquine enantiomers in the (a) standard at 150 ppm and (b) pharmaceutical (tablet) sample at 150 ppm. Acetonitrile:aquabidest:dimethylamine (47:52:1, v/v), amylose tris (3,5-dimethyl phenyl carbamate)-based chiral column (Lux® 5 Amylose-1, 250 4,6 mm) as chiral stationary phase, 343 nm UV detection, 0.6 mL/min flow rate, and injection sample volume of 1 μL.

3.4. Molecular Docking

Identification of enantiomers with different retention times has been carried out using molecular docking. Preparation was carried out on receptors and ligands. The receptor, namely tris amylose, 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 so that the R-hydroxychloroquine and S-hydroxychloroquine structures are stabilized at lowest energy.

Molecular docking in this study was carried out using the Command Prompt (CMD) to carry out the docking process. The docking process is carried out by removing the H2O molecule at the receptor because it will inhibit the docking process. After that, it is continued by adding polar hydrogen atoms to the receptor for further docking. The grid boxes used in this docking are grid centers in the form of X = 19,977, Y = 20,069, and Z = 25,901; with a grid size 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. In another inclusion complex, namely tris amylose/S-hydroxychloroquine, the ΔG value was obtained in the range -5.5 to -5.1. This ΔG value indicates the conformational stability of the inclusion complex formed. A small ΔG value indicates that the complex conformation is stable, while a large ΔG value indicates that the complex conformation is less stable (Jiang et al, 2018). In this case, that S-hydroxychloroquine has a greater ΔG value compared to R-hydroxychloroquine, this means that at chiral receptors, R-hydroxychloroquine has a more stable conformation while S-hydroxychloroquine has a less stable conformation. In HPLC, this causes S-hydroxychloroquine to elute first from its chiral column, then followed by R-hydroxychloroquine.

4. Conclusion

The development of the chiral hydroxychloroquine analysis method using this method obtained a value of Rs = 2.23 with a retention time of 14.77 minutes for S-

hydroxychloroquine and 16.3 minutes for R-hydroxychloroquine. This method is validated and has obtained r^2 values of 0.9997 and 0.9986; LOD of 3.82 and 12.72; LOQ of 8.99 and 29.98; the standard deviation of 1.8 and 1.51; coefficient of variation of 1.43% and 0.960%; HORRAT of 0.13 and 0.09; % recovery of 92.43%; the method ranges at 12.72-200.73 ppm and 29.98-197.68 ppm; and selectivity of 2.65. The results obtained were used to determine the levels of hydroxychloroquine in the hydroxychloroquine drug sample 150 ppm resulting % recovery of 98.47%. The chiral hydroxychloroquine analysis method used in this study resulted in higher resolution and shorter analysis time.

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