3. Analytical Method Development

3.1 Introduction

Selecting or developing an appropriate analytical method is crucial for accurate, precise, and convenient drug analysis. For Luliconazole UV-Vis spectrophotometric and HPLC methods were developed. UV method of Luliconazole was used for drug estimation during solubility studies and entrapment efficiency. HPLC method of Luliconazole was used for assessing in-vitro drug release, conducting in vivo studies, evaluating ex vivo skin deposition/permeation. For Tavaborole only HPLC method was developed which was used for evaluation of solubility studies, entrapment efficiency, in vitro drug release, ex vivo drug release and pharmacokinetic study.

3.2 Analytical Method Validation:

Linearity: Linearity refers to an analytical method's ability to produce results that are directly proportional to the analyte concentration within a specified range. This concept aligns with Lambert Beer's law, where the concentration of the analyte affects its absorbance.

Accuracy: Accuracy represents the closeness between the measured value and the actual amount of the substance present in the sample. To assess accuracy, one can spike the sample with a known concentration of an analyte standard and analyze it using the validated method.

Precision: Precision quantifies the agreement (or scatter) among measurements obtained by repeatedly sampling the same homogeneous sample under specific conditions. Three levels of precision exist: reproducibility, moderate precision, and repeatability. Variance, standard deviation, or coefficient of variation is commonly used to express an analytical method's precision.

The Limit of Detection (LOD) refers to the smallest amount of a substance that can be identified with precision and reliability using a specific testing method. Meanwhile, the Limit of Quantification (LOQ) represents the minimum concentration of a substance that can be measured with acceptable accuracy and precision. These limits are typically expressed in measurements such as micrograms per milliliter (μg/ml), nanograms per milliliter (ng/ml), or picograms per milliliter (pg/ml). It's important to note that LOD values are dependent on the particular conditions under which the test is conducted.

Table 3.1: Analytical techniques reported for Tavaborole and Luliconazole

API	Analytical method	References
Luliconazole	UV Spectroscopy	4,5
	HPLC	6,7
Tavaborole	HPLC	8,9

In this study, analytical methodologies are employed to measure key parameters of the formulation, including the rate of drug release, bioavailability in vivo, percentage of entrapment efficiency, skin deposition/permeation ex vivo, and the drug's concentration during stability assessments. The analytical methods applied for the quantitative evaluation of Luliconazole and Tavaborole within the formulation (as mentioned in table 3.1) are outlined subsequently.

3.3 List of Material and Instruments

Table 3.2 List of Materials

Materials	Manufacturer
Luliconazole	Sun Pharmaceutical Industries Ltd., Vadodara
Tavaborole	Symed labs limited, Hyderabad
Methanol (HPLC grade)	Spectrochem Pvt. Ltd., Mumbai
Acetonitrile (HPLC grade)	Spectrochem Pvt. Ltd., Mumbai
Ortho-Phosphoric Acid	Spectrochem Labs Ltd
Double distilled filtered water	Prepared in-house

Table 3.3 List of Instruments

Equipment/Instrument	Manufacturer
pH meter	Lab India Pvt. Ltd., Mumbai
Digital Analytical Balance	Shimadzu, Japan
RP-HPLC with UV detector (gradient)	Agilent, Germany
UV visible spectrophotometer (UV-1800)	Shimadzu, Japan
Bath Sonicator	Sartorious, Mumbai

Materials and instruments used in analytical method development are mentioned in table 3.2 and 3.3 respectively.

3.4 Development of Analytical Methods for Luliconazole

3.4.1 Luliconazole Estimation Using UV Spectrophotometry

3.4.1.1 Preparation of Luliconazole drug stock solution

In a 10 ml volumetric flask, precisely 10 mg of Luliconazole was weighed and dissolved by adding a small amount of methanol. The volume was then adjusted to the mark with methanol, resulting in a stock solution with a concentration of 1000 μg/ml. Subsequently, 1 ml of this stock solution was transferred to another 10 ml volumetric flask, yielding a solution with a concentration of 100 μg/ml. Finally, 1 ml of the stock solution was again transferred to a 10 ml volumetric flask and volume made up with methanol, resulting in a solution with a concentration of 10 μg/ml.

3.4.1.2 Detection of λmax:

A UV-Visible spectrophotometer, with methanol serving as the reference, was utilized to scan a prepared solution of Luliconazole at a concentration of 10 μg/ml across a wavelength range of 200 to 400 nm. The wavelength corresponding to the peak absorbance was determined and selected as the analytical wavelength.

3.4.1.3 Luliconazole Calibration plot in Methanol:

Volumes of 1, 1.5, 2, 2.5, and 3 ml from a Luliconazole stock solution (100 μg/ml in methanol) were carefully pipetted out using a pipette. Each aliquot was transferred into separate 10 ml volumetric flasks and diluted with methanol to achieve concentrations of 10, 15, 20, 25, and 30 μg/ml respectively. The absorbance for each resulting solution was recorded at the λ_{max} of 295 nm against methanol as blank.

3.4.1.4 Validation method for Luliconazole by UV Spectrophotometry

3.4.1.4.1 Accuracy: The method's accuracy was evaluated using the standard addition method to test recovery at three different Luliconazole concentration levels: 80%, 100% (20 μg/ml) and 120%. Accuracy was quantified by calculating the average percentage of recovery.

3.4.1.4.2 Precision: The precision of the method was determined by analyzing the consistency of responses within the same day (intraday) and across consecutive days (interday). Luliconazole concentrations of 10, 20, and 30μg/ml were examined in triplicate for this precision study. The method's precision was assessed by computing the average and percentage relative standard deviation (%RSD).

3.4.1.4.3 Determination of LOD and LOQ:

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for Luliconazole were calculated using the calibration plot and following formula,

$$LOD = 3.3\ x\ \sigma/S$$

$$LOQ = 10\ x\ \sigma/S$$

Where, σ = Standard deviation of the y-intercept

S = Slope

3.4.1.5 Analytical Interference study:

Interference studies were conducted to ascertain whether excipients affect drug detection accuracy. For this, a solution of Luliconazole was prepared in methanol at a concentration of 100 µg/ml and further diluted to 10 µg/ml. Following solutions were prepared using the drug solution of 10 µg/ml i) 1 ml drug solution and 1 ml Capmul MCM C8 ii) 1 ml drug solution and 1 ml Cremophore EL iii) 1 ml drug solution and 1 ml solution of Pluronic F 127 (10 µg/ml) iv) 1 ml drug solution, 1 ml Capmul MCM C8, 1 ml Cremophore EL and 1 ml solution of Pluronic F 127 (10 µg/ml). The absorbance was measured at 295 nm using methanol as a blank to evaluate any interference from these substances.

3.4.2. Analytical method development of Luliconazole on HPLC

Table 3.4 HPLC process parameters (6,7)

Parameter	Value
Instrument	Agilent Technologies 1220 Infinity II
Column	Welchrom®, C18, 5 µm, 4.6x250 mm
Mobile phase	Methanol: Water: ACN (75:25:10)
Run time	10 min
Injection volume	20 µl
Flow rate	1 ml/min
λ_{max}	295 nm
Retention time	7.1 min

3.4.2.1 Mobile Phase Composition:

As mentioned in table 3.4, the mobile phase was composed by mixing Methanol (HPLC grade), double-distilled water (filtered through a 0.45 µm vacuum filter), and Acetonitrile (ACN) in a 75:25:10 ratio. This mixture was then sonicated for three cycles of 5 minutes to ensure thorough degassing and proper mixing of solvents.

3.4.2.2 Stock Solution Preparation:

Luliconazole, weighing 10 mg, was placed into a 10 ml volumetric flask, to which 3 ml of methanol was added. The flask was shaken vigorously to ensure complete dissolution of Luliconazole, and then the volume was made up to 10 ml with methanol. From this stock solution, 1 ml was transferred into a 10 ml volumetric flask to prepare a 100 µg/ml solution. 1 ml was transferred from 100 µg/ml solution into a 10 ml volumetric flask and volume was made upto 10 ml with methanol to prepare a 10 µg/ml solution.

3.4.2.3 Preparation of calibration Plot:

Subsequent aliquots of 0.2 ml to 1.2 ml were taken from 10 µg/ml solution and added to different 10 ml volumetric flasks, which were then filled up with the mobile phase to achieve dilutions ranging from 200 ng/ml to 1200 ng/ml. These samples were then introduced into the HPLC system using a syringe of 20 µl to prepare a calibration plot.

3.4.2.4 Sample Processing in Skin Homogenate:

Rat skin was procured following approval (Protocol No.- MSU/IAEC/2021-22/2108) from the institutional animal ethics committee at Maharaja Sayajirao University of Baroda, Gujarat, India. The skin was cleaned with PBS pH 7.4 and carefully dissected to separate the full thickness using forceps and a scalpel, ensuring all fat was removed. The skin was then minced and homogenized. To each of six 10 ml volumetric flasks, 2 ml of skin homogenate was added, followed by aliquots ranging from 0.2 ml to 1.2 ml from the Luliconazole stock solution (10 µg/ml). After adding 100 µl of methanol to each flask, the mixture was vortexed for two minutes before being filled up with methanol to reach volumes of 10 ml for dilutions from 200 ng/ml to 1200 ng/ml. Each sample underwent centrifugation at 10°C and 3500 rpm for ten minutes, after which the supernatant was collected for analysis.

3.4.2.5 Plasma Sample Preparation:

Aliquots from the Luliconazole standard stock solution (10 µg/ml) were placed into separate centrifuge tubes in volumes ranging from 0.2 ml to 1.2 ml, followed by the addition of 4 ml of

rat plasma. After adding 100 µl of methanol to each flask, the mixture was vortexed for two minutes for protein precipitation purposes. The volume was brought up to 10 ml with methanol, resulting in final concentration ranges between 200–1200 ng/ml after vortexing for five minutes and centrifuging at 25 °C at 5000 rpm for fifteen minutes to separate the precipitated proteins. The supernatant was then filtered through a 0.22 µm PVDF syringe filter and analyzed via HPLC at a wavelength of 295 nm under identical chromatographic conditions as specified in Table 3.4, with each experiment conducted in triplicate for constructing a calibration curve for Luliconazole in ex-vivo release studies.

3.4.2.6 Validation method for Luliconazole by HPLC

3.4.2.6.1 Accuracy: The method's accuracy was evaluated using the standard addition method to test recovery at three different Luliconazole concentration levels: 80%, 100% (800 ng/ml) and 120%. Accuracy was quantified by calculating the average percentage of recovery.

3.4.2.6.2 Precision: The precision of the method was determined by analyzing the consistency of responses within the same day (intraday) and across consecutive days (interday). Luliconazole concentrations of 200, 800, and 1200 ng/ml were examined in triplicate for this precision study. The method's precision was assessed by computing the average and percentage relative standard deviation (%RSD).

3.4.2.6.3 Determination of LOD and LOQ:

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for Luliconazole were calculated using the calibration plot and following formula,

$$LOD = 3.3\ x\ \sigma/S$$

$$LOQ = 10\ x\ \sigma/S$$

Where, σ = Standard deviation of the y-intercept

S = Slope

3.5 Analytical method development of Tavaborole on HPLC (8,9)

Table 3.5 HPLC process parameters

Parameter	Value
Instrument	Agilent Technologies 1220 Infinity II
Column	Welchrom®, C18, 5 µm, 4.6x250 mm
Mobile phase	Phosphoric acid solution (10 mM, pH 2.0): ACN(60:40)
Run time	10 min

Injection volume	20 µl
Flow rate	1 ml/min
λ_{max}	214 nm
Retention time	7.18 min

3.5.1 Mobile Phase Composition:

As mentioned in table 3.5, a mixture of ACN and 10 mM phosphoric acid in a 60:40 ratio was combined with double distilled water, adjusted to pH 2.0, to formulate the mobile phase. The solution underwent sonication for degassing and was then filtered using a 0.45 µ vacuum filter for complete homogenization.

3.5.2 Stock Solution preparation:

10 mg tavaborole was dissolved in a 10 ml volumetric flask with an initial addition of 5 ml ACN. After complete solubilization of tavaborole, volume was made up to 10 ml with ACN, achieving a concentration of 1000 µg/ml. From this stock solution, 1 ml was transferred into a 10 ml volumetric flask and made up the volume upto 10 ml with the mobile phase to obtain concentrations of 100 µg/ml. From the 100 µg/ml solution, 1 ml was transferred into a 10 ml volumetric flask and made up the volume upto 10 ml with the mobile phase to obtain concentrations of 10 µg/ml.

3.5.3 Preparation of calibration Plot:

Further dilutions were prepared by taking aliquots of 0.5 ml, 1 ml, 1.5 ml, 2 ml, and 2.5 ml from the 10 µg/ml solution into separate 10 ml volumetric flasks and diluting with the mobile phase to achieve final concentrations of 500 ng/ml, 1000 ng/ml, 1500 ng/ml, 2000 ng/ml, and 2500 ng/ml respectively. For HPLC analysis, 20 µl of each concentration was injected using an HPLC syringe.

3.5.4 Sample Preparation in Skin Homogenate:

Rat skin samples were procured following approval (Protocol No.- MSU/IAEC/2021-22/2108) from the IAEC at Maharaja Sayajirao University's Faculty of Pharmacy in Gujarat, India. The skins were meticulously cleaned with PBS at pH 7.4 and then dissected to remove the full thickness using surgical tools while ensuring no fat remained. The skin pieces were then homogenized into fine particles using a homogenizer. To each of six volumetric flasks (10 ml), 2 ml of skin homogenate was added followed by aliquots from the Tavaborole stock solution (10

μg/ml) in volumes corresponding to desired final concentrations after methanol addition and vortexing for uniform mixing for two minutes. Post-centrifugation at 3500 RPM and 10°C for ten minutes, the supernatant was collected for analysis and further dilution if necessary.

3.5.5 Plasma Sample Preparation:

Aliquots from the Tavaborole standard stock solution (10 μg/ml) were used to prepare plasma samples with concentrations ranging from 0.5μg/ml to 2.5μg/ml by transferring into separate volumetric flasks and diluting with mobile phase accordingly. Volumes ranging from 0.2 ml to 1.2 ml of these solutions were then combined with rat plasma (4 ml) in centrifuge tubes followed by ACN addition up to a total volume of 10 ml to precipitate plasma proteins. After vortexing and centrifugation at 5000 rpm for fifteen minutes at a temperature of 25 °C, the supernatant containing Tavaborole was filtered through a PVDF syringe filter (0.22 μ) for HPLC analysis at an absorbance of 214 nm using the previously mentioned mobile phase composition.

3.5.6 Validation method for Tavaborole by HPLC

3.5.6.1 Accuracy: The method's accuracy was evaluated using the standard addition method to test recovery at three different Tavaborole concentration levels: 80%, 100% (1500 ng/ml) and 120%. Accuracy was quantified by calculating the average percentage of recovery.

3.5.6.2 Precision: The precision of the method was determined by analyzing the consistency of responses within the same day (intraday) and across consecutive days (interday). Tavaborole concentrations of 500, 1500, and 2500 ng/ml were examined in triplicate for this precision study. The method's precision was assessed by computing the average and percentage relative standard deviation (%RSD).

3.5.6.3 Determination of LOD and LOQ:

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for Tavaborole were calculated using the calibration plot and following formula,

$$LOD = 3.3\ x\ \sigma/S$$

$$LOQ = 10\ x\ \sigma/S$$

Where, σ = Standard deviation of the y-intercept

S = Slope

3.6 Result and Conclusion:

3.6.1 Determination of Luliconazole via UV Spectrophotometry:

The UV absorption profile of Luliconazole at a standard concentration of 10µg/ml is depicted in Figure 3.1. The peak absorbance for Luliconazole was observed at 295 nm, which was selected as the optimal wavelength for quantitative analysis due to its maximum absorbance.

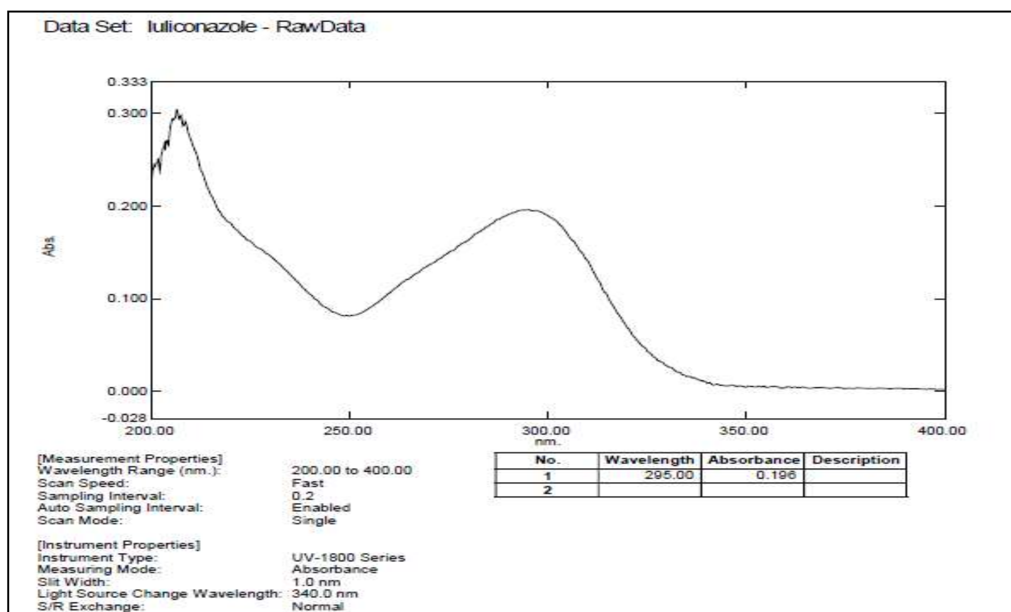

Figure 3.1: UV Spectrum of Luliconazole in Methanol

Table 3.6: Calibration data for estimation of Luliconazole in Methanol:

Sr. No.	Conc. (µg/ml)	Absorbance ± SD (n=3)
1	0	0
2	10	0.201±0.001
3	15	0.304±0.001
4	20	0.408±0.001
5	25	0.532±0.007
6	30	0.664±0.025

*Every experiment was carried out three times.

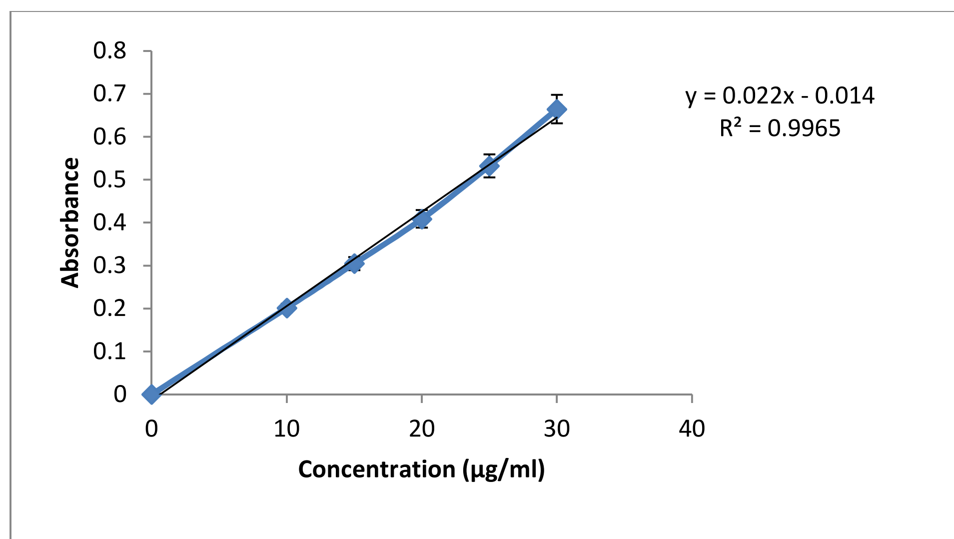

Figure 3.2: Calibration plot of Luliconazole in Methanol

As per table 3.6 and the calibration plot for Luliconazole in methanol demonstrated linearity within the 10–30 µg/ml concentration span, evidenced by a robust correlation coefficient (R^2 = 0.996). This suggests adherence to Beer's law for Luliconazole in the specified concentration window.

Table 3.7 Parameters from calibration plot of Luliconazole in Methanol

λ_{max}	**Solvent**	**Concentration Range**	**Regression Equation**	**Correlation Coefficient**	**LOD** (µg/ml)	**LOQ** (µg/ml)
295 nm	Methanol	10-30 µg/ml	0.023x-0.039	0.996	0.15	0.45

The low values of LOD and LOQ indicate the sensitivity of the method which is shown in table 3.7.

3.6.2 Validation of UV spectrophotometric methods for Luliconazole

3.6.2.1 Accuracy

Table 3.8 Accuracy in methanol

Drug Spiked (%)	Expected concentration (µg/ml)	Observed Concentration (µg/ml) ±SD (n=3)	% Drug recovered
80%	16	16.04±0.35	100.25
100%	20	19.90±0.18	99.50
120%	24	24.13±0.07	100.54

Table 3.8 shows the percentage recoveries at lower, intermediate, and higher concentrations. Their findings demonstrated that the suggested analytical method could accurately determine and quantify even a slight variation in the drug concentration in the solution.

3.6.2.2 Precision:

Table 3.9: Intraday and interday precision analysis of Luliconazole

Concentration (µg/ml)	Observed Concentration (µg/ml) ±SD (n=3)		%Recovery		%RSD	
	Intraday	Interday	Intraday	Interday	Intraday	Interday
10	9.97±0.24	10.03±0.17	99.7	100.3	0.30	1.72
20	19.90±0.18	20.05±0.06	99.5	100.25	0.88	0.30
30	30.06±0.12	29.96±0.05	100.2	99.87	0.40	0.16

Uniform testing conditions were applied to assess both intraday and interday precision. The precision and consistency of these methods are reflected in the %RSD values obtained, which are below 2.0%, indicating high reproducibility. As demonstrated in table 3.9, the method yields consistent and accurate outcomes.

3.6.3 Analytical interference Study

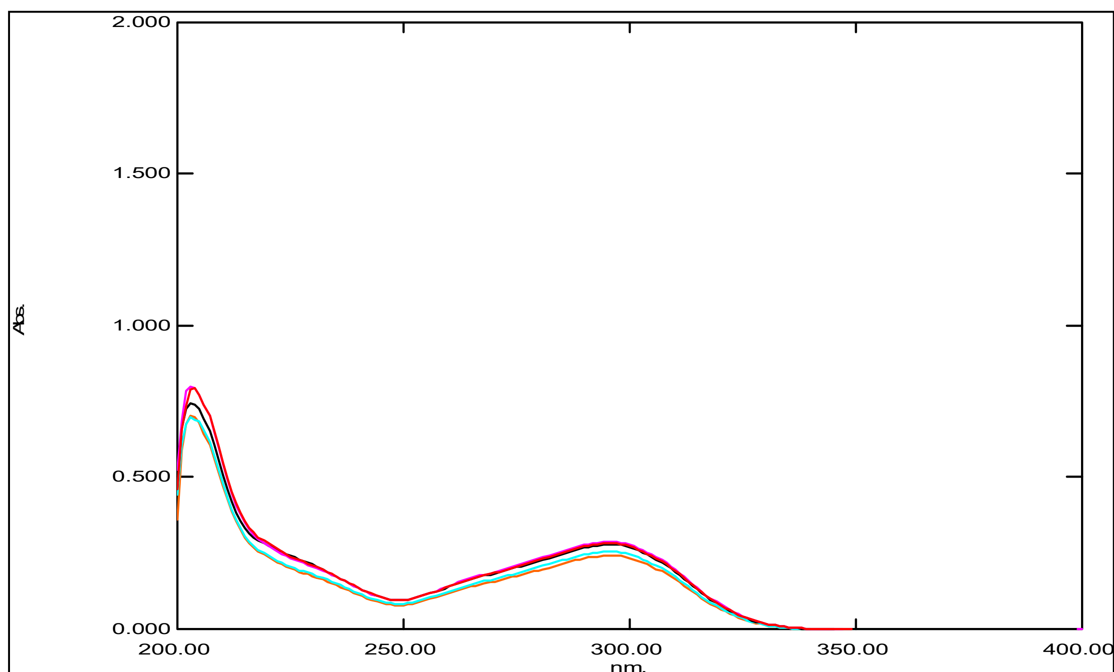

Figure 3.3: Interference study of drug and excipients

Table 3.10: Drug Excipients interference study

Sr. No.	Name of Ingredients	Absorbance
1	Luliconazole	0.279
2	Luliconazole + Capmul MCM C8(1:1)	0.283
3	Luliconazole + Cremophore EL(1:1)	0.287
4	Luliconazole + Pluronic F 127(1:1)	0.242
5	Luliconazole + Mixture	0.255

As shown in table 3.10, there is no significant difference in absorbance of drug and mixture. Therefore, it can be concluded that there is no analytical interference of excipients in the estimation of drug.

3.6.4 Estimation of Luliconazole using HPLC

Representative chromatograms from the RP-HPLC method using a C18 column are presented in Figure 3.3. The analysis resulted in well-defined, symmetrical peaks with an average retention time of 7.1 minutes, utilizing a detection wavelength of 295 nm and a flow rate set at 1.0 ml/min.

Figure 3.4: Overlay Chromatogram of Luliconazole by HPLC

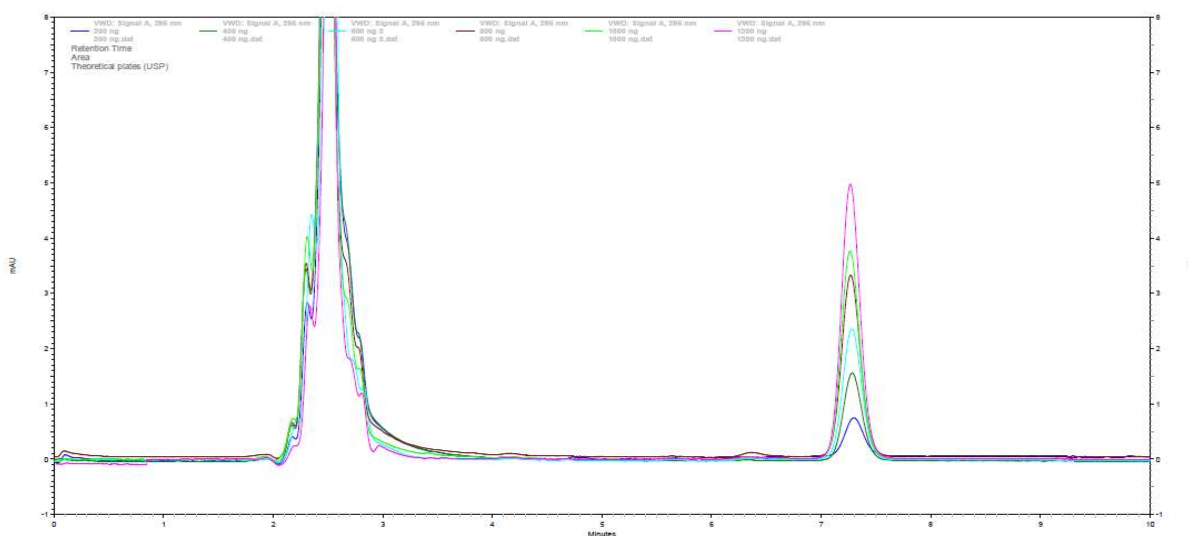

Figure 3.5: Overlay Chromatogram of Luliconazole by HPLC in skin homogenate

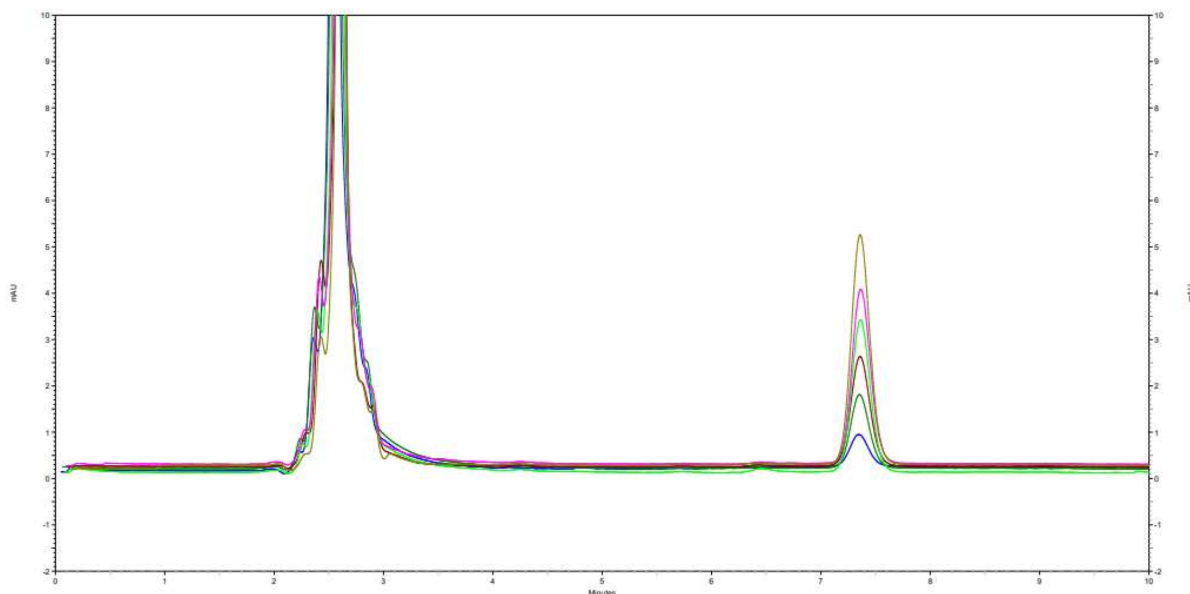

Figure 3.6: Overlay Chromatogram of Luliconazole by HPLC in rat plasma

Table 3.11: Data of Calibration plot in Methanol:Water:ACN (75:25:10)

Sr. No.	Concentration of sample (ng/ml)	Peak Area±S.D (n=3)		
		Analytical method	Bioanalytical method	
			In skin homogenate	In rat plasma
1	0	0	0	0
2	200	1,22,049±135.05	1,42,496±118.05	1,38,159±96.21
3	400	2,55,080±83.44	2,95,080±88.48	3,06,229±103.56
4	600	3,95,765±105.63	4,77,613±102.26	4,84,210±100.32
5	800	5,67,735±95.18	6,67,028±86.28	6,67,378±90.26
6	1000	6,74,499±125.77	7,98,818±105.87	8,50,159±101.64
7	1200	8,48,506±100.06	10,07,422±95.06	10,52,932±88.31

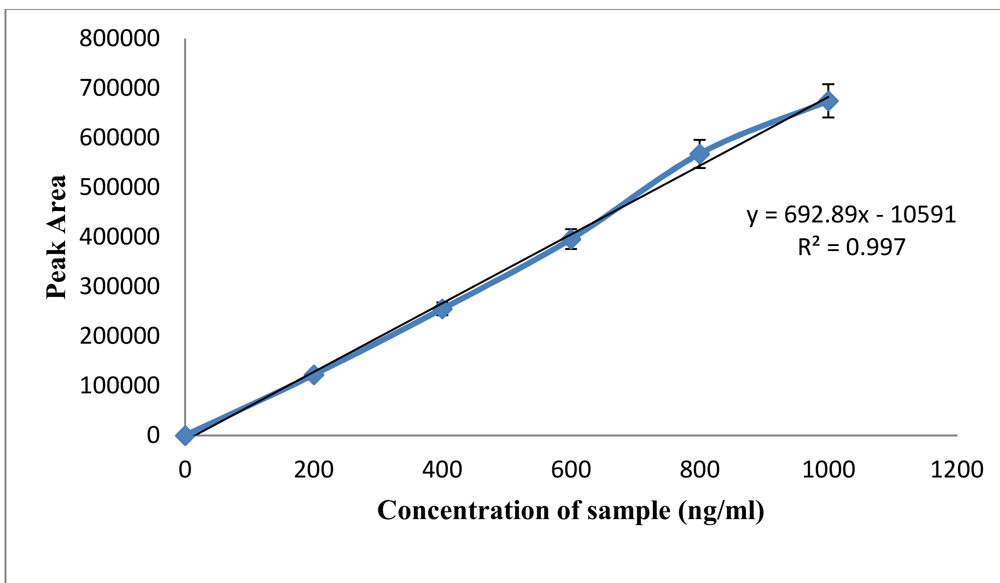

Figure 3.7: Calibration plot of Luliconazole by HPLC

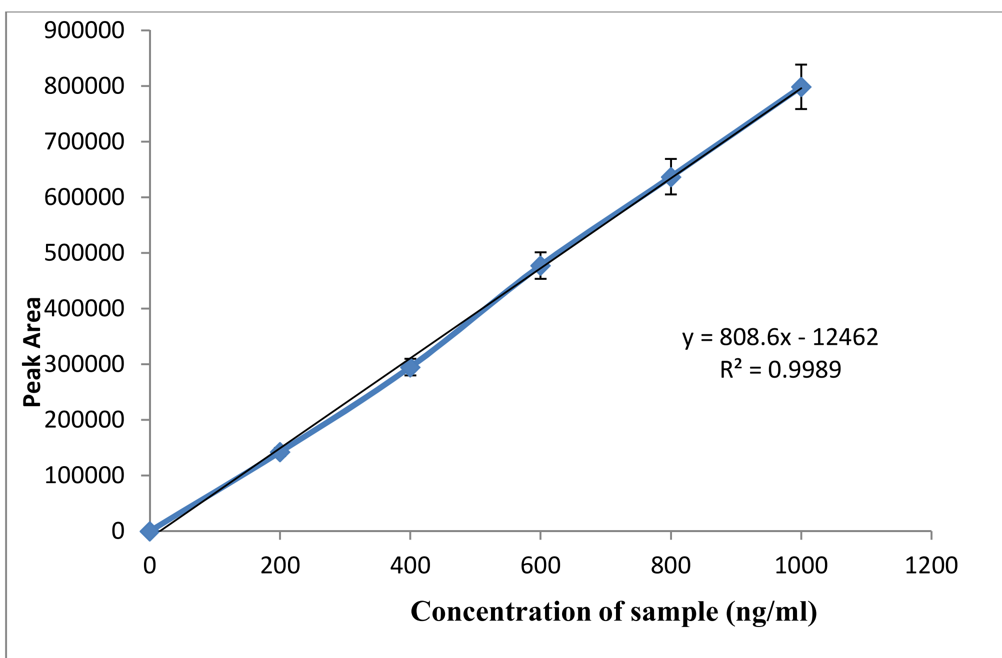

Figure 3.8: Calibration plot of Luliconazole by HPLC (Skin Homogenate)

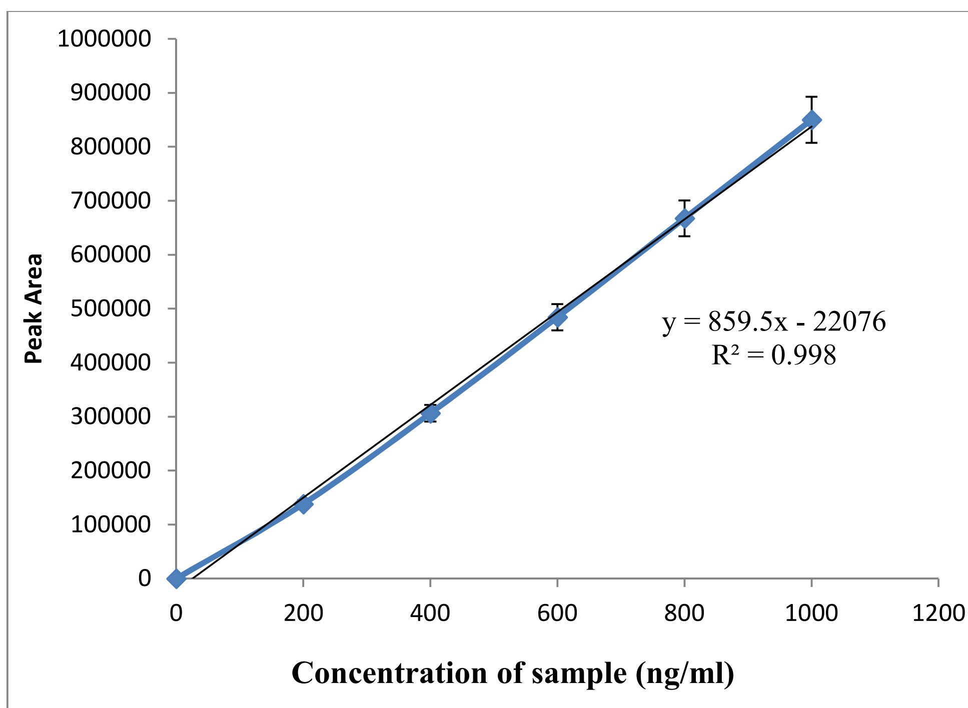

Figure 3.9: Calibration plot of Luliconazole by HPLC (Rat Plasma)

As per table 3.11 and the calibration plot for Luliconazole in mobile phase, skin homogenate and rat plasma demonstrated linearity within the 200-1200 ng/ml concentration span. This suggests adherence to Beer's law for Luliconazole in the specified concentration window.

Table 3.12: Parameters from calibration plots

Methods	λ_{max}	Mobile Phase	Linearity Range	Regression Equation	Correlation Coefficient	LOD (ng/ml)	LOQ (ng/ml)
Analytical method	295 nm	Methanol: Water: ACN	200-1200 ng/ml	y = 707.6x - 15524	0.997	1.61	4.88
Bioanalytical method in skin homogenate				y = 838.7x - 20598	0.997	0.74	2.23
Bioanalytical method in rat plasma				y = 859.5x - 22076	0.998	0.14	0.42

The low values of LOD and LOQ indicate the sensitivity of the methods which is shown in table 3.12.

Table 3.13 Accuracy of the method

Drug Spiked (%)	Expected concentration (ng/ml)	Observed Concentration (ng/ml) ±SD (n=3)	% Drug recovered
Analytical Method			
80%	640	640.25±0.07	100.04
100%	800	800.06±0.22	100.01
120%	960	959.84±0.42	99.98
For skin homogenate			
80%	640	639.94±0.19	99.99
100%	800	799.80±0.16	99.98
120%	960	960.17±0.23	100.02
For Bioanalytical method			
80%	640	639.68±0.61	99.95
100%	800	799.66±1.35	99.96
120%	960	960.48±0.36	100.05

Table 3.13 shows the percentage recoveries at lower, intermediate, and higher concentrations. Their findings demonstrated that the suggested analytical method could accurately determine and quantify even a slight variation in the drug concentration in the solution.

Table 3.14: Intraday and interday precision analysis of Luliconazole

Concentration (ng/ml)	Observed Concentration (ng/ml) ±SD (n=3)		%Recovery		%RSD	
	Intraday	Interday	Intraday	Interday	Intraday	Interday
Analytical method						
200	199.83±0.32	200.05±0.10	99.92	100.03	0.16	0.05
800	800.06±0.22	799.79±0.95	100.01	99.97	0.03	0.12
1200	1199.44±1.23	1199.87±0.40	99.95	99.99	0.10	0.03
Bioanalytical method in skin homogenate						

200	200.22±0.23	199.96±0.06	100.11	99.98	0.11	0.03
800	799.80±0.16	799.82±0.72	99.98	99.98	0.03	0.09
1200	1199.74±0.87	1200.19±0.14	99.98	100.02	0.07	0.02
Bioanalytical method in rat plasma						
200	199.98±0.44	199.97±0.11	99.92	99.98	0.22	0.06
800	799.66±1.35	799.60±0.63	99.96	99.95	0.17	0.08
1200	1199.98±0.87	1199.73±0.36	99.99	99.98	0.07	0.03

Uniform testing conditions were applied to assess both intraday and interday precision. The precision and consistency of these methods are reflected in the %RSD values obtained, which are below 2.0%, indicating high reproducibility. As demonstrated in Table 3.14, the method yields consistent and accurate outcomes.

3.6.5 Estimation of Tavaborole by HPLC method

Table 3.15: Data of Calibration plot in OPA:ACN (60:40)

Sr. No.	Concentration (ng/ml)	Peak Area ± SD (n=3)		
		Analytical method	Bioanalytical method	
			In skin homogenate	In rat plasma
1	0	0	0	0
2	500	406760±80.83	431770±78.23	426782±98.21
3	1000	724286±90.91	882793±89.27	863218±65.32
4	1500	1150094±112.18	1382153±91.18	1298106±87.17
5	2000	1499271±85.91	1737159±80.31	1797203±79.08
6	2500	2034394±98.04	2284726±88.17	2201325±102.87

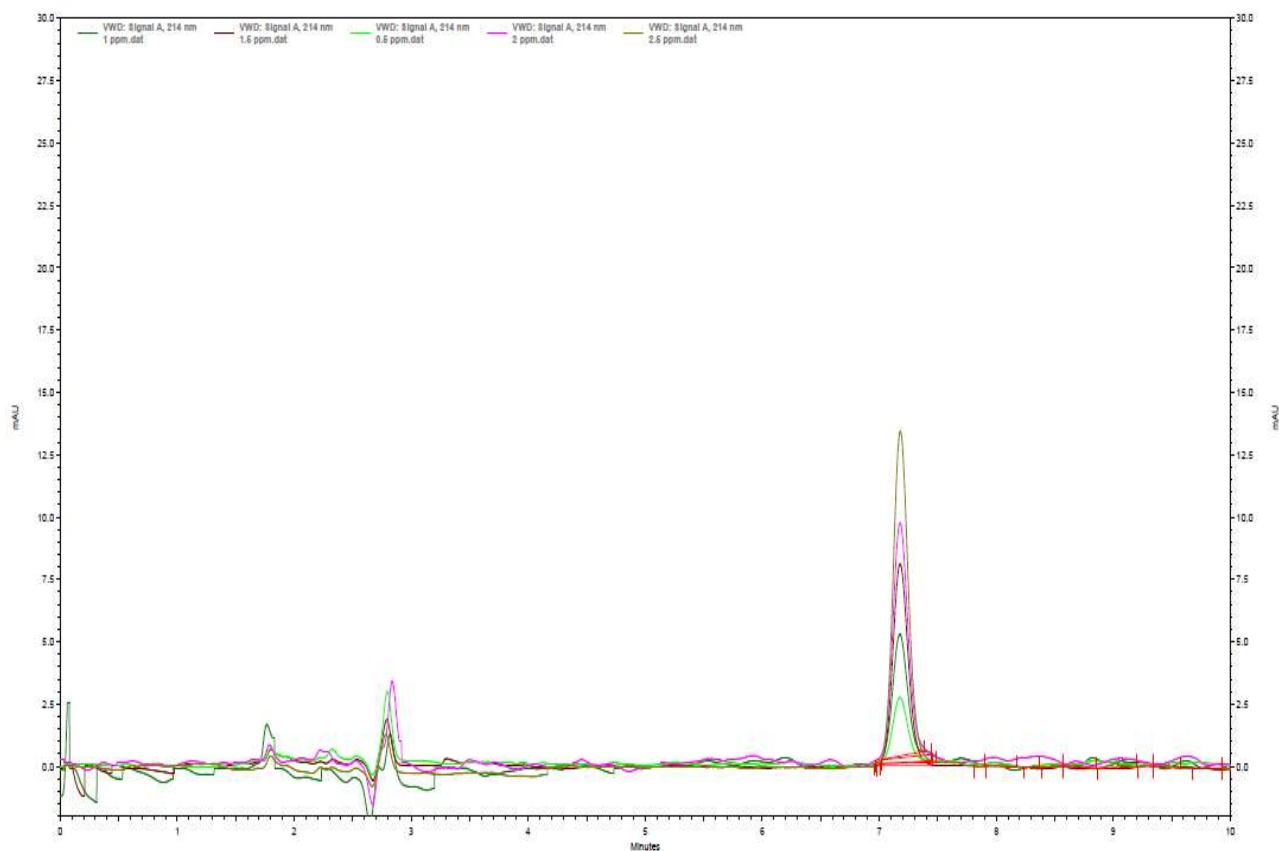

Figure 3.10: Overlay Chromatogram of Tavaborole by HPLC

Figure 3.10 presents an overlay chromatogram of Tavaborole in the mobile phase. The retention time for Tavaborole is 7.18 minutes, while the other peaks represent solvent signals. These solvent peaks are sufficiently separated from the drug peak, indicating that they will not interfere with the Tavaborole peak. The peaks observed at 7.18 minutes correspond to a concentration range of 500-2500 ng/ml. This implies that the drug's concentration in the sample can be determined by measuring the height or area of this peak, as its intensity is directly proportional to the amount of Tavaborole present.

.

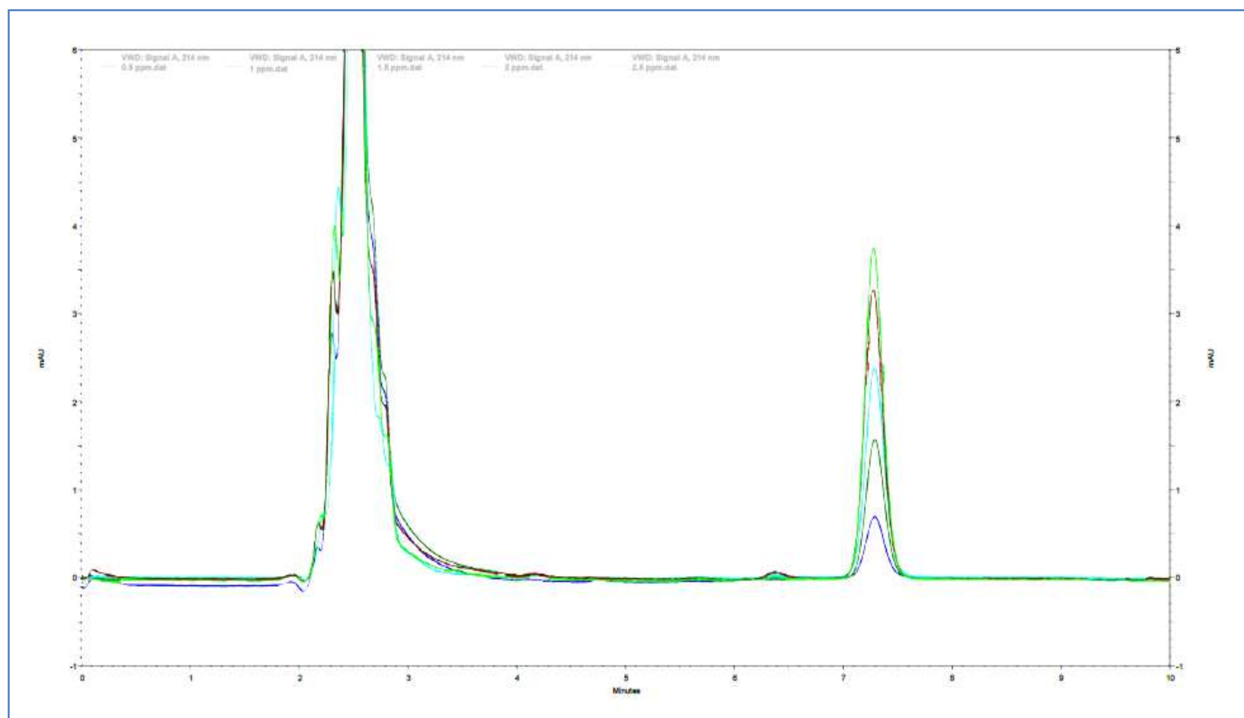

Figure 3.11: Overlay Chromatogram of Tavaborole by HPLC in skin homogenate

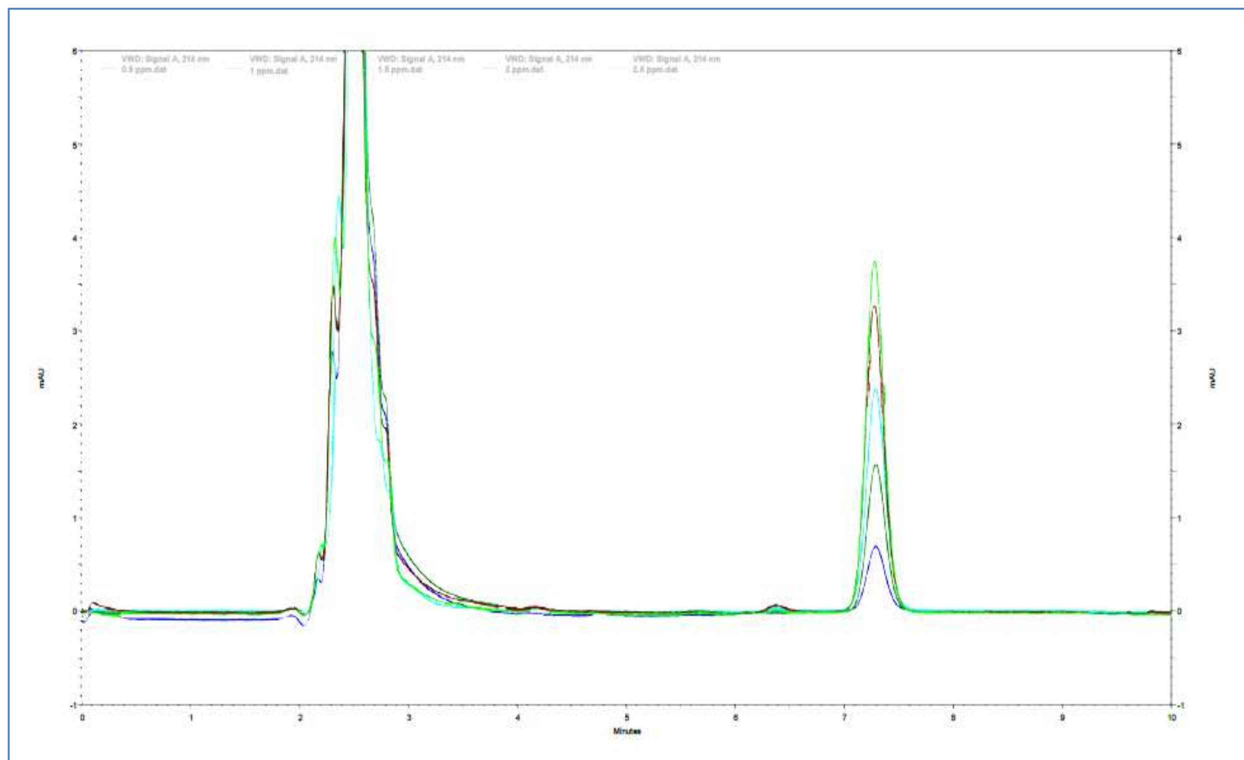

Figure 3.12: Overlay Chromatogram of Tavaborole by HPLC in rat plasma

Figure 3.11 and figure 3.12 show the overlay chromatogram of Tavaborole by HPLC in skin homogenate and rat plasma respectively. The ranges of chromatograms show are 500-2500

ng/ml. It shows that concentration of Tavaborole and area (response) have linear relationship as well as retention time remain constant at different concentrations.

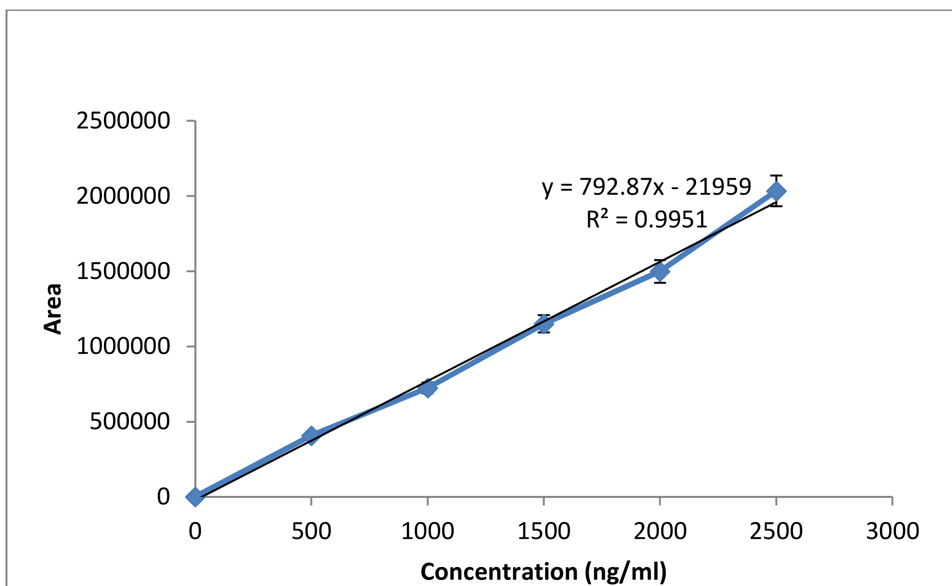

Figure 3.13: Calibration plot of Tavaborole by HPLC

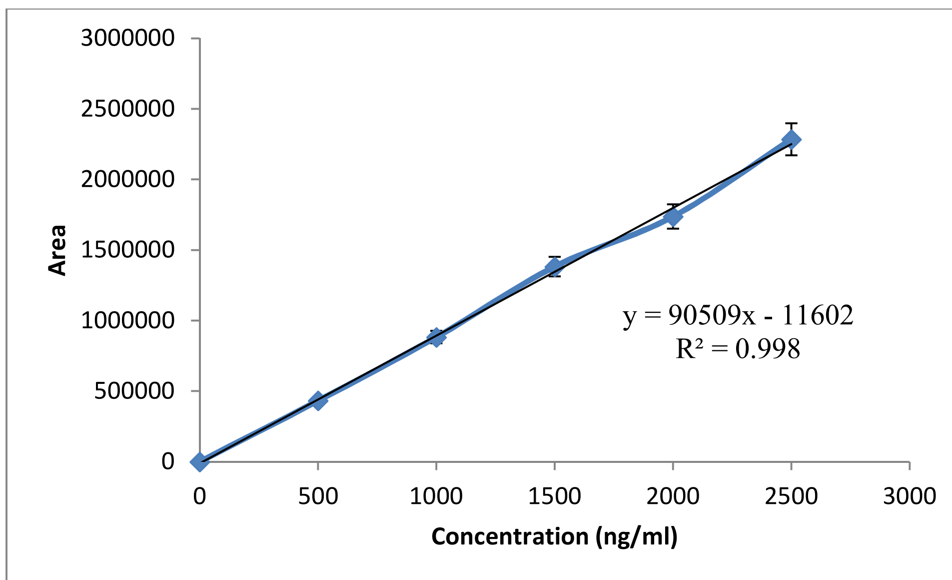

Figure 3.14: Calibration plot of Tavaborole by HPLC in skin homogenate

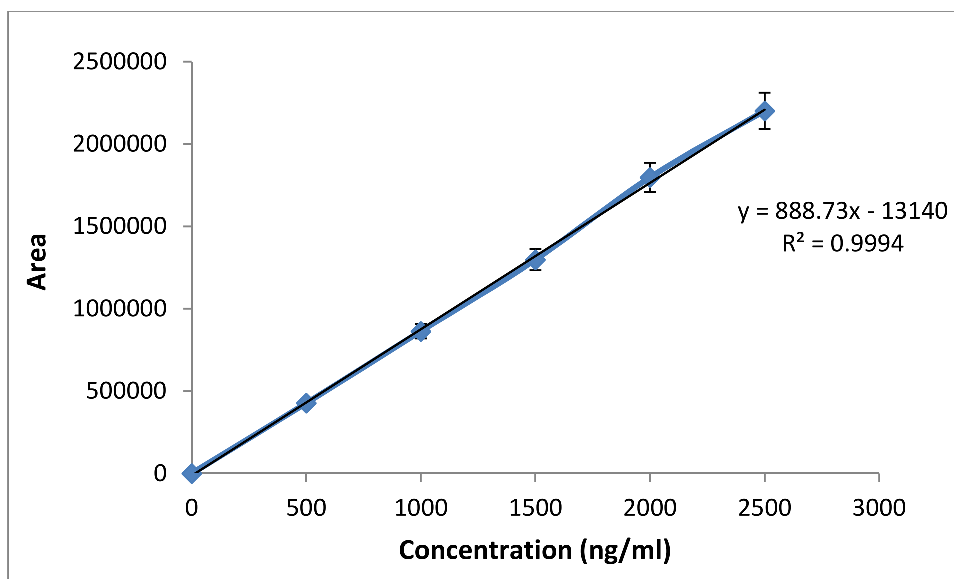

Figure 3.15: Calibration plot of Tavaborole by HPLC in rat plasma

As per table 3.15 and the calibration plot for Tavaborole in mobile phase, skin homogenate and rat plasma demonstrated linearity within the 500-2500 ng/ml concentration span. This suggests adherence to Beer's law for Tavaborole in the specified concentration window.

Table 3.16: Parameters from calibration plot

Methods	λ_{max}	Mobile Phase	Linearity Range	Regression Equation	Correlation Coefficient	LOD (ng/ml)	LOQ (ng/ml)
Analytical method	214 nm	Phosphoric acid (10 mM, pH 2.0): ACN (60:40)	500-2500 ng/ml	y = 79287x - 21959	0.995	3.5	1.1
Bioanalytical method in skin homogenate				y = 90509x - 11602	0.998	2.7	8.3
Bioanalytical method in rat plasma				y = 88873x - 13140	0.999	2.4	7.2

The low values of LOD and LOQ indicate the sensitivity of the methods which is shown in table 3.16.

Table 3.17 Accuracy of the method

Drug Spiked (%)	Expected concentration (ng/ml)	Observed Concentration (ng/ml) ±SD (n=3)	% Drug recovered
Analytical Method			
80%	1200	1199.66±0.58	99.97
100%	1500	1500.33±1.39	100.04
120%	1800	1800.07±0.13	100.00
Bioanalytical method in skin homogenate			
80%	1200	1200.18±0.31	100.01
100%	1500	1500.15±0.49	100.02
120%	1800	1799.74±0.65	99.99
Bioanalytical method in rat plasma			
80%	1200	1199.18±1.05	99.93
100%	1500	1499.57±0.52	99.97
120%	1800	1800.67±1.15	100.04

Table 3.17 shows the percentage recoveries at lower, intermediate, and higher concentrations. Their findings demonstrated that the suggested analytical method could accurately determine and quantify even a slight variation in the drug concentration in the solution.

Table 3.18: Intraday and interday precision analysis of Tavaborole

Concentration (ng/ml)	Observed Concentration (ng/ml) ±SD (n=3)		%Recovery		%RSD	
	Intraday	**Interday**	**Intraday**	**Interday**	**Intraday**	**Interday**
Analytical method						
500	499.69±0.29	499.85±0.37	99.94	99.97	0.06	0.07
1500	1500.37±0.83	1500.43±0.77	100.02	100.03	0.05	0.05
2500	2499.66±0.58	2500.05±0.45	99.99	100.00	0.02	0.02
Bioanalytical method in skin homogenate						
500	499.67±0.58	499.91±0.87	99.93	99.98	0.11	0.17
1500	1500.33±1.53	1500.27±0.64	100.02	100.02	0.10	0.04
2500	2499.85±0.79	2500.70±1.52	99.99	100.03	0.03	0.06
Bioanalytical method in rat plasma						
500	500.26±0.39	500.51±0.34	100.05	100.01	0.08	0.07
1500	1501.03±1.01	1501.23±1.32	100.07	100.08	0.07	0.09
2500	2500.64±1.18	2500.97±1.01	100.03	100.04	0.05	0.04

Uniform testing conditions were applied to assess both intraday and interday precision. The precision and consistency of these methods are reflected in the %RSD values obtained, which are below 2.0%, indicating high reproducibility. As demonstrated in Table 3.18, the method yields consistent and accurate outcomes.

3.7 References:

1. Ravisankar, Panchumarthy, et al. "A review on step-by-step analytical method validation." *IOSR j pharm* 5.10 (2015): 7-19.
2. Lavanya, G., et al. "Analytical method validation: An updated review." *International journal of pharmaceutical sciences and research* 4.4 (2013): 1280.
3. Boqué, Ricard, et al. "Validation of analytical methods." *Grasas y aceites* 53.1 (2002): 128-143.
4. Chaudhari, Mansi J., et al. "Application of area under curve technique for UV-Spectrophotometric determination of Luliconazole in bulk and pharmaceutical formulation." *Asian journal of pharmaceutical analysis* 8.1 (2018): 45-48.
5. Kumar, Manish, Nithya Shanthi, and Arun Kumar Mahato. "Qualitative and Quantitative Methods for Determination of Drug Luliconazole." *International journal of research in advent technology* 6.10 (2018): 2321-9637.
6. Malasiya, Aditi, and Anju Goyal. "Method development and validation of RP HPLC method for assay and related substances of luliconazole in topical dosage form." *International journal of pharmaceutical chemistry and analysis* 4.2 (2017): 46-50.
7. Panthi, Vijay Kumar, and Utsav Nepal. "Formulation and Development of a Water-in-Oil Emulsion-Based Luliconazole Cream: In Vitro Characterization and Analytical Method Validation by RP-HPLC." *International journal of analytical chemistry* 2022 (2022).
8. Tampucci, Silvia, et al. "Development and validation of an HPLC–UV method to quantify Tavaborole during in vitro transungual permeation studies." *Journal of AOAC international* 101.2 (2018): 437-443.
9. Sheela, Avuthu Sai, Mukthinuthalapati Mathrusri Annapurna, and Rangisetty Spandana Yasaswini. "New validated analytical methods for the determination of Tavaborole (An anti-fungal agent)." *Research journal of pharmacy and technology* 13.4 (2020): 1895-1900.