Chapter: 3

Materials and analytical method

3 Materials and analytical method

3.1 Materials

Table 3.1 List of materials used for the study

S.No.	Material Name	Procured from
1.	Acetic acid	SD Fine-Chem Ltd., Mumbai, India
2.	Acetonitrile	SD Fine-Chem Ltd., Mumbai, India
3.	Acetone	Himedia, Mumbai, India
4.	Antibiotic solution 100X liquid	Himedia, Mumbai, India
5.	Aluminium foil (fresh wrap)	Hindalco Industries Ltd., Mumbai, India
6.	Disposable syringes	Hindustan Syringes & Medical Devices Ltd., Faridabad, India
7.	Chitosan	Sigma-Aldrich, Bangalore, India
8.	Disodium hydrogen orthophosphate	Merck Ltd., Mumbai, India
9.	Dulbecco's modified eagle medium (DMEM)	Thermo Fisher Scientific, Mumbai, India.
10.	D-a-tocopheryl polyethylene glycol 1000 succinate	ANTARES Health Product, Mumbai, India
11.	Dichloromethane	SD Fine-Chem Ltd., Mumbai, India
12.	Ehrlich's solution	Sigma-Aldrich, Bangalore, India
13.	Ethanol	SD Fine-Chem Ltd., Mumbai, India
14.	Fetal bovin serum (FBS)	Thermo Fischer Scientific, Mumbai, India
15.	Formic acid	SD Fine-Chem Ltd., Mumbai, India
16.	Gelatin from porcine skin, Type A	Himedia, Mumbai, India
17.	Glutathione	Sisco Research Laboratories Pvt. Ltd., Mumbai, India
18.	Hydrochloric acid	Thermo Fischer Scientific, Mumbai, India
19.	Human epidermal keratinocytes (HaCaT)	National Centre for Cell Science, Pune, India
20.	Luligel™ (Luliconazole marketed cream)	Anthus Pharmaceuticals Pvt Ltd, Kerala, India

21.	Methanol	Merck Ltd., Mumbai, India
22.	MTT	SRL Pvt Ltd, Mumbai, India
23.	Polyvinyl alcohol	Sigma-Aldrich, Bangalore, India
24.	Poly (D,L-lactide-co-glycolide) (Mw = 7000-17000)	Sigma-Aldrich, Bangalore, India
25.	Poly (ε- caprolactone) (Mw =120000)	Corbion, Netherlands
26.	Potassium dihydrogen orthophosphate	Qualigens Chemicals, Mumbai, India
27.	Sodium hydroxide	Qualigens Chemicals, Mumbai, India
28.	Silver nitrate	Sisco Research Laboratories Pvt. Ltd., Mumbai
29.	Trypsin-EDTA solution 1X	Himedia, Mumbai, India
30.	1,1,1,3,3,3-hexafluoro-iso-propanol (HFIP)	Spectrochem Pvt. Ltd, Mumbai, India
31.	Well plates (6, 32 & 96 wells)	Genetix Biotech Asia Pvt Ltd, New Delhi, India

3.2 Instruments

Table 3.2 List of instruments used for the study

S. No.	Instruments	Source
1.	Camera assisted optical microscope	Dewinter Optical, Inc., New Delhi, India
2.	Cooling centrifuge	REMI C20, Mumbai, India
3.	Contact angle Instrument	Kruss^{TM} DSA30E , Germany
4.	Digital electronic balance	Shimadzu, Japan
5.	Digital pH meter	Mettler-Toledo, Mumbai, India
6.	Dissection box	Camlin Ltd., Mumbai, India
7.	Fourier transform infrared spectrophotometer	Shimadzu FTIR-8400S, Japan
8.	High voltage power supply	Goldstar, New Delhi, India
9.	High-resolution scanning electron microscopy	FEI, Quanta 200F, Japan
10.	Hot air oven	IKA, Germany
11.	Magnetic stirrer	Decibel Instruments, Chandigarh, India
12.	Microplate reader	Synergy HTX multi-mode reader (Bio Tek, USA)
13.	Powder X-Ray diffractometer	Mini Flex 600, Rigaku, Japan
14.	Probe sonicator	Labman Scientific Instruments Pvt. Ltd. Chennai, India
15.	Scanning electron microscope	ZEISS EVO 18 Research, USA
16.	Sonicator (bath type)	IKA, Germany
17.	Syringe pump	AYRA N801 New era pumps, USA

18.	High performance liquid chromatography	Shimadzu LC20AD Prominence, Japan

3.3 Software

Table 3.3 List of software used for the study

S. No.	Name of Software	Developer
1	Adobe Acrobat reader 11.0	Adobe Systems Inc. USA
2	Zotero 6.0.27	Wintertree Software Inc., USA
3	Grammarly	Corporation of Digital Scholarship
4	GraphPad Prism 5.03	Graph Pad Software Inc., USA
5	Microsoft Office 2016	Microsoft Corp., USA
6	OriginPro 2024	Origin Lab Corp., USA
7	Image J 1.52a	National Institute of Health, USA

3.4 Analytical method

3.4.1 Development of HPLC method for the estimation of luliconazole and naringenin.

The amount of luliconazole and naringenin were analysed using high performance liquid chromatography (Shimadzu LC20AD Prominence Liquid Chromatography). The HPLC system consist of binary pump with Rheodyne7725I manual injector, DGU-20A_3 Prominence Degasser, Quasar-C18 reverse phase column (5µm particle, 250x4.6mm) and SPD-20A Prominenece photodiode array detector. The mobile phase of methanol and 0.1% aqueous orthophosphoric acid in 70:30 was used with flow rate of 1mL/min. The column temperature was maintained at 30^{o}C during whole process and 290nm wavelength and retention time 5.4 and 8.1 min was used the AUC calculation using LC solution 1.25 software. The standard calibration of luliconazole and naringenin were plotted from a range of 4-160µg/mL.

3.4.2 Analytical method validation

3.4.2.1 Linearity and range

According to ICH) guideline Q2 (R1), linearity refers to the capability of the analytical method to generate test outcomes that are directly proportional to the quantity of the substance being analysed. This signifies the method's capacity to produce results, that increase or decrease in a consistent manner as the concentration of the analyte changes. It is a fundamental characteristic that demonstrates the method's ability to measure analyte concentrations accurately across a range of values. Moreover, range denotes, both upper and lower concentration limits within which the method demonstrates a linear response [ICHQ2 (R1) 2005].

3.4.2.2 Accuracy and precision

The accuracy of the analytical method defines the ability of an analytical procedure to produce test results close to the reference values. For this a known amount of standard stock solution of luliconazole and naringenin was added at three different levels, i.e. 80%, 100% and 120% in the pre-analysed solution of both drugs. And reanalysed to calculate accuracy in the term of % recovery.

$$\%\, Recovery = \frac{Practical\ Concentration}{Theoritical\ Concentration} * 100$$

Similarly, precision defines degree of scattering between a series of measurements obtained from multiple samplingof the same homogeneous sample under the prescribed conditions. It's calculated in form of Relative standard deviation [ICHQ2 (R1) 2005].

$$\%\, Relative\ standard\ Devaition = \frac{Standard\ deviation\ of\ values}{Mean\ of\ values} * 100$$

3.4.2.3 Limit of detection and limit of quantification

The limit of detection (LOD) refers to the minimum concentration of an analyte in a sample that can be identified, although not necessarily precisely quantified. On the other hand, the limit of quantitation (LOQ) is the minimum concentration of an analyte in a sample that can be accurately and precisely quantified. The determination of LOD and LOQ for the analytical method was carried out in accordance with established protocols and using specific equations [ICHQ2 (R1) 2005].

$$Limit\ of\ Detection = 3.3 * \frac{\sigma}{S}$$

$$Limit\ of\ Quantification = 10 * \frac{\sigma}{S}$$

σ = Standard deviation of intercept
S =Slope of the calibration curve.

3.4.3 Results

The calibration curves of the luliconazole, naringenin, luliconazole in combination and naringenin in combination in the range of 4-160µg/mL were plotted (Figure 3.1-3.4), and showed different regression parameters i.e. slope, intercept, correlation coefficient as shown in Table 3.1.

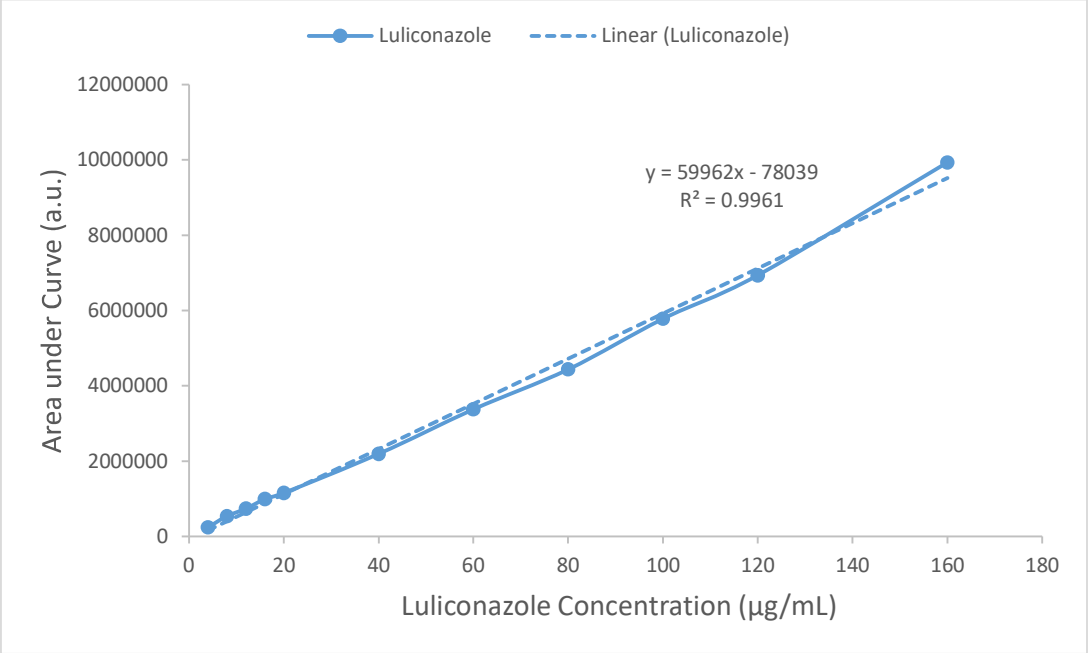

Figure 3.1 Calibration curve of luliconazole

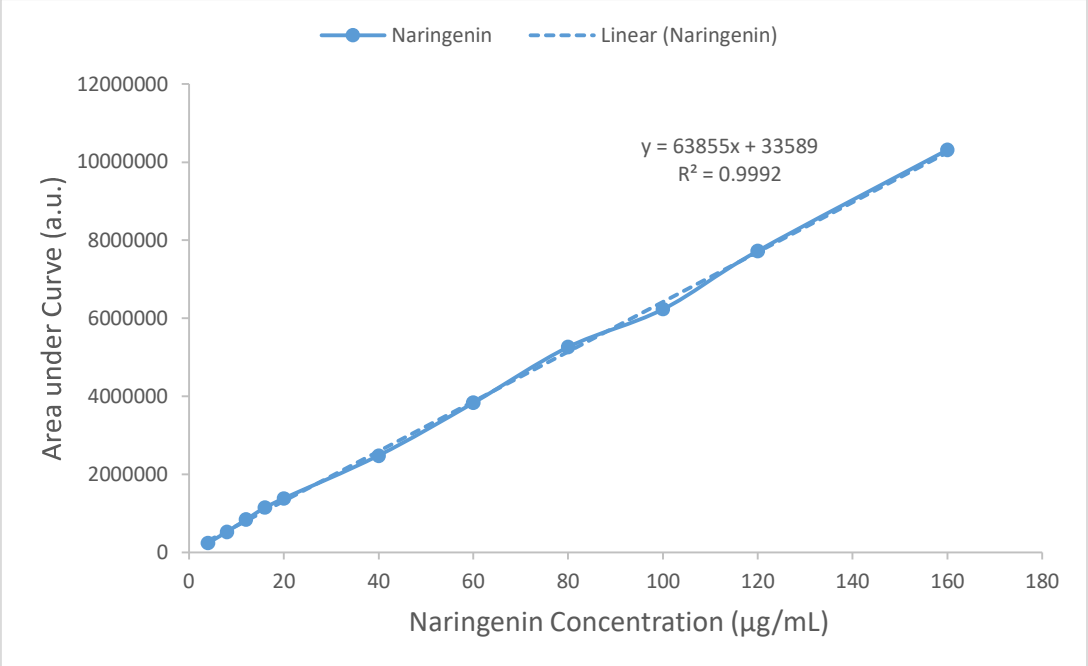

Figure 3.2 Calibration curve of Naringenin

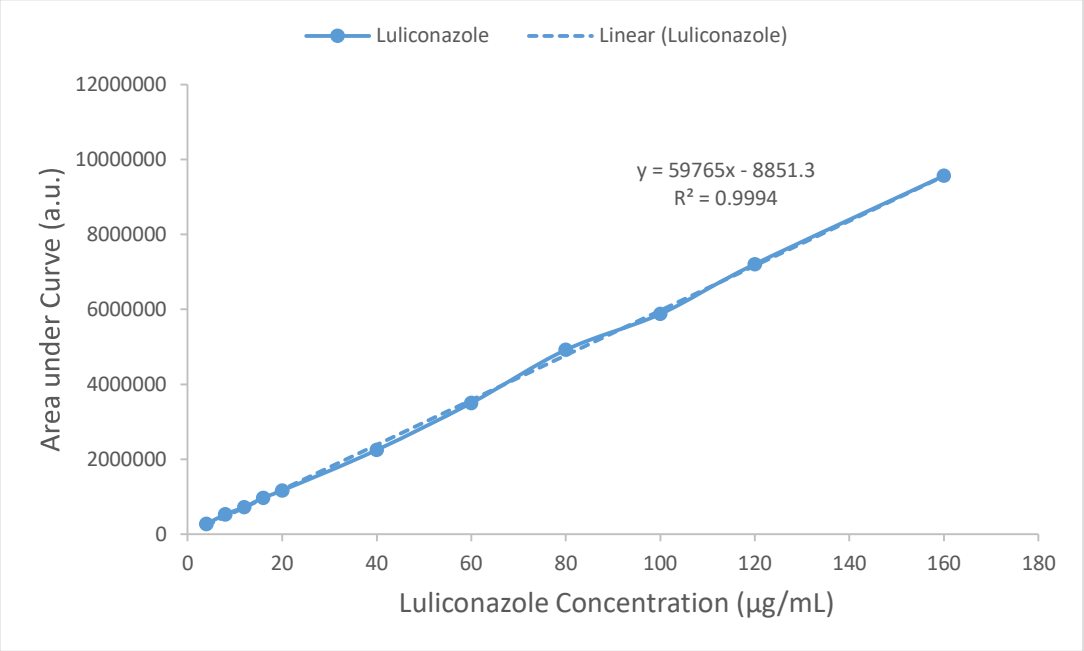

Figure 3.3 Calibration curve of Luliconazole in combination with naringenin

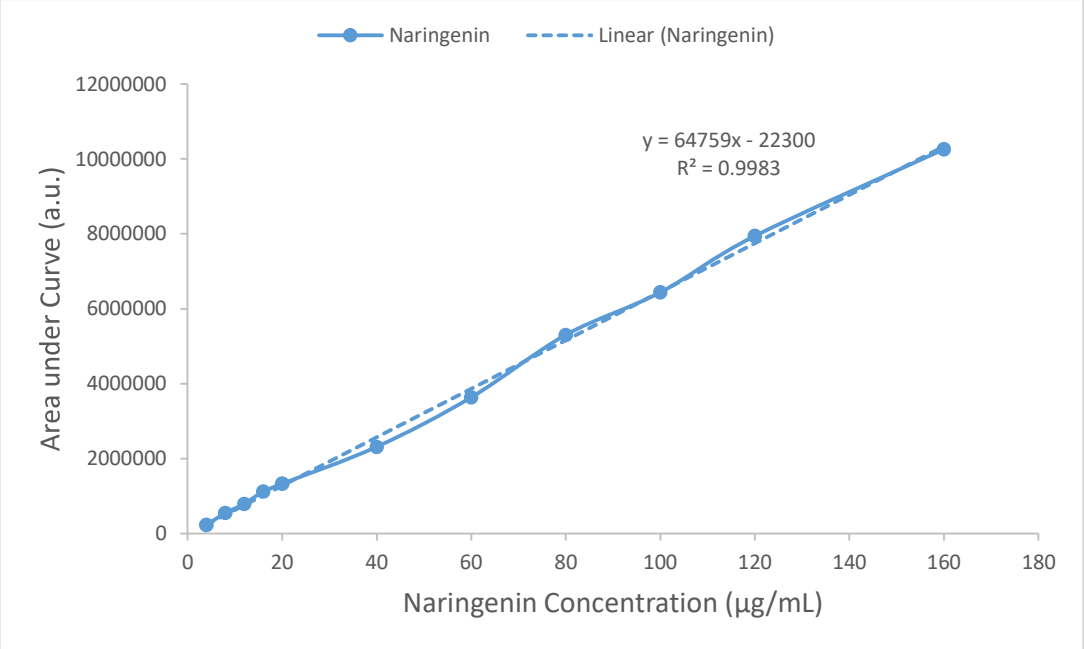

Figure 3.4 Calibration curve of Naringenin in combination with luliconazole

Table 3.4 Regression parameter from calibrations curve

Parameters	Drugs		Combination	
	Luliconazole	Naringenin	Luliconazole	Naringenin
Lambda max (λ)	296	291	296	291
Correlation coefficient	0.9961	0.9992	0.9994	0.9983
Slope	59962	63855	59765	64759
Intercept	-78039	33589	-8851	22300
Range (µg/mL)	4-160	4-160	4-160	4-160
Retention Time (min)	5.34	8.13	5.42	8.07

Further the accuracy of the method was found to be in the limit of 100±2.5% (Table 3.5) for both drug indicating acceptable of the method. The interday and intraday precision (RSD value) was found to be <3%, indicating repeatability of the developed method.

Table 3.5 Accuracy and precision of the developed method

Drug	Pre-Analysed Sample	Level of Recovery	Drug added (µg/mL)	Drug recovered (mean± S.D)	% Drug recovered	Precision	
						Inter day	Intra day
LTZ	10 µg/mL	80%	8.00	8.13±0.03	101.6	0.47	0.41
		100%	10.00	10.03±0.1	100.27	0.86	1.04
		120%	12.00	12.08±0.3	100.66	0.73	2.02
NAR	10 µg/mL	80%	8.00	7.91±0.2	98.93	1.04	2.11
		100%	10.00	9.93±0.23	99.31	1.71	2.31
		120%	12.00	11.92±0.33	99.33	2.68	2.09
LTZ in combination	10 µg/mL	80%	8.00	7.94±0.12	99.3	1.23	1.56
		100%	10.00	10.05±0.21	100.46	2.46	2.26
		120%	12.00	11.88±0.23	98.99	1.78	1.96
NAR in combination	10 µg/mL	80%	8.00	7.87±0.04	98.36	1.44	0.56
		100%	10.00	10.02±0.22	100.21	2.47	2.22
		120%	12.00	11.90±0.27	99.15	1.53	2.3

The developed method also found to specific and selective for both drugs. The developed method shows no interfering peaks (Figure 3.5-3.8) of excipients at respective retention time of drugs indicating specificity of the developed method.

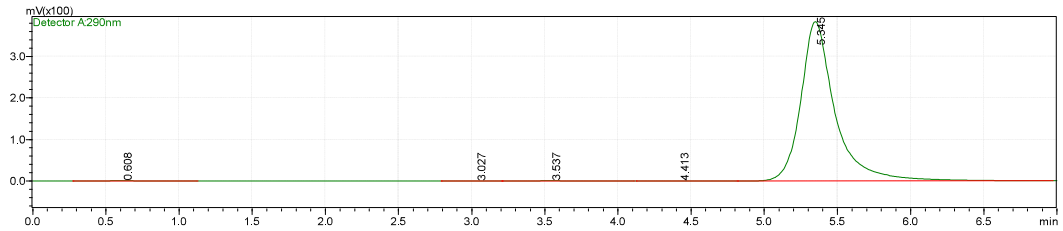

Figure 3.5 Luliconazole chromatogram

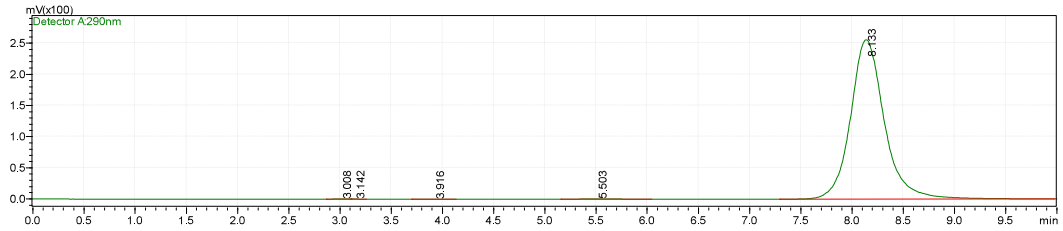

Figure 3.6 Naringenin chromatogram

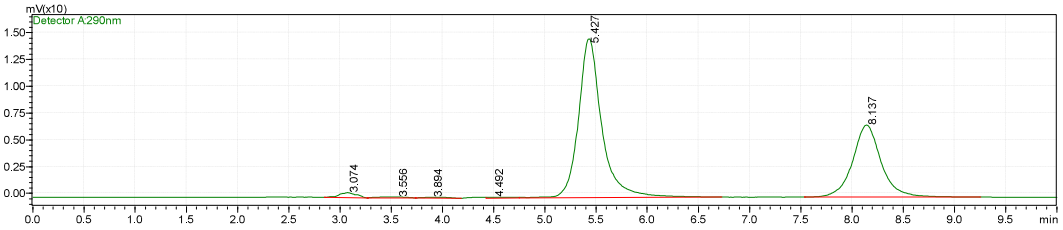

Figure 3.7 Luliconazole chromatogram in combination with naringenin

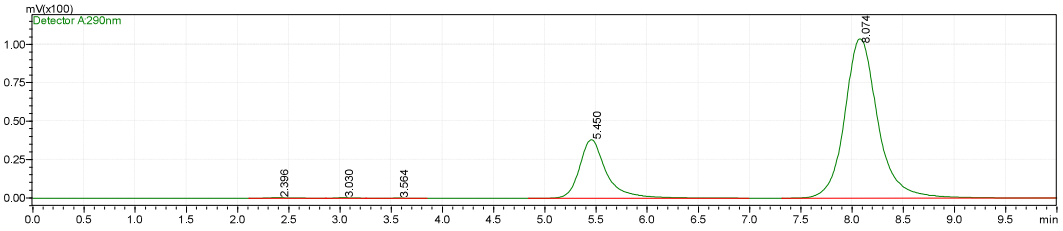

Figure 3.8 Naringenin chromatogram in combination with luliconazole

Further, developed analytical method showed limit of detection and quantification value (Table 3.3) of 107.1 & 321.15 ng/mL, 321.57 & 964.81 ng/mL, 533.03 & 1599.93 ng/mL and 303.39 & 910.26 ng/mL for luliconazole, naringenin, luliconazole in combination and

naringenin in combination, respectively; indicating selectivity of the method for luliconazole and naringenin.

Table 3.6 Limit of detection and Limit of quantification of luliconazole and naringenin

Drug	**Limit of detection (ng/mL)**	**Limit of Quantification (ng/mL)**
LTZ	107.1	321.15
NAR	321.57	964.81
LTZ in combination	533.03	1599.93
NAR in combination	303.39	910.26