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Research Article

Pharmaceutical analysis for Novel drug formulation

A Simultaneous Estimation of Clotrimazole and Its Impurities

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Abstract: A simple, efficient and reproducible method for the simultaneous determination of Clotrimazole and its Impurities (Imidazole and (2-chlorophenyl) phenyl methanone (2-chlorobenzophenone)) has been developed using reversed phase high performance liquid chromatography. The separation was carried out using a mobile phase consisting of Potassium dihydrogen phosphate buffer (pH 7.2): Acetonitrile (25:75 %v/v). Column used was SHIMADZU C_{18} (4.6x250 mm, 5 µm) with a flow rate of 0.8 ml/min and the column temperature was maintained at 40 °C. The detection wavelength used was at 220 nm. The retention time of Clotrimazole, Imidazole and 2-chlorophenyl phenyl methanone (2-chlorobenzophenone)was 8.12, 7.56 and 3.02 min respectively. Linearity of Clotrimazole and its Impurity are in range of 0.013-0.076 mg/mL. For System Suitability % RSD was found to be 1.60 for Clotrimazole, 1.29 for Imp-D, 1.15 for Imp-E..The Mean% Recoveries were found to be within the range of 98-102 %.Analytical parameters were calculated and a statistical evaluation was included.

Keywords: Clotrimazole; Imidazole; 2-chlorophenyl phenyl methanone (2-chlorobenzophenone); HPLC; Simultaneous; estimation; Impurity.

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1. INTRODUCTION

Clotrimazole (CLO) is a broad spectrum antimycotic drug, andit works by inhibiting the fungal cytochrome P450 3A enzyme, lanosine 14α-demethylase, which is responsible for converting lanosterol to ergosterol, the main sterol in fungal cell membrane. Chemically, it is 1-[(2-Chlorophenyl)(diphenyl)methyl]-1H-imidazole (Fig. 1).[1-2]

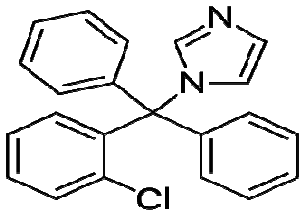

Fig 1.Chemical structure of Clotrimazole

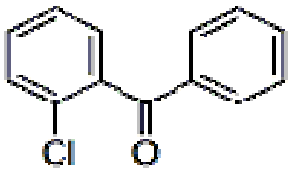

Fig 2.Clotrimazole Impurity-E (2-chlorophenyl phenylmethanone (2-chlorobenzophenone)

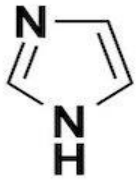

Fig 3.Clotrimazole Impurity D (Imidazole)

Literature survey reveals that few methods reported for identification and quantification of active substance Clotrimazole(Fig.1) such as UV[3], HPLC[4-13] and HPTLC[4-16]. It is important to detect the impurities not only in the active ingredient but in the final formulation. To the best of our knowledge, there is no reported method on simultaneous estimation of Clotrimazole and its impurities-D (Fig. 3) and E (Fig. 2). Thus, the main objective of the studyis to develop and validate the method of Clotrimazole and its impurities-D and E by RP-HPLC. The validation parameters were performed according to the International Conference on Harmonization (ICH) guidelines [17-18]

2. MATERIAL AND METHODS

2.1 Chemical Resources

The drugs Clotrimazole, Clotrimazole Impurity-D and Clotrimazole Impurity-E were procured from Indian Pharmacopoeia Council, India. Ortho phosphoric Acid (OPA), Triethylamine were purchased from LOBA Chemical Laboratories Pvt. Ltd. HPLC grade water, Acetonitrile, Ammonium acetate (reagent grade), methanol, Potassium dihydrogen orthophosphate, ethyl amine,Tetrahydrofuranwere purchased from Thermo Fisher Scientific Pvt. Ltd, India.

2.2 Instrument Resources

An HPLC system SHIMADZU (Prominence-i LC-2030, Singapore), data acquisition and processing were accomplished using Lab Solutions software with PDA detector. Micro balance (Teraoka Pvt. Ltd), pH meter (LAB INDIA), variable range micro pipette (Cyberpet pro, ANM Amkette Industries), variable size glass bottles, graduated measuring cylinders, volumetric flasks (Borosil), ultrasonic water bath (LOBA Chem Pvt. Ltd, Mumbai), Vortexer (Remi equipment Pvt. Ltd), deep freezer (-48°C Ilshn lab Co.Ltd), refrigerator (Godrej). Pipettes ranging from 10-1000 µl, 12x7.5 mm Tarson made riavials, polypropylene tubes, pipette tips ranging from 10-1000 µl and surgical gloves were employed in this present work.

2.3 Preparation of potassium dihydrogen ortho phosphate buffer

Approximately 13.6 g of potassium dihydrogen ortho phosphate was weighed and taken into 100 ml volumetric flask. To that, 60 ml of HPLC grade water was added and sonicated to dissolve properly. Then the volume was made upto the mark with water.[1-4]

2.4 Preparation of Standard stock solutions

Weighed approximately 10 mg of Clotrimazole, Imp-D and E working standards individually into a 10 ml volumetric flask , dissolved and diluted to volume with diluent and sonicated.[5-6]

2.5 Selection of wavelength for detectionby scanning in UV

The working standard solution of Clotrimazole, Impurity-D and E were scanned in the UV region 200-400 nm and spectrum was recorded. Distilled water was used as the diluent. Maximum absorbance was found at 220 nm. In HPLC, the proper peak response was observed at 220 nm. Hence, the same wavelength was selected for HPLC estimation.

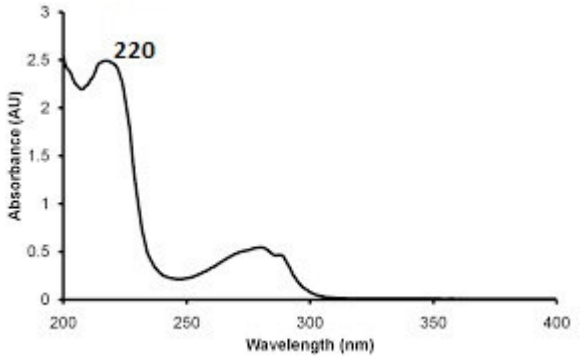

Fig 4. UV Scan spectrum for wavelength selection

2.6 Selection of Mobile Phase

The pure drug Clotrimazole, Imp-D and E were injected into the HPLC system and run in different solvent systems. Acetonitrile: Triethylamine, Acetonitrile: Water; Water: Acetonitrile: Phosphate buffer with varying mobile phase ratios and pH were tried in order to find the best conditions for the separation of Clotrimazole, Imp-D and E. From the various chromatograms obtained with different mobile phases, Potassium dihydrogen phosphate buffer (pH 7.2): Acetonitrile (25:75 %v/v) was selected for the separation of Clotrimazole, Imp-D and E.[7-10]

2.7 Preparation of Mobile Phase

Potassium dihydrogen phosphate buffer (pH 7.2): Acetonitrile were filtered through 0.45 µm, 47 mm membrane filter paper and then ultrasonicated for 20 minutes on ultrasonicator. Mobile phase was prepared by mixing 75 mL of Potassium dihydrogen phosphate buffer (pH 7.2), 25 ml of Acetonitrile. (Justify here, why the ratio of the solvent has varied from the above said ratio).[11-12]

2.8 Chromatographic Separation

Mixed standards of Clotrimazole, Imp-D and E were injected into column with 20 µL of micro-syringe. The chromatogram was run for appropriate minutes with the selected mobile phase which was previously degassed. The flow rate was set to 0.8 mL/min and detection was carried out at 220 nm. After complete separation of two drugs, data related to peak area, height, retention time, resolution were recorded using software (mention the software version details).[13-16]

3. RESULTS

The method was quantified and validated as per International Conference on Harmonization Guideline on Validation of Analytical Procedures. Text and Methodology, Q2 (R1).2005 and International Conference on Harmonization Guideline on Stability Testing of New Drug Substances and Products; Q1 A (R2). 2003 17-[18]

3.1 System Suitability

3.1.1 System suitability test was performed to confirm that the developed chromatographic conditions are suitable for the present work.

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (Rt), number of theoretical plates (N) and tailing factor (T) were evaluated for six replicate injections of the drug (Table-1).

Table 1. System suitability solution data

Name of Reference solution	Clotrimazole	Imp-D	Imp-E
Injection-1	11485	336509	457726
Injection-2	11145	326158	447302
Injection-3	11260	335703	456952
Injection-4	11286	337487	462807
Injection-5	10940	335790	459867
Injection-6	11242	337262	457996
Mean	**11226**	**334818**	**457108**
STDEV	**179.14**	**4305.34**	**5242.96**
% RSD	**1.60**	**1.29**	**1.15**

3.1.2 Selectivity/ specificity

The selectivity of the method is verified by injecting each of known impurity and API individually and also as a mixture. Individual impurities and Clotrimazole were injected at 0.05 mg/mL concentration level and a mixture of impurities and Clotrimazole were injected at 0.05 mg/mL (Specification level) for checking the resolution. (Fig. 5-9)

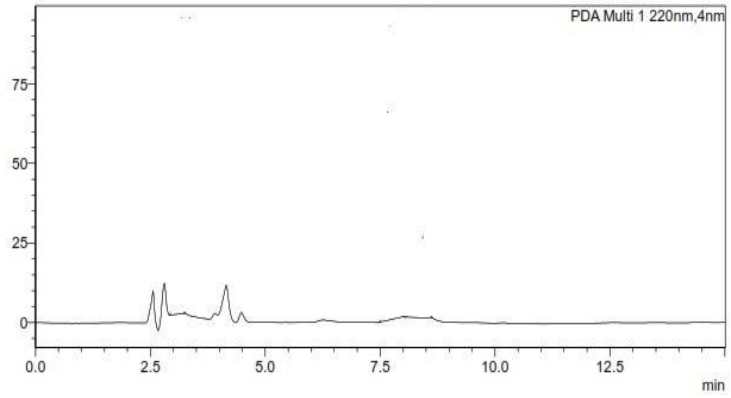

Fig 5.Selectivity chromatogram

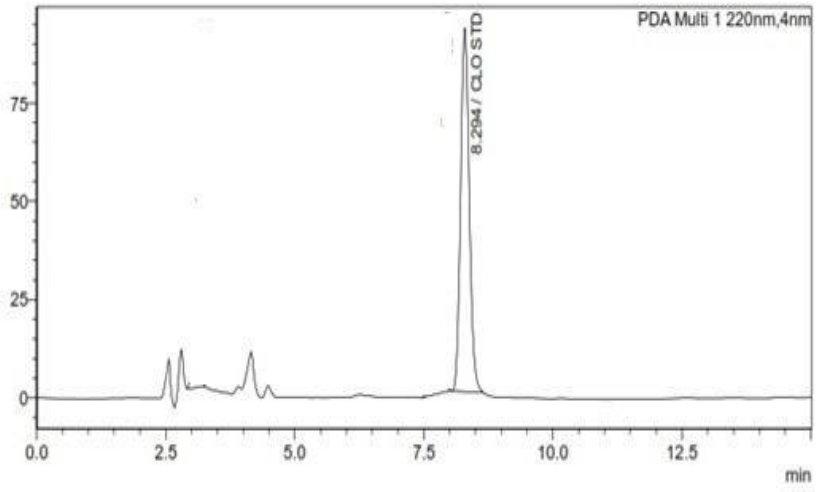

Fig 6.Clotrimazole Chromatogram

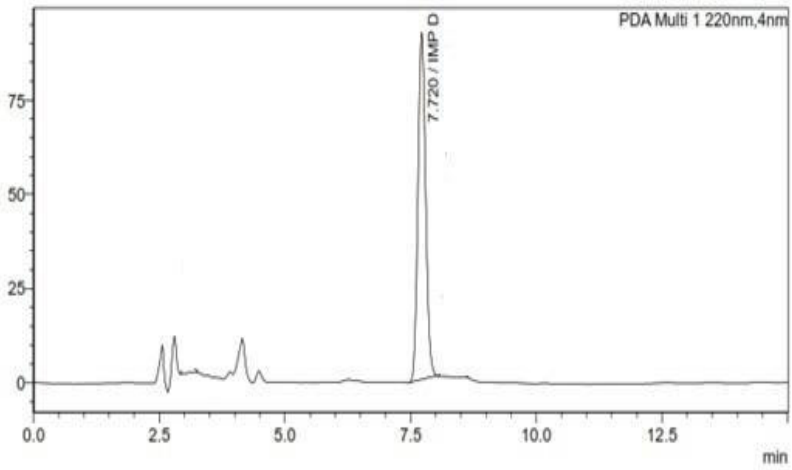

Fig 7.Clotrimazole-Imp-D Chromatogram

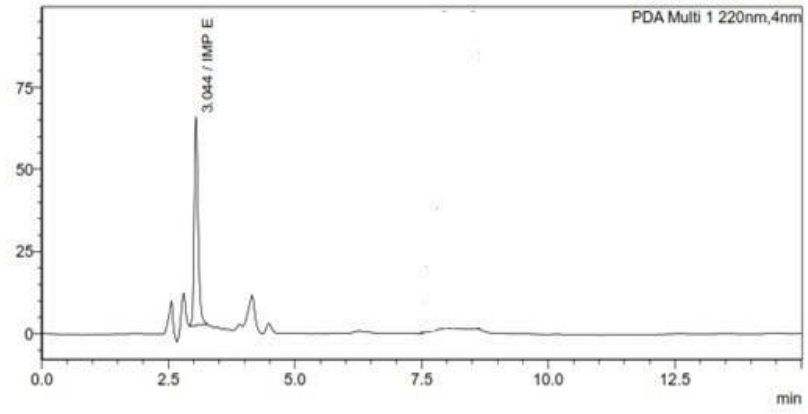

Fig 8.Clotrimazole-Imp-E Chromatogram

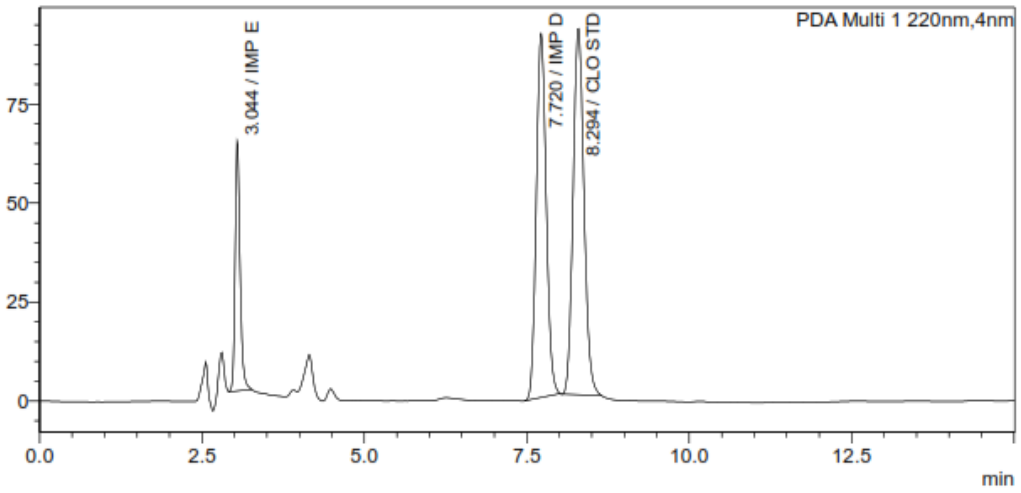

Fig 9.Standard Chromatogram Clotrimazole and its impurities (Imp-D & E)

By performing selectivity any Interference was not observed with the standard peaks and the chromatograms of Standard and Sample were identical with same retention time.

3.2 Precision

Precision is the measurement of the degree of reproducibility of the analytical method derived by analyzing a sufficient number of aliquots of a homogenous solution of Clotrimazole having known concentration of each of the impurities. A Resolution solution at 0.05 % level of each impurity and 0.05 % level of Clotrimazole with respect to the test concentration (15.0 mg/ml) has been prepared and injected in six replicatetimes.

Table 2. Method precision

Name of the Reference solution	Clotrimazole	Imp-D	Imp-E
Precision sol-1	11485	338317	453862
Precision sol-2	11145	336205	457739
Precision sol-3	11260	338137	460698
Precision sol-4	11286	342306	465321
Precision sol-5	10940	337648	458109
Precision sol-6	11242	337397	454542
Mean	**11226**	**334818**	**457108**
STDEV	**179.14**	**4305.34**	**5242.96**
% RSD	**1.60**	**1.29**	**1.15**

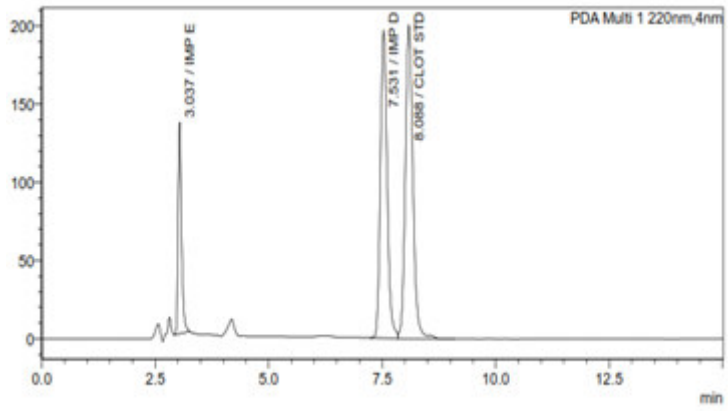

Fig 10.Precision Chromatogram

The % RSD for peak area and retention time for all the impurities and Clotrimazole in the six replicate injections were calculated and summarized in Table 2.

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

LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantisation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Limit of Detection (LOD)

LOD can be defned as the detection of the least amount of the analyte.

1. Based on Signal-to-Noise for LOD (3:1), LOQ (10:1)
2. Based on the Standard Deviation of the Response and the Slope

Limit of Quantization (LOQ)

LOQcan be quantitatively determined with suitable precision and accuracy using the following formula.

$$LOD = \frac{3.3\,\sigma}{S}, \quad LOQ = \frac{10\,\sigma}{S}$$

σ = standard deviation of the response
S = slope of the calibration curve of the analyte

A solution containing 0.015 % (test concentration) of impurities and Clotrimazole was prepared and injected in six replicates to demonstrate that impurities and Clotrimazole peak is quantifiable at 0.015 % level. Results were summarized in the table-3.

Table 3. LOD and LOQ data

Name of the solution	LOQ		LOD	
	Conc (%)	S/N	Conc (%)	S/N
Clotrimazole	0.013	9.46	0.00429	2.57
Impurity-D	0.0013	10.45	0.000429	2.87
Impurity-E	0.0013	9.54	0.000429	2.24

3.3 Precision at LOQ

The precision at LOQ expresses the precision under the same operating conditions over a short interval of time at LOQ level and it was determined by analyzing six replicate injections of LOQ solution. Results of peak area of Clotrimazole (Fig 9) and its known impurities are summarized in table 4.

Table 4. Precision at LOQ level

Name of the solution	Area of Clotrimazole	Area of Imp-D	Area of Imp-E
LOQ Solution inj-1	3990	8041	10902
LOQ Solution inj-2	3243	8136	9952
LOQ Solution inj-3	3522	8588	10444
LOQ Solution inj-4	3177	8228	9544
LOQ Solution inj-5	3659	8223	10595
LOQ Solution inj-6	3802	8377	10542
Mean Area	**3566**	**8266**	**10330**
STDEV	**316.77**	**193.30**	**493.16**
% RSD (NMT 10)	**8.9**	**2.3**	**4.8**

The % RSD is less than 10 % for the peak area shows that the above method is capable of quantification of impurities 0.0150 % level. A solution containing 0.0050 % (. test concentration) of impurities and Clotrimazole was prepared and injected in duplicate to demonstrate that Clotrimazole peak is detectable at 0.0045 % level. The detection of Clotrimazole and impurities peak in two replicate injections show that the above method is capable of detecting impurities 0.0050 % level.

3.4 Linearity

Linearity can be checked by injecting different concentrations of a analyte. Linearity of Clotrimazole and impurities are established by injecting different concentrations in the range of 50 % to 150 % (Table-5).

Table 5. Linearity data

Name of the solution	Clotrimazole		Imp-D		Imp-E	
	Conc. (%)	Area	Conc. (%)	Area	Conc. (%)	Area
LOQ Level	0.013	3617	0.0013	8089	0.0013	10427
50% Level	0.0254	6134	0.0253	167051	0.0254	228794
75% Level	0.0381	9163	0.0379	247047	0.0381	336896
100% Level	0.0508	12337	0.0505	334108	0.0507	455280
125% Level	0.0635	15299	0.0632	418357	0.0634	567512
150% Level	0.0762	18168	0.0758	489022	0.0761	666630
Y-Intercept	**396.2**		**1528.1**		**22221.9**	
Slope	**233481.36**		**6519789.6**		**8832176.5**	
r^2	**0.9992**		**0.9992**		**0.9995**	

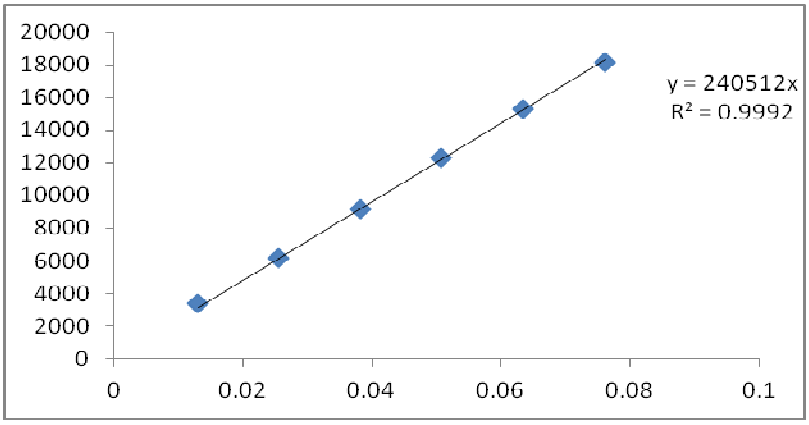

Fig 11.Linearity Plot for Clotrimazole

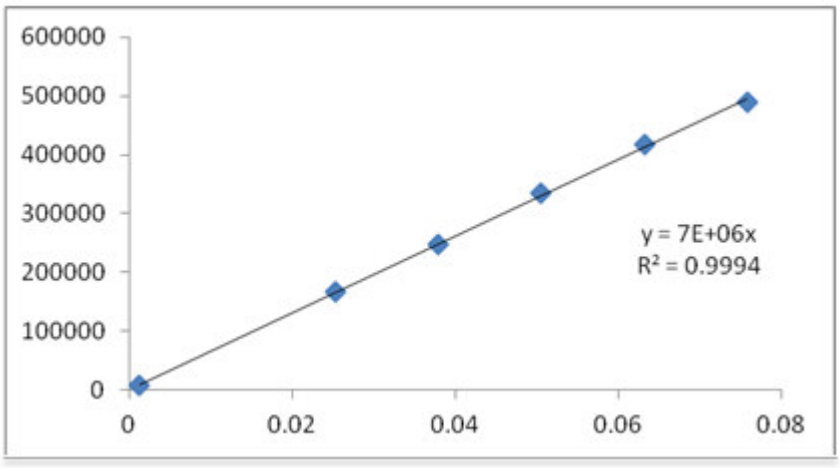

Fig 12.Linearity Plot for Imp-D

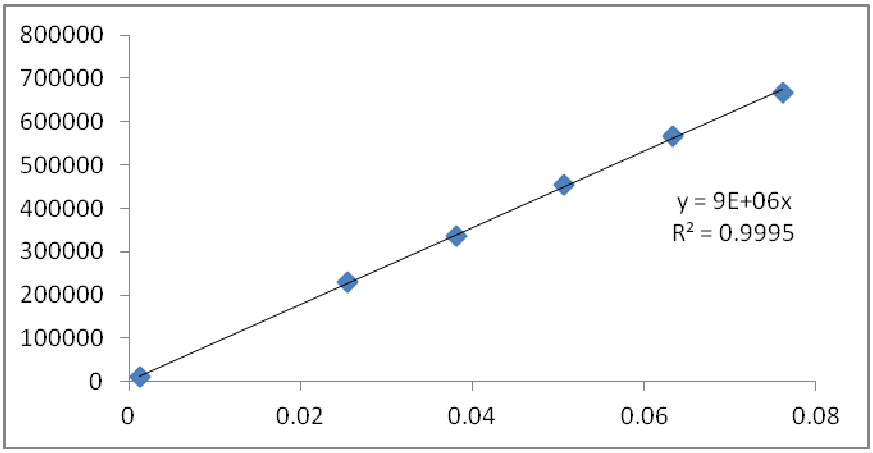

Fig: 13 Linearity Plot for Imp-E

The correlation coefficient is calculated from a plot of concentration versus peak area by regression analysis using the line of best fit. The % R.S.D for clotrimazole and impurities (D and E) were found to be 0.9992, 0.9994 and 0.9995 which are within limits. [Fig.10-12]

3.5 Accuracy

Accuracy is the measurement of exactness of the analytical method which is determined by adding a known amount of each analyte to pre-analysed sample and calculating the percent recovery of each of the impurity from the spiked solution. Each impurity spiked in the sample solution and injected in two replicates. Percentage recovery has been calculated from recovered amount of impurities. Results are summarized in table-6-8.

Table 6. Accuracy – Clotriamazole (LOQ -150 %)

Sample ID	Area	Amount Added (% w/w)	Amount Found (% w/w)	% Recovery	Mean (% Recovery)
50% Level Sample-1	225960	0.0252	0.0253	100.4	101.6
50% Level Sample-2	229706		0.0257	102.0	
50% Level Sample-3	229552		0.0258	102.4	
100% Level Sample-1	450662	0.0504	0.0503	99.8	100.0
100% Level Sample-2	450663		0.0505	100.2	
100% Level Sample-3	451031		0.0504	100.0	
150% Level Sample-1	675998	0.0755	0.0755	100.0	100.0
150% Level Sample-2	675936		0.0754	99.9	
150% Level Sample-3	675319		0.0755	100.0	

Table 7. Accuracy – Impurity-D (LOQ -150%)

Sample ID	Area	Amount Added (% w/w)	Amount Found (% w/w)	% Recovery	Mean (% Recovery)
50% Level Sample-1	170735	0.0251	0.0256	102.0	102.3
50% Level Sample-2	171296		0.0257	102.4	
50% Level Sample-3	170992		0.0257	102.4	
100% Level Sample-1	339049	0.0502	0.0507	101.0	101.0
100% Level Sample-2	338642		0.0508	101.2	
100% Level Sample-3	337706		0.0506	100.8	
150% Level Sample-1	506081	0.0753	0.0757	100.5	100.7
150% Level Sample-2	506857		0.0758	100.7	
150% Level Sample-3	506618		0.0759	100.8	

Table 8. Accuracy – Impurity-E (LOQ - 150 %)

Sample ID	Area	Amount Added (% w/w)	Amount Found (% w/w)	% Recovery	Mean (% Recovery)
50% Level Sample-1	336095	0.0252	0.0266	105.6	106.1
50% Level Sample-2	337108		0.0268	106.3	
50% Level Sample-3	336644		0.0268	106.3	
100% Level Sample-1	494621	0.0504	0.0515	102.2	102.3
100% Level Sample-2	494682		0.0517	102.6	
100% Level Sample-3	493699		0.0514	102.0	
150% Level Sample-1	645473	0.0756	0.0754	99.7	99.3
150% Level Sample-2	645960		0.0753	99.6	
150% Level Sample-3	645387		0.0755	98.7	

System met the system suitability criteria. The % recovery for impurity-D, impurity-E, impurity-C, impurity-D and impurity-E are within the limit between 70 and 130% for LOQ level and between 80 and 120% for 50 to 150% level. Hence the method is accurate for the quantification of related substances in clotrimazole.

3.6 Robustness

Robustness of the method is performed by altering the chromatographic conditions such as the pH of the buffer, Wavelength, Mobile phase composition and observed the variation of the results which should be within the acceptance criteria. For Clotriamazole, the obtained values % RSD was found to be within the range of 0.43 %-0.86 % which states the method is acceptable. For Impurity-D the obtained values % RSD was found to be within the range of 0.74 %-1.05 % which states the method is acceptable. For Impurity-E, the obtained values % RSD was found to be within the range of 0.58 %-0.95 % which states the method is acceptable.

4. DISCUSSION

In HPLC method, the conditions were optimized to obtain, an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to get optimum results. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, and capacity factor), run time etc.Themethd was quantified and validated as per International Conference on Harmonization Guideline on Validation of Analytical Procedures. Text and Methodology, Q2 (R1). 2005 and International Conference on Harmonization Guideline on Stability Testing of New Drug Substances and Products; Q1 A (R2). 2003 [17,18] The system with Buffer and Solvent Mixture with 0.8 ml/min flow rate (flow rate discrepancy with the flow rate mentioned in abstract) is quite robust.The optimum wavelength for detection was at 220 nm for the determination. The linearity was observed in the range of 50-150 % for Clotrimazole and its related substances with a correlation coefficient of nearer to 1.00 respectively. The low values of % R.S.D. indicate the method is precise and accurate. The mean recoveries were

found in the range of 80-120 %.[19]In Selectivity, no interference should be observed at the retention time of all known impurities and Clotrimazole peaks. Peak purity of Clotrimazole and known impurity peaks should pass for the individual solutions and spiked solution. In system precision and method, precision %RSD less than 10.0.LOQ &LOD S/N ratio was found to be 9.46 and 2.57, 10.45 and 2.87, 9.54 and 2.24 for Clotrimazole, IMP-D and IMP-E, respectively. The correlation coefficient is more than 0.99 for Clotrimazole, impurity-D and impurity-E. The percentage y-intercept to average response of 100 % standard below the limit (±5 %) for each analyte. Hence, the method is linear for related substances in Clotrimazole and impurities. In robustness, the obtained values % RSD was found to be within the range of 0.43 %-1.05 % which states the developed method was robust to changes in chromatographic conditions. [17,18]

5. CONCLUSION

The method includes development and validation of RP-HPLC method for simultaneous estimation of Clotrimazole and its impurities (D and E) in bulk mixture. The method was validated using various parameters like linearity, precision, accuracy, robustness etc. and the method was found to be specific, accurate, precise, repeatable and reproducible.

6. AUTHORS CONTRIBUTION STATEMENT

Ms. P. Abhinandana has conceptualized, designed and executed current research work. Dr. Rama RaoNadendla has supervised the work.

7. ACKNOWLEDGEMENT

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8. CONFLICT OF INTEREST

Conflict of interest declared none.

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