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ANALYTICAL METHODS DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF PREDNISOLONE AND ITRACONAZOLE IN SYNTHETIC MIXTURE

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ABSTRACT

The objective of this study was to develop and validate a precise, accurate, and reliable RP-HPLC method for the simultaneous estimation of Prednisolone and Itraconazole in a synthetic mixture according to ICH guidelines. Identification tests such as melting point, solubility, FT-IR, and UV analysis were performed for both drugs. In the RP-HPLC method, a mobile phase consisting of acetonitrile, phosphate buffer, and methanol was used, providing well-resolved peaks with suitable retention times. The method was developed and optimized for effective separation and analysis. The method showed excellent linearity, precision, and accuracy, with a correlation coefficient close to 1. Recovery studies were within acceptable limits, and low LOD and LOQ values indicated high sensitivity. The percentage assay value was close to 100%, confirming the reliability of the method. No interference from excipients was observed. The developed RP-HPLC method was found to be accurate, precise, economical, and reproducible. This method is suitable for routine quality control analysis of Prednisolone and Itraconazole in synthetic mixtures.

KEYWORDS: Prednisolone, Itraconazole, Synthetic Mixture, and RP-HPLC Methods Development and Validation.

INTRODUCTION[1-4]

Bronchial asthma is a chronic inflammatory disorder of the airways characterized by reversible airflow obstruction and bronchial hyperresponsiveness. Additionally, aspergillosis is a fungal infection caused by *Aspergillus* species, which primarily affects the lungs and may lead to allergic bronchopulmonary aspergillosis (ABPA). The management of such conditions often requires a combination of corticosteroids and antifungal agents for effective treatment.

Prednisolone is a corticosteroid that reduces inflammation and suppresses immune response, making it effective in the treatment of asthma and allergic conditions. Itraconazole is a triazole antifungal drug used for the treatment of various fungal infections including aspergillosis. The combination of these drugs has been found to be more effective in reducing exacerbation rates in ABPA compared to monotherapy.

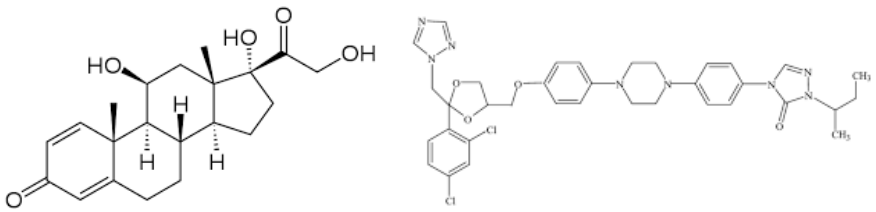

Fig. 1: Structure of Prednisolone and Itraconazole.[5,6]

Literature review reveals that various analytical methods such as RP-HPLC[7-12] have been reported for individual drugs. However, no validated method has been reported for the simultaneous estimation of Prednisolone and Itraconazole in synthetic mixture.

Therefore, the present study aims to develop and validate simple, precise, and accurate RP-HPLC methods for simultaneous estimation of Prednisolone and Itraconazole in synthetic mixture, suitable for routine quality control analysis.

MATERIALS AND METHODS

Instruments and apparatus

- A Systronic RP-HPLC (LC-20-AD) (SPD-20 A) Instrument [Clarify]
- Column: Kromstar C_{18} (250 × 4.6 mm, 5 µm)
- Digital Analytical Balance: Wensa r DA 13-220 (India)
- pH meter (Systronics, Naroda, Ahmedabad)

- Sonicator (Equitron, India)
- Volumetric flask: 10, 50 and 100 ml (Borosil)
- Pipettes: 1, 2, 5 and 10 ml (Borosil)
- Beaker: 50, 100 and 150 ml (Borosil)
- Hamilton syringe

Chemicals and Materials

- Acetonitrile, Methanol and Water (HPLC grade) (Finar Chemicals Pvt. Ltd., India)
- Ortho Phosphoric Acid and Methanol (AR Grade) (Astron Chemical India)
- Prednisolone (Zydus Pharmaceuticals, Ahmedabad)
- Itraconazole (Resonant Pharmaceuticals Pvt. Ltd., Kheda)

Selection of Detection Wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. At 240 nm both drugs gave remarkable absorbance, good peak height and shape. So, 240 nm was selected for simultaneous estimation of Prednisolone and Itraconazole in synthetic mixture.

Mobile phase selection

The composition and flow rate of mobile phase were changed to optimize the separation condition using combined solution. After number of trial experiments, it was established that the mobile phase ACN: Phosphate Buffer: Methanol (pH 3.2 adjusted with 10% ortho phosphoric acid) (40:35:25 %v/v/v) shows good peak shape and resolution.

Chromatographic condition

Column: Kromstar C_{18} (250 mm × 4.6 mm, 5 µm)

Mobile phase: ACN: Phosphate Buffer: Methanol (pH 3.2 adjusted with 10% ortho phosphoric acid) (40:35:25 %v/v/v)

Flow rate: 1 ml/min

Run time: 10 min

Detection wavelength: 240 nm

Detector: U.V Detector

Injection volume: 20 µL

Syringe: Hamilton.

Preparation of Mobile phase

- **Preparation of 10% Orthophosphoric acid:** 10% ortho phosphoric acid was prepared by diluting 1.0 ml of concentrated ortho phosphoric acid in 10 ml HPLC grade water.

- **Preparation of buffer (10 mM phosphate buffer):** Accurately weighed 0.272 gm potassium dihydrogen phosphate (KH_2PO_4) was transferred it in 200 ml HPLC grade water and allowed it to dissolve. It was filtered through 0.45 µm membrane filter and sonicated for about 10 min. Buffer pH was adjusted to 3.2 with 10% ortho phosphoric acid.

- **Preparation of Mobile phase:** ACN: Phosphate Buffer: Methanol (pH 3.2 adjusted with 10% ortho phosphoric acid) (40:35:25 %v/v/v) Mobile phase was used after filtered it through 0.45 µm membrane filter and sonication.

Preparation of standard stock solution

- **Prednisolone (100 µg/ml)**

Accurately weighed Prednisolone (10 mg) was transferred to a 100 ml volumetric flask, and diluted up to the mark with mobile phase to obtain a standard stock solution (100 µg/ml).

- **Itraconazole (1000 µg/ml)**

Accurately weighed Itraconazole (100 mg) was transferred to a 100 ml volumetric flask, and diluted up to the mark with mobile phase to obtain a standard stock solution (1000 µg/ml).

Preparation and analysis of synthetic mixture

- The synthetic mixture of Prednisolone and Itraconazole was prepared in the ratio of 0.5:260.
- Accurately weighed equivalently weight of Prednisolone (0.5 mg) and Itraconazole (260 mg) and transferred in 100 ml volumetric flask and allow to sonicate and made up to mark with Methanol.
- Common excipients such as MCC (Micro Crystalline Cellulose) (2.37 mg), Lactose Monohydrate (27.65 mg), Polyvinyl Pyrrolidine (7.9 mg), Talc (0.79 mg) and Magnesium Stearate (0.79 mg) were added in the motor pestle along with the drug Prednisolone (0.5 mg) and Itraconazole (260 mg).
- This solution was filtered through Whatmann filter paper. The filtrate was diluted to the mark with Methanol. The mixture contains 5 µg/ml of Prednisolone and 2600 µg/ml of Itraconazole.

Preparation of sample solution

- Accurately 0.4 ml of the above mixture solution of (Prednisolone 5 µg/ml and Itraconazole 2600 µg/ml) was pipetted out into 10 ml volumetric flask and the volume was adjusted up to the mark with Methanol.
- Final concentration of Prednisolone was 0.2 µg/ml and Itraconazole 104 µg/ml.

Method Validation

The developed method was validated with respect to specificity, linearity, range, accuracy, precision, limit of detection and limit of quantification in accordance with the ICH Q2 R2 guideline.

a. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc.

b. Linearity & Range

The linearity of Prednisolone and Itraconazole was found to be in the range of 0.1-0.5 µg/ml and 52-260 µg/ml, respectively. Plot the calibration curve of Peak area Vs Concentration (µg/ml). Linearity of both the drugs was checked in term of slope, intercept and correlation coefficient.

Preparation of calibration curve

Aliquots of stock solution of Prednisolone (100 µg/ml) (0.01, 0.02, 0.03, 0.04 and 0.05 ml and Itraconazole (1000 µg/ml) 0.52, 1.04, 1.56, 2.08 and 2.60 ml were pipetted out in five different 10 ml volumetric flasks and further diluted with mobile phase to obtain the concentration of about 0.1, 0.2, 0.3, 0.4 and 0.5 ml µg/ml for Prednisolone and 52, 104, 156, 208 and 260 ml µg/ml for Itraconazole. 20 µL of each solution were injected into RP-HPLC system by Hamilton syringe and analysed. Calibration curve was obtained by plotting respective Peak area Vs Concentration in µg/ml and Regression equation was obtained.

c. Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels.

1) Intraday Precision: Solutions containing 0.1, 0.2, 0.3 µg/ml of Prednisolone and 52, 104, 156 µg/ml of Itraconazole were analyzed three times on the same day and % R.S.D was calculated.

2) Interday Precision: Solutions containing 0.1, 0.2, 0.3 µg/ml of Prednisolone and 52, 104, 156 µg/ml of Itraconazole were analyzed on three different successive days and % R.S.D was calculated.

3) Repeatability: Solutions containing 0.2 µg/ml of Prednisolone and 104 µg/ml of Itraconazole were analyzed for six times and % R.S.D. was calculated.

d. Limit of Detection (LOD)

Limit of detection can be calculated using following equation as per ICH guidelines.

LOD = 3.3 x (σ/ S)

where, σ = standard deviation of the Y intercept of calibration curve

S = Mean slope of the corresponding calibration curve.

e. Limit of Quantification (LOQ)

Limit of quantification can be calculated using following equation as per ICH guidelines.

LOQ = 10 x (σ/ S)

where, σ = standard deviation of the Y intercept of calibration curve

S = Mean slope of the corresponding calibration curve.

f. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy of the developed method was confirmed by doing recovery study as per ICH guideline at three different concentration levels 50%, 100%, 150% and the values were measured for **Prednisolone (0.2 µg/ml) and Itraconazole (104 µg/ml)**. This performance was done in triplicate.

The amounts of Prednisolone and Itraconazole were estimated by applying obtained values to the respective regression line equations. The experiment was repeated for 3 times (n=3).

g. Robustness

- The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an

indication of its reliability during normal usage.

- In case of liquid chromatography, examples of typical variations are:

- Influence of variations of pH in mobile phase;
- Influence of variations in mobile phase composition;
- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate.

h. System suitability tests

- A system suitability test is an integral part of liquid chromatography.
- They are used to verify that resolution and reproducibility of chromatography system are adequate for the analysis to be done.
- The test includes the Resolution, Column efficiency, Tailing factor and Theoretical plates.

RESULTS AND DISCUSSION

Selection detection wavelength

- The sensitivity of RP-HPLC method that uses UV detection depends upon proper selection of detection wavelength. At 240 nm both drugs give good peak height and shape. So, 240 nm was selected for simultaneous estimation of Prednisolone and Itraconazole in synthetic mixture.
- Overlain UV spectra of Prednisolone (0.2 µg/ml) and Itraconazole (104 µg/ml) in Methanol has been shown in Figure 2.

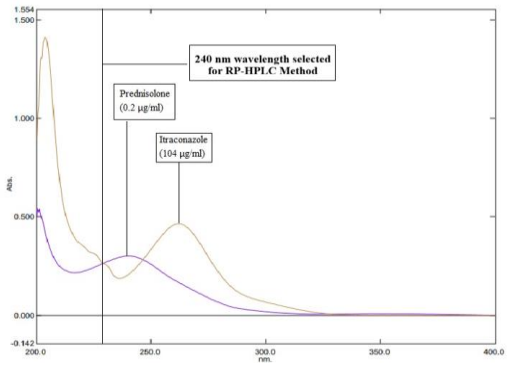

Figure 2: Overlain UV Spectra of Prednisolone (0.2 µg/ml) and Itraconazole (104 µg/ml).

Optimization of Chromatographic conditions

The mobile phase Acetonitrile: Phosphate Buffer: Methanol (pH 3.2 adjusted with 10% ortho

phosphoric acid) (40:35:25 %v/v/v) was selected because it was found to ideally resolve the peaks with retention time 3 min and 7 min for Prednisolone and Itraconazole, respectively. Kromstar C_{18} (250×4.6 mm, 5 µm) column was used for separation of Prednisolone and Itraconazole with flow rate of 1.0 ml/min at 240 nm.

Table 1: Mobile phase optimization trials for Prednisolone and Itraconazole.

Trial	Mobile Phase	Ratio (%v/v)	Remark
1	Methanol: Water at 240 nm	70:30	No peak was obtained
2	ACN: Water at 240 nm	60:40	One peak was obtained but peak shape was not good
3	ACN: Phosphate Buffer (pH 4) at 240 nm	55: 45	Only one peak was obtained
4	ACN: Phosphate Buffer: Methanol (pH 4) at 240 nm	50: 40:10	Two peaks were obtained; One Sharp peak was observed but one peak shape was not proper
5	ACN: Phosphate Buffer: Methanol (pH 3.2) at 240 nm	45: 35:20	Peak separation was not proper
6	ACN: Phosphate Buffer: Methanol (pH 3.2) at 240 nm	40:35:25	**Good resolution and Sharp peak was observed. Rt: PRED: 3 min ITRA: 7 min**

Where, Phosphate Buffer = Potassium dihydrogen Ortho phosphate buffer

Trial 1:

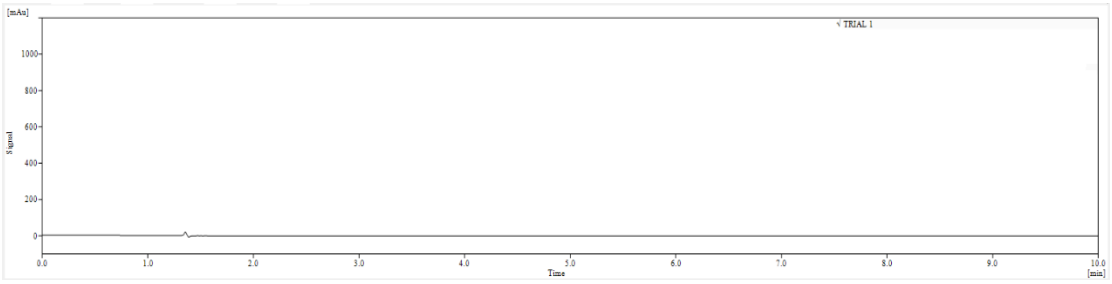

Figure 3: RP-HPLC Chromatogram of Prednisolone (0.2 µg/ml) and Itraconazole (104 µg/ml) in Methanol: Water (70:30%v/v) at 240 nm {Run time: 10 min, Flow rate: 1 ml/min}.

Trial 2:

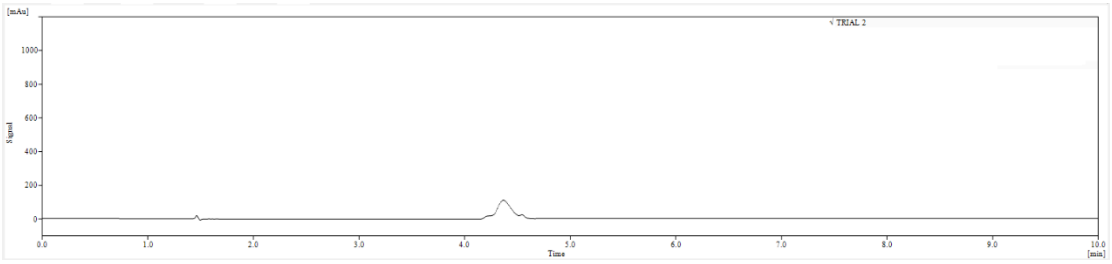

Figure 4: RP-HPLC Chromatogram of Prednisolone (0.2 µg/ml) and Itraconazole (104 µg/ml) in ACN: Water (60:40 %v/v) at 240 nm {Run time: 10 min, Flow rate: 1 ml/min}.

Trial 3:

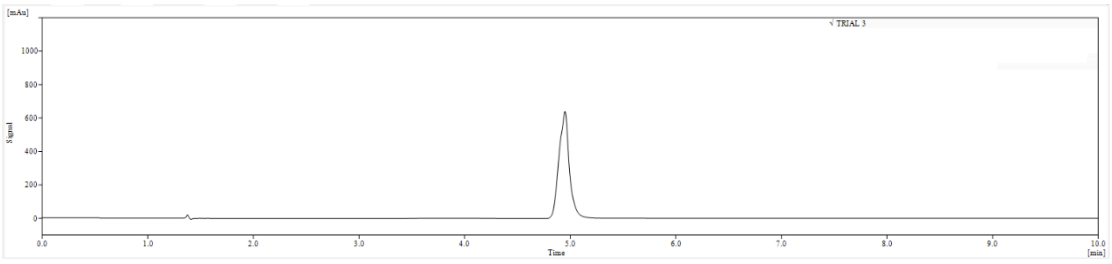

Figure 5: RP-HPLC Chromatogram of Prednisolone (0.2 µg/ml) and Itraconazole (104 µg/ml) in ACN: Phosphate Buffer (pH 4) (55:45 %v/v) at 240 nm {Run time: 10 min, Flow rate: 1 ml/min}.

Trial 4:

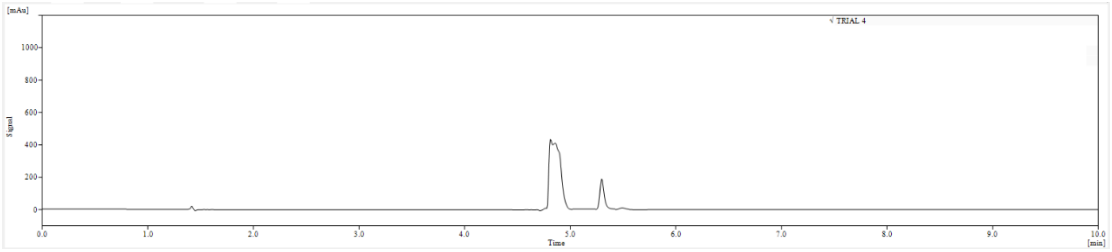

Figure 6: RP-HPLC Chromatogram of Prednisolone (0.2 µg/ml) and Itraconazole (104 µg/ml) in ACN: Phosphate Buffer: Methanol (pH 4) (50: 40:10 %v/v/v) at 240 nm {Run time: 10 min, Flow rate: 1 ml/min}.

Trial 5:

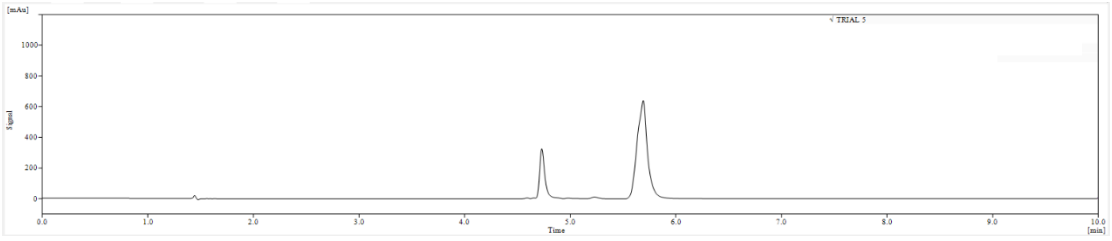

Figure 7: RP-HPLC Chromatogram of Prednisolone (0.2 µg/ml) and Itraconazole (104 µg/ml) in ACN: Phosphate Buffer: Methanol (pH 3.2 adjusted with 10% ortho phosphoric acid) (45: 35:20 %v/v/v) at 240 nm {Run time: 10 min, Flow rate: 1 ml/min}.

Trial 6:

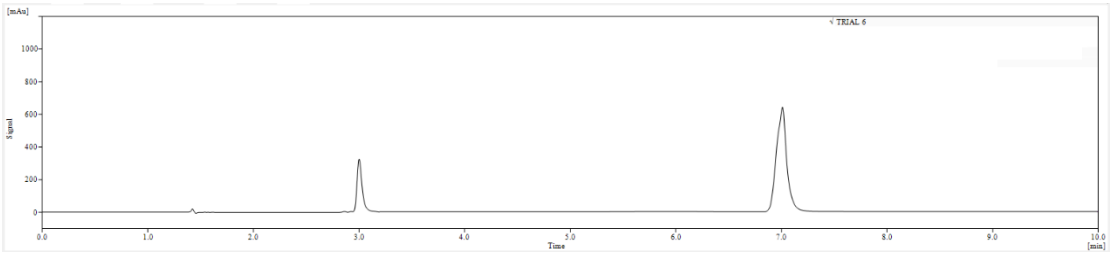

Figure 8: RP-HPLC Chromatogram of Prednisolone (0.2 µg/ml) and Itraconazole (104 µg/ml) in Acetonitrile: Phosphate Buffer: Methanol (pH 3.2 adjusted with 10% OPA) (40:35:25 %v/v/v) at 240 nm {Run time: 10 min, Flow rate: 1 ml/min}.

Final Chromatogram

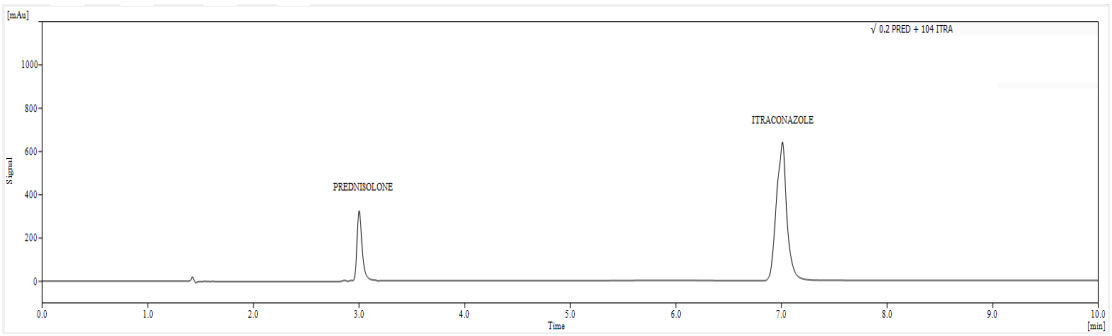

Figure 9: RP-HPLC Chromatogram of Prednisolone (0.2 µg/ml) and Itraconazole (104 µg/ml) in Acetonitrile: Phosphate Buffer: Methanol (pH 3.2 adjusted with 10% ortho phosphoric acid) (40:35:25 %v/v/v) at 240 nm {Run time: 10 min, Flow rate: 1 ml/min}.

- System suitability parameter has been shown in Table 2.

Table 2: System suitability parameter.

Parameters	Retention Time	Tailing Factor	Number of Theoretical plate	Resolution
Prednisolone	3 min	0.8	9948	4.0
Itraconazole	7 min	1.4	7813	

Method Validation of RP-HPLC method

a. Specificity

- Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.
- Typically, these might include impurities, degradants, matrix, etc. It was proved by comparing the chromatogram of mobile phase, test preparation solution to show that there was no interference of mobile phase and excipients peaks with peak of Prednisolone and Itraconazole shown in figure.

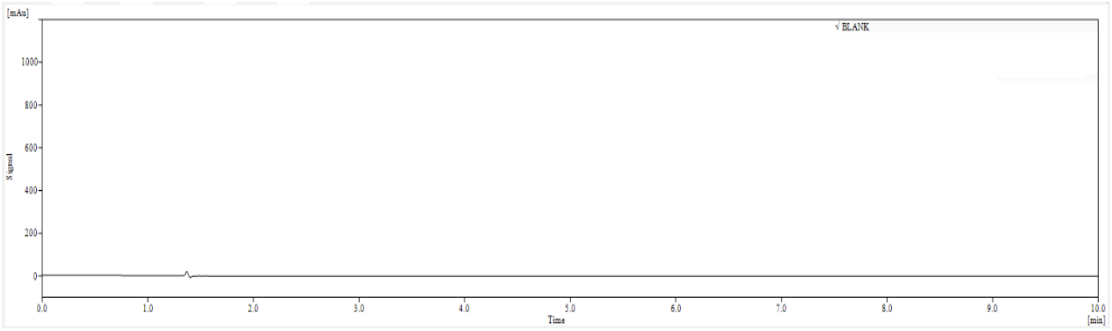

Figure 10: Blank RP-HPLC Chromatogram in Acetonitrile: Phosphate Buffer: Methanol (pH 3.2 adjusted with 10% ortho phosphoric acid) (40:35:25 %v/v/v) at 240 nm {Run time: 10 min, Flow rate: 1 ml/min}.

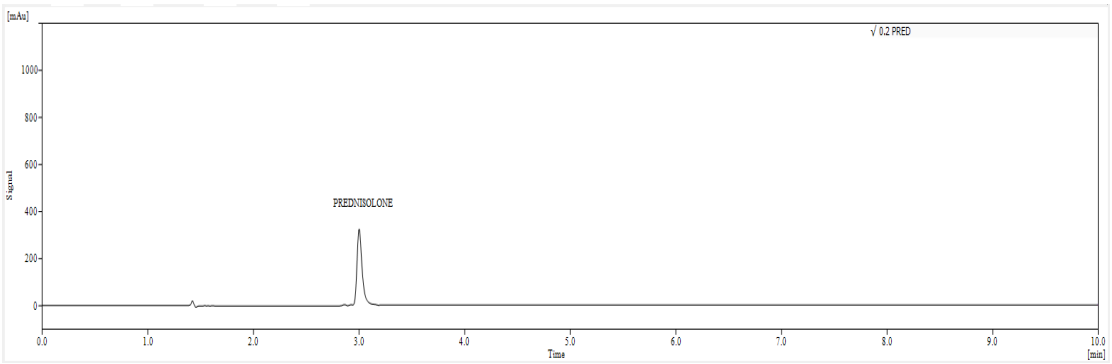

Figure 11: RP-HPLC Chromatogram of Prednisolone (0.2 µg/ml).

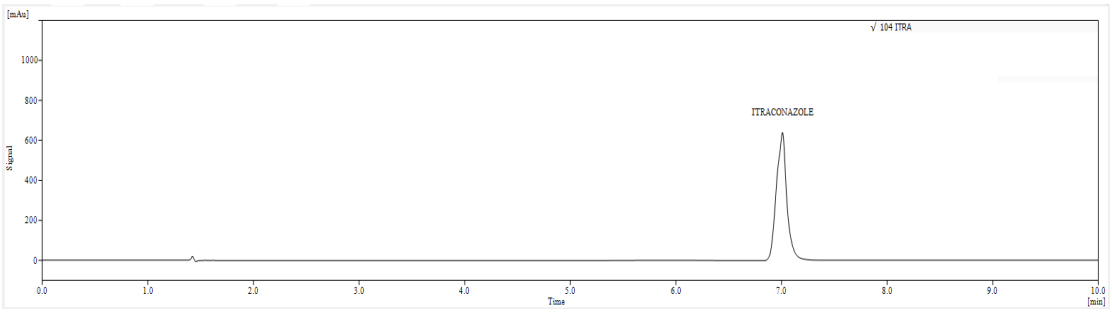

Figure 12: RP-HPLC Chromatogram of Itraconazole (104 µg/ml)}.

b. Linearity

The linearity of Prednisolone and Itraconazole was found to be 0.1-0.5 µg/ml and 52-260 µg/ml, respectively.

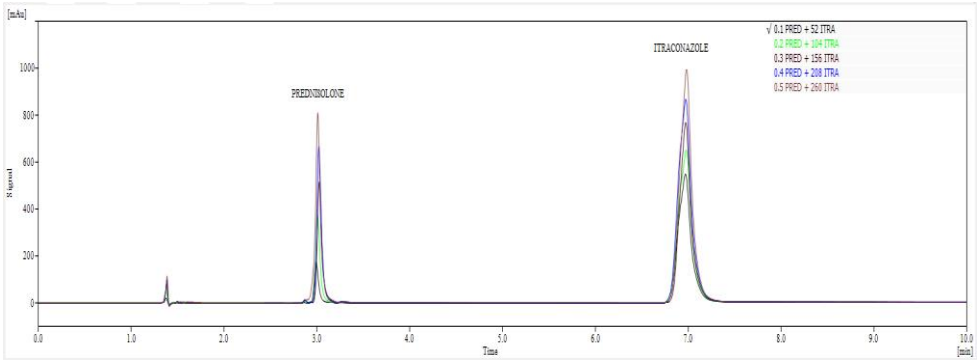

Figure 13: Overlain RP-HPLC Chromatogram of Prednisolone (0.1-0.5 µg/ml) and Itraconazole (52-260 µg/ml)}.

Table 3: Calibration data for Prednisolone (0.1-0.5 µg/ml) and Itraconazole (52-260 µg/ml).

Sr. No	Concentration (µg/ml)		Mean Peak area (mAu*sec) ± S. D. (n=6)		% RSD	
	PRED	ITRA	PRED	ITRA	PRED	ITRA
1	0.1	52	10715.0±55.157	15536.3±111.058	0.51	0.71
2	0.2	104	17210.5±71.976	26306.8±127.293	0.42	0.48
3	0.3	156	24899.3±57.854	36386.5±54.579	0.23	0.15
4	0.4	208	31544.5±39.816	46135.7±43.117	0.13	0.09
5	0.5	260	39142.8±33.581	55814.1±9.095	0.09	0.02

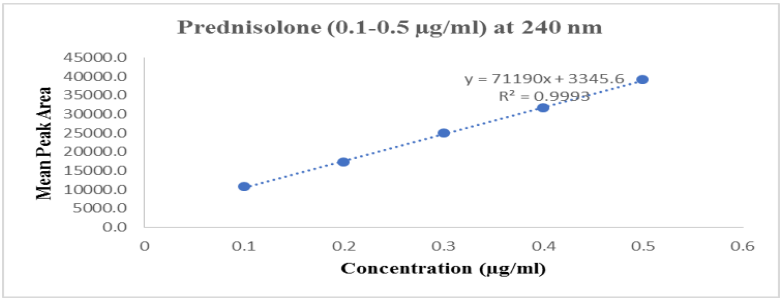

Figure 14: Calibration curve of Prednisolone (0.1-0.5 µg/ml) at 240 nm.

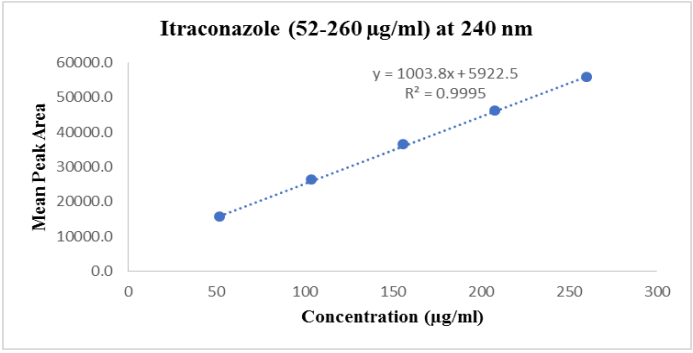

Figure 15: Calibration curve of Itraconazole (52-260 µg/ml) at 240 nm.

c. Precision

Table 4: Precision study for Prednisolone.

Prednisolone (240 nm)		
Intraday precision of Prednisolone		
Conc. (µg/ml)	**Mean peak area (mAu*sec) ± S.D (n=3)**	**% RSD**
0.1	10709.2 ± 51.1609	0.48
0.2	17199.3 ± 67.4983	0.39
0.3	24907.2 ± 55.3993	0.22
Interday precision of Prednisolone		
Conc. (µg/ml)	**Mean peak area (mAu *sec) ± S.D (n=3)**	**% RSD**
0.1	10711.6 ±54.0701	0.50
0.2	17198.3±68.7829	0.40
0.3	24910.9±59.9471	0.24
Repeatability of Prednisolone		
Conc. (µg/ml)	**Mean peak area (mAu *sec) ± SD (n=6)**	**% RSD**
0.2	17208.0±69.8353	0.41

Table 5: Precision study for Itraconazole.

Itraconazole (240 nm)		
Intraday precision of Itraconazole		
Conc. (µg/ml)	**Mean peak area (mAu*sec) ±SD (n=3)**	**% RSD**
52	15545.6±105.6976	0.68
104	26316.9±120.0998	0.46
156	36339.9±35.2784	0.10
Interday precision of Itraconazole		
Conc. (µg/ml)	**Mean peak area (mAu *sec) ±SD (n=3)**	**% RSD**
52	15544.6±106.8787	0.69
104	26312.9±124.8859	0.47
156	36332.2±40.0101	0.11
Repeatability of Itraconazole		
Conc. (µg/ml)	**Mean peak area (mAu *sec) ±SD (n=6)**	**% RSD**
104	26309.0±128.528	0.49

d. Accuracy (Recovery study)

Table 6: Recovery study data.

Name of Drug	%Level Of Recovery	Test Amount (µg/ml)	Amount of drug Spiked (µg/ml)	Total Std Amount (µg/ml)	Total amount Recovered (µg/ml)	% Recovery ±S. D (n=3)
Prednisolone	50	0.2	0.1	0.3	0.2997	99.90±0.1231
	100	0.2	0.2	0.4	0.3997	99.92±0.2853
	150	0.2	0.3	0.5	0.4998	99.96±0.1534
Itraconazole	50	104	52	156	155.97 9688	99.98±0.0246
	100	104	104	208	207.98	99.99±0.0116
	150	104	156	260	260.02	100.01±0.0631

e. LOD and LOQ data

Table 7: LOD and LOQ data.

Drug Name	Prednisolone	Itraconazole
LOD (µg/ml)	0.003	0.365
LOQ (µg/ml)	0.008	1.106

f. Analysis of synthetic mixture

Table 8: Analysis of synthetic mixture.

Drug Name	Amount in synthetic mixture (µg/ml)	Amount found (µg/ml)	% Assay ± S.D. (n=3)
Prednisolone	0.2	0.1998	99.90±0.6789
Itraconazole	104	103.958	99.96±0.5142

g. Robustness

Table 9: Robustness data.

Condition	Variation	Prednisolone	Itraconazole
		% Assay ± SD (n=3)	% Assay ± SD (n=3)
Flow rate (1 ml ± 0.2 ml/ min)	0.8 ml/min	99.91±5.6624	99.54±5.7038
	1.0 ml/min	99.86±7.1345	99.68±7.4567
	1.2 ml/min	99.75±9.7370	99.92±8.2661
Detection wavelength (240 nm ± 2 nm)	242	99.63±6.1368	99.91±3.4674
	240	99.98±4.5267	99.45±5.2855
	244	99.87±8.1256	99.76±3.3621
Mobile Phase Acetonitrile: Phosphate Buffer: Methanol (pH 3.2) (40:35:25 ±2 %v/v/v)	38:32:30	99.51±4.0831	99.50±6.1769
	40:35:25	99.38±3.9732	99.95±7.5056
	42:38:20	99.93±1.0937	99.98±9.5445

Table 10: Summary of Validation Parameters.

Sr. No.	Parameters	Prednisolone	Itraconazole
1	Detection wavelength (nm)	240 nm	
2	Linearity Range (µg/ml)	0.1-0.5	52-260
3	Regression equation (y = mx +c)	y = 71190x + 3345.6	y = 1003.8x + 5922.5
4	Correlation Coefficient (r^2)	0.9993	0.9995
5	Intraday Precision (%RSD, n=3)	0.22-0.48	0.10-0.68
6	Interday Precision (% RSD, n=3)	0.24-0.50	0.11-0.69
7	Repeatability (% RSD, n=6)	0.41	0.49
8	Accuracy (% Recovery, n=3)	99.99%-99.96%	99.98%-100.01%
9	LOD (µg/ml)	0.003	0.365
10	LOQ (µg/ml)	0.008	1.106
11	% Assay	99.90%	99.96%

CONCLUSION

A rapid, sensitive, accurate and precise RP-HPLC method has been developed and validated for routine analysis of Prednisolone and Itraconazole in Synthetic mixture. The RP-HPLC method is suitable for simultaneous estimation of Prednisolone and Itraconazole in Synthetic mixture in without interference of each other. The developed method was successfully applied in Synthetic mixture. The proposed method can be utilized for the routine analysis of Prednisolone and Itraconazole in Synthetic mixture.

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