Development and Validation of the RP-HPLC Method for Cis- Bromo Benzoate Determination in Itraconazole API and its Pharmaceutical Dosage forms

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Abstract

An Isocratic RP-HPLC method was developed for the quantitative determination of Cis-Bromobenzoate (CBB) in Itraconazole API and its pharmaceutical formulations.The baseline separation for Itraconazole and Cis-Bromobenzoate was achieved by utilizing a Symmetry C18 (100 mm × 4.6 mm) 3.5 µm column particle size and an Isocratic elution method. The mobile phase contains a mixture of 0.1% formic acid in Water and Acetonitrile in the ratio of 35:65 v/v, respectively. The flow rate of the mobile phase was 1.0 ml/min with a column temperature of 25°C and detection wavelength at 227 nm.Themethod was validated for limit of detection, limit of quantification, linearity, accuracy and reproducibility with the help of the exhibit and simulated samples. The limit of detection for CBB was 0.30 µg/mL respectively. The limit of quantification (LOQ) for CBB was 0.92 µg/mL respectively. The correlation coefficient obtained for impurity was > 0.999. The recovery was obtained for impurity was 100±10%. The developed method was validated as per ICH guidelines with respect to specificity, precision, linearity, Accuracy, limit of detection and quantification, Ruggedness, robustness and Solution stability.

Keywords: *Itraconazole, Cis-Bromobenzoate (CBB), RP- HPLC, Method development and validation.*

I. INTRODUCTION

Itraconazole (figure.1) is a 4-(4-(4-(4-(((2R,4S)-2-((1H-1,2,4-triazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)methoxy)phenyl)piperazin-1-yl)phenyl)-1-(sec-butyl)-1H-1,2,4-triazol-5(4H)-one is member of the drug class known as anti-fungal.

Molecular Formula is $C_{35}H_{38}Cl_2N_8O_4$ and Molecular Weight is 705.63 g/mol.It is used for the inhibition of fungal cytochrome p450 enzyme "lanosterol 4 demethylase", used in the conversion of lanosterol to ergosterol, which is a main sterol in fungal cell membrane, thus inhibits replication and promotes cell death in case of the yeast cells transformation into hypothetically invasive hyphae[1].

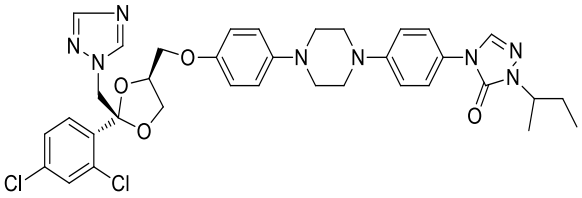

Fig.1:Chemical Structure of Itraconazole

Literature survey revealed that very few methods have been reported for the analysis of Cis-Bromobenzoate in Itraconazole API and its pharmaceutical dosage forms. Which include UV spectroscopy, Reverse Phase High Performance Liquid Chromatography, Ultra Pressure Liquid Chromatography, LCMS, GC-MS methods. The present studies illustrate development and validation of simple, economical, selective, accurate, precise GC-HS method forthe determination of Cis-Bromobenzoate in Itraconazole API as per ICH guidelines. In the present work a successful attempt had been made to develop a method for the determination of Cis-Bromobenzoate in Itraconazole API and validate it. The method would help in estimation of the Cis-Bromobenzoate in single run which reduces the time of analysis and does not require separate method for the drug. This paper reports an economical, simple and accurate RP-HPLC method for the above said API'S and pharmaceutical dosage forms.

Cis-bromobenzoate (figure.2) is designated chemically((2R,4R)-2-(bromomethyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)methyl benzoate. Molecular Formula is $C_{18}H_{15}BrCl_2O_4$ and Molecular Weight is 446.119 g/mol. In literature survey reveals that the drug can be estimated by spectrophotometric methods, ionic spectrophotometric methods and Chromatographic methods[2-6]. In the present investigation RP-HPLC method is a carried out with simple solvent system 0.1% Formic acid in Water: Acetonitrile (35:65). Cis-bromobenzoate is an Anti-Fungal drug because it interferes with the fungal synthesis of ergo sterol, a constituent of fungal cell membranes, as well as certain enzymes[7-12].

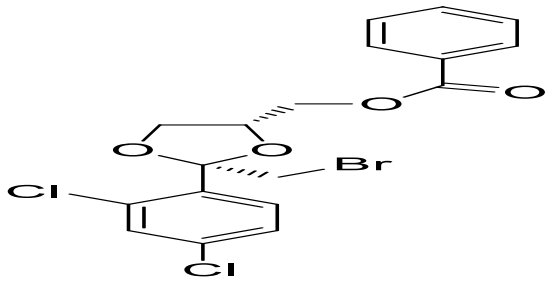

Fig.2: Chemical Structure of Cis-bromobenzoate

II. METERIALS AND METHODS

A. *Chemicals and reagents*

The samples of Itraconazole API and Cis-Bromobenzoate supplied by local well known laboratories. The HPLC grade methanol, Formic acid and acetonitrile were purchased from Sigma Aldrich. High purity water was prepared by using Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

B. *Experimental Conditions*

The chromatography analysis was performed using Waters Alliance 2695 separation module (Waters Corporation, Milford, USA) equipped with 2489 UV/visible detector and 2998 PDA detector (for peak purity), degasser, quaternary pump, and auto sampler system. The output signals were monitored and processed using Empower 2 software

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C. *Chromatographic conditions*

The method was developed using an Symmetry C18 (100 mm × 4.6 mm) 3.5 µmcolumn with the mobile phase containing a Isocratic mixture of 0.1% Formic acid in water and Acetonitrile in the ratio of 35:65v/v. The mobile phase were filtered through nylon 0.45 µm membrane filters and degassed. The flow rate of the mobile phase was 1.0 mL/min. The column temperature was maintained at 25°C, and the eluted compounds were monitored at the wavelength of 227 nm. The sample injection volume was 20 µl.The total runtime is 20 minutes.

III. PREPARATIONS

A. Diluent:0.1% Formic acid in Methanol and Acetonitrile in the ratio of 50:50v/v were used as diluent.

B. Preparation of Itraconazole API solution (5mg/mL)

Accurately weighted 100 mg Itraconazole and transfer into 20 ml volumetric flask. Then Sonicated for 10 to 15 min. After dissolved the final volume make up with diluent.

C. *Preparation of Cis-Bromobenzoate standard solution (1000ppm or 0.1%)*

Accurately weighted 100 mg Cis-Bromobenzoate and transfer it to 100 mL volumetric flask. Dissolve it with 50 mL of diluent. After dissolving the final volume make upwith diluent. Take 2.5mL of this solution in to 50mL volumetric flask and diluted up to the mark with diluent. Again take 5.0mL of above solution in to 50mL volumetric flask and diluted up to the mark with diluent. The final concentration (1000ppm) was prepared with respect to Itraconazole API sample concentration(5mg/mL).

D. *Preparation of Tablet solution*

Twenty tablets were taken, Average weight was determined and mix well fine powder. Amount equivalent to 100 mg Itraconazole was taken into 20 mL of volumetric flask. This powder dissolved in 10 mL of diluent and sonicate for 15 Min. The volume was made up to 20 mL with diluent. Shaken vigorously for 15 minutes and filtered through the Whatman filter paper. Again this solution is filtered by vacuum filtration through 0.45 membrane filter paper.

IV. METHOD DOVELOPMENT

A. *Selection of Analytical Wavelength*

The stock solution of Cis-Bromobenzoate were separately diluted with diluent to get a concentration of 1000ppm of Cis-Bromobenzoate respectively and scanned in the wavelength range of 200-400 nm on the HPLC system through PDA detector. From the spectra of Cis-Bromobenzoate, the wavelength (λ max) observed at 227nm.The sharp peak with minimum consumption of time are obtained at 227 nm, and peak purity was passed for Cis-Bromobenzoate (Figure 3). Therefore this wavelength is selected for the present work.

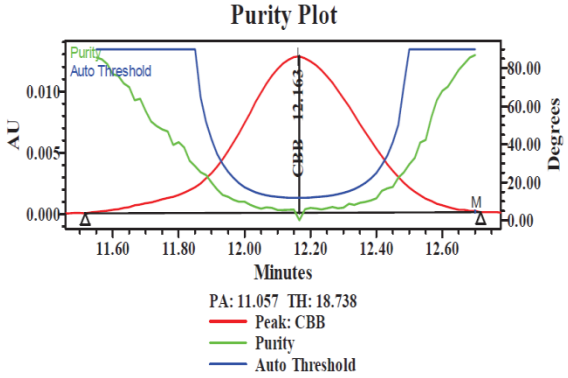

Fig.3:PDA Spectrum for CBB

B. *Selection of mobile phase and Column*

The aim of the study was to separate the both Itaconazole API and Cis-Bromobenzoate. And the Cis-Bromobenzoate determination in Itaconazole API and pharmaceutical dosage forms. Various attempts were made to separate 0.1% formic acid in water and composition of acetonitrile in the mobile phase using C-18 and C-8 stationary phase columns. To ensure

great resolution between the C-18 stationary phase with an end-capping was used. In this case, the optimized mobile phase was constituted by the 0.1% formic acid in water and Acetonitrile in ratio of 35:65,v/v. The total program is Isocratic. The flow rate of the mobile phase was 1.0 mL/min. The column temperature was maintained at 25°C, and the eluted compounds were monitored at the wavelength of 227 nm.

V. RESULT AND DISCUSSION

A. *Method validation*

The proposed method was validated as per ICH guidelines [13-14]. The following validation characteristics were addressed.Those are Specificity, accuracy, precision, limit of detection and quantification, linearity, solution stability, ruggedness and robustness.

A. *Specificity*

Interference of Cis-Bromobenzoate from Itraconazole peak was ensured as part of specificity. Cis-Bromobenzoate eluted at about 12.226 minutes while Itraconazole eluted at about 3.725 minutes indicating that the method has good resolving capability for these analytes. The specificity data and chromatograms are shown in table 1 and figure 4.

Table 1:Specificitydata for Cis-Bromobenzoate and Itraconazole

Name	RT	Area	USP Resolution	USP Plate Count	USP Tailing	Height(µV)
Itraconazole	3.725	117260742	----	2515	0.80	3622107
CBB	12.226	323013	7.56	6223	0.96	12531

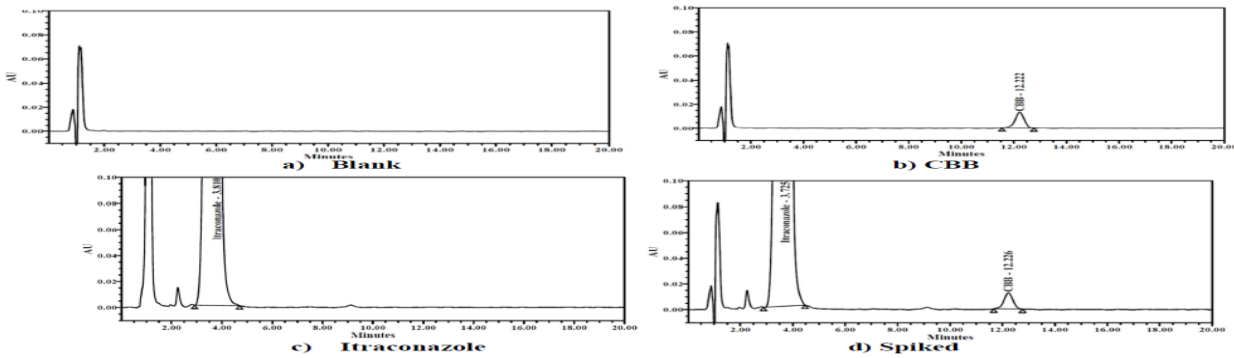

Fig.4: Specificity for a) Blank b)Cis-Bromobenzoatec) Itraconazole and d) Spiked

B. *System suitability*

System suitability shall be checked for the conformance of suitability and reproducibility of chromatographic system for analysis. System suitability was determined before sample analysis from six injections of the standard solution containing 1000ppm of Cis-Bromobenzoate. The acceptance criteria of USP tailing factor not more than 2.0 and % RSD for the RT and area of six replicate injections is not more then 2.0%.All critical parameters tested met the acceptance criteria.The data and chromatograms are shown in table 2 and figure 5.

Table 2:System suitabilitydata for CBB

Injection	RT	Area
1	12.209	313926
2	12.216	318481
3	12.219	317358
4	12.218	312370
5	12.217	313376
6	12.221	312006
Mean	**12.22**	**314586**
STDV	**0.004**	**2695.25**
%RSD	**0.03**	**0.86**

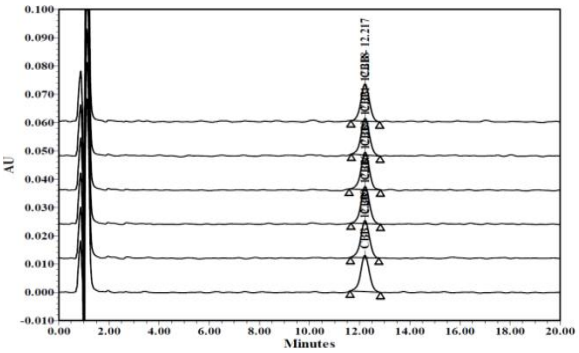

Fig.5: SST Chromatogram for the CBB

C. *Linearity*

For linearity checking Stock solution (1mg/mL) containing a Cis-Bromobenzoate was further diluted with diluent to give the linearity concentrations 25, 50, 75,100,150 and 200% (250, 500, 750, 1000, 1500, 2000ppm). And these solutions were injected (n=3) into the HPLC system and the

resultants peak areas of each component were recorded. The correlation coefficient obtained for impurity was not less than 0.999. The linearity data and graph are shown in table 3 and figure 6.

Table 3:Linearitydata for Cis-Bromobenzoate

Con (%)	Con(ppm)	Area
25	250	75217
50	500	151602
75	750	232055
100	1000	316150
150	1500	480627
200	2000	624257
Correlation coefficient(r2)		**0.9997**
STEYX		**5844**
SLOPE		**317**
LOD(ppm)		**61**
LOQ(ppm)		**184**

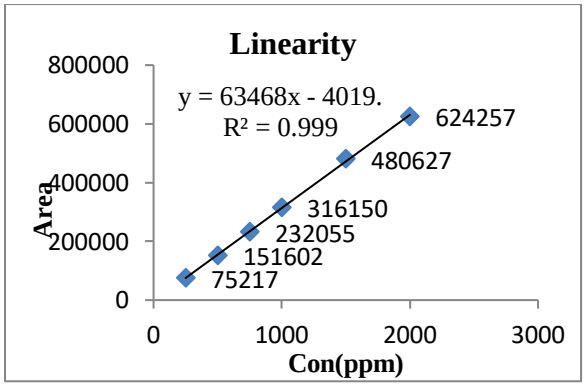

Fig.6: Linearity graph for the Cis-Bromobenzoate

D. Limits of detection and Quantification

The detection and quantification limit were determined based on linearity by injecting linearity solutions 250, 500, 750, 1000, 1500, 2000ppm into the HPLC system. Limit of detection (LOD) was calculated as 3.3× STEYX/SLOPE where as limit of quantification (LOQ) was calculated as 10× STEYX/SLOPE. LOD value for Cis-Bromobenzoate 61 ppm (0.30µg/mL), LOQ value for Cis-Bromobenzoate 184 ppm (0.92µg/mL) respectively. Prepare the standard solution of Cis-Bromobenzoate impurity at LOD and LOQ concentrations. The correlation coefficient obtained for impurity with LOQ was not less than 0.999.The corresponding linearity data and graphs at LOD and LOQ concentration are presented in table 4 and figure 7 & 8.

Table 4:LOD and LOQdata for CBB

Con(ppm)	Area
184(LOQ)	54606
250	75217
500	151602
750	232055
1000	316150
1500	480627
2000	624257
Correlation coefficient(r2)	**0.9997**

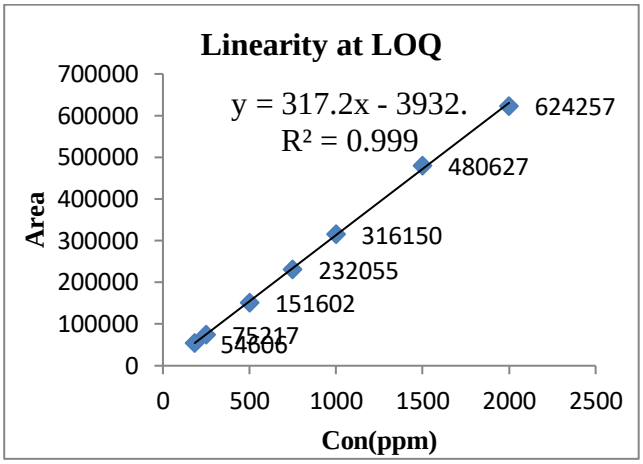

Fig.7: Linearity graph for the CBB with LOQ

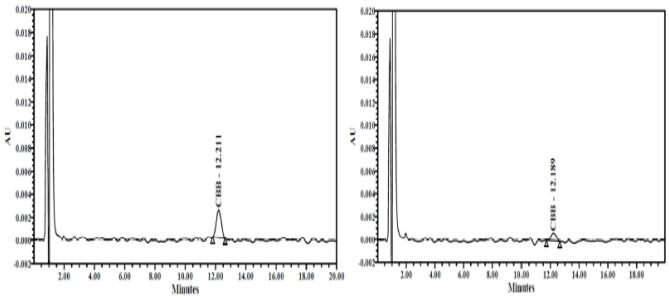

Fig.8: a) LOD b) LOQ Chromatograms for CBB

E. Accuracy

The accuracy of the method was expressed as the percentage recovery of Cis-Bromobenzoate. Recovery studies were carried out by standard addition method was three different concentrations (50%, 100% and 150%). The above said components were prepared with in the calibration range of Cis-Bromobenzoate. The sample of Itraconazole, standard Cis-Bromobenzoate impurity and spiked samples at 50%, 100% and 150% levels in triplicate are injected. From accuracy data, the % recovery of standard Cis-Bromobenzoate impurity was found within the limits (100±10%). The results indicate that the method has an acceptable level of accuracy. The recovery data is presented in table 5.

Table 5:Recovery data forCis-Bromobenzoate

No.of Injections	Sample	50%(n=3)	100%(n=3)	150%(n=3)
Inj-1	Not detected	150077	302919	463721
Inj-2	Not detected	150650	306575	469423
Inj-3	Not detected	146588	307599	461944
Average	-----	**149105**	**305698**	**465029**
STD 100% CBB Avg area		**314586**		
%Recovery		**94.79**	**97.17**	**98.55**

F. LOQ Precision

Prepare the standard Cis-Bromobenzoate solution at LOQ concentration (184 ppm) and injected in six replicates. The %RSD (n=6) values obtained for the average area of Cis-Bromobenzoate54439. The acceptance criteria of %RSD for RT and area of impurity was not more than 2.0%. The LOQ precision data and chromatograms are shown in table 6 and figure 9.

Table 6: LOQ Precision data for CBB

Injection	RT	Area
1	12.221	54733
2	12.213	54812
3	12.217	54107
4	12.211	54442
5	12.215	54266
6	12.194	54276
Mean	**12**	**54439**
STDV	**0.01**	**280.11**
%RSD	**0.08**	**0.51**

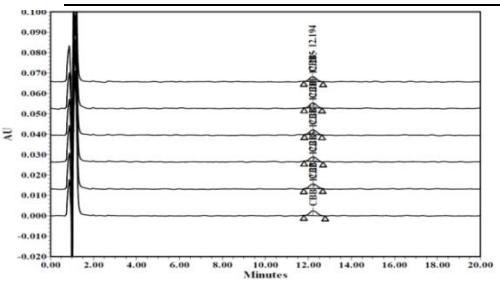

Fig.9: LOQ precision Chromatogram for the CBB

G. LOQ Accuracy

Weighed accurately about 50.0mg of the Itraconazole API into three different 20 ml of volumetric flasks and spiked with LOQ level of Cis-Bromobenzoate standard solution. Then the inject sample and Cis-Bromobenzoate impurity in triplicate. From accuracy data at LOQ level, the %recovery of Cis-Bromobenzoate was found within the limits (100±10%). The results are presented in table 7.

Table 7: LOQ Accuracy data for Cis-Bromobenzoate

No. of Injections	Sample	LOQ%(n=3)
Inj-1	Not detected	52448
Inj-2	Not detected	50260
Inj-3	Not detected	51645
Average	----	**51451**
LOQ STD Avg. area	**54439**	
%Recovery	**94.51**	

H. Robustness

Robustness is a measure of a method's immunity to small but deliberate variations in the conditions used. 0.1% formic acid in Water and Acetonitrile was in the ratio of 33:67 and 37:63 and flow rate 0.8 and 1.2 mL/min were deliberately changed and effects were monitored. The results are presented in table8.

Table 8: Robustness data for Cis-Bromobenzoate

No of Injections	Flow 0.8 ml/min		Flow 1.2ml/min		M.P 33:67		M.P 37:63	
	RT	Area	RT	Area	RT	Area	RT	Area
1	15.148	388555	10.166	268077	10.340	321005	14.443	317359
2	15.143	382315	10.181	261456	10.325	325866	14.446	318240
3	15.145	386357	10.180	266333	10.352	324284	14.448	314381
4	15.147	381494	10.165	265552	10.339	319863	14.450	318136
5	15.143	382315	10.181	261011	10.352	325486	14.448	312659
6	15.148	381139	10.180	264939	10.339	315229	14.446	313927
Mean	**15.146**	**383696**	**10.176**	**264561**	**10.341**	**321956**	**14.447**	**315784**
STDV	**0.002**	**3030**	**0.008**	**2788**	**0.010**	**4092**	**0.002**	**2418**
%RSD	**0.02**	**0.79**	**0.08**	**1.05**	**0.10**	**1.27**	**0.02**	**0.77**

I. Ruggedness

The ruggedness of the method was evaluated by performing the sample analysis in six replicates using different analysts on different days. The %RSD for Individual days, analysts and cumulative days, analysts were not less than 2.0%.The Cis-Bromobenzoate content indicate that the method adopted is rugged. The results are summarized as shown in table 9.

Table 9: Ruggednessdata for Cis-Bromobenzoate

Individual Days and Analysts	Mean(n=6)		STDV(n=6)		%RSD(n=6)	
	RT	Area	RT	Area	RT	Area
Day-1(analyst-1)	12.204	311875	0.003	1109.49	0.03	0.36
Day-1(analyst-2)	12.205	311291	0.002	1577.39	0.02	0.51
Day-2(analyst-1)	12.204	320158	0.002	2712.54	0.02	0.85
Day-2(analyst-2)	12.202	320941	0.003	2389.06	0.02	0.74
Cumulative Days and Analysts	**Mean(n=12)**		**STDV(n=12)**		**%RSD(n=12)**	
	RT	Area	RT	Area	RT	Area
Day-1(analyst-1&2)	12.205	311583	0.003	1335.47	0.02	0.43
Day-2(analyst-1&2)	12.203	320549	0.003	2471.12	0.02	0.77
Analyst-1(day1&2)	12.204	316016	0.003	4755.32	0.02	1.50
Analyst-2(day1&2)	12.204	316116	0.003	5396.60	0.03	1.71

J. Solution stability

Stability of Cis-Bromobenzoate 0.1% Formic acid in Methanol and Acetonitrile in the ratio of 50:50v/v was checked by keeping them in an autosampler and observing the variations in their peak areas. Each stage (at 0 h,12 h,24 h and 48 h) standard solution was injected in six times. Then the calculated the % of RSD and % of Solution stability. The %RSD is NMT 2.0% and the % of Solution stability is 100±2%. From the stability results, we found that Cis-Bromobenzoate was stable up to 48 h. The corresponding data is presented in table 10.

Table 10: Solution stabilitydata for Cis-Bromobenzoate

No of Injections	At 0 hors	At 12 hors	At 24 hors	At 48 hors
Injection-1	314937	312876	313258	312256
Injection-2	312455	313256	315869	314256
Injection-3	318462	313256	312863	313658
Injection-4	313362	314586	312897	312586
Injection-5	313458	314589	311258	312689
Injection-6	313115	313442	312358	310368
Average	**314298**	**313668**	**313084**	**312636**
STDV	**2196.59**	**736.04**	**1531.77**	**1338.84**
%RSD	**0.70**	**0.23**	**0.49**	**0.43**
% Solution Stability	----	**99.80**	**99.61**	**99.47**

K. Formulation analysis

The prepared tablet solution (5mg/mL) was injected and run on HPLC instrument.TheCis-Bromobenzoate content in Itraconazole tablets was Not more than 0.1%(1000ppm). Results were summarized in table 11. Typical chromatogram of Itraconazole is shown in figure 10.

Table 11: Formulation analysisdata for Itraconazole

Name of Drug	Lable claim	Amount of found	RT
Itraconazole	200mg	100.0%	3.810
CBB	---	Not detected	----

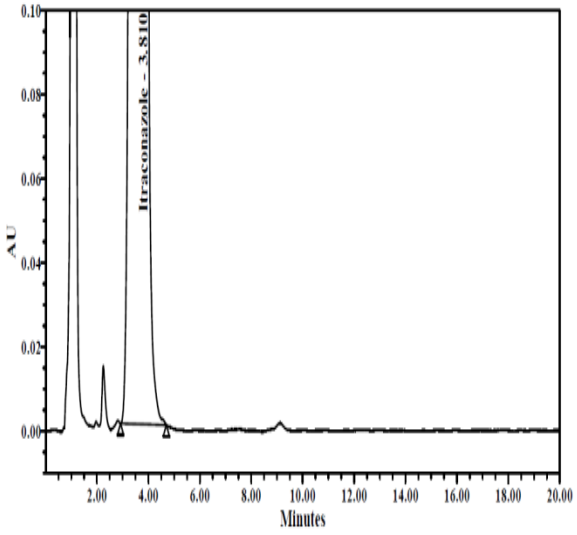

Fig.10: Chromatogram of Tablet analysis for the Itraconazole tablet

VI. DISCUSSION

The LOD and LOQ value for the Cis-Bromobenzoate obtained, demonstrate the suitability of the system for the analysis of the drug. System suitability parameter may fall within ±2% range during routine performance of the method. To study the accuracy and reproducibility of the proposed method recovery experiment study were carried out, in this a fixed amount of pre analyzed sample taken and standard drug was added at 50%, 100%, and 150% level. Each level was repeated for three times. The recovery of Cis-Bromobenzoate was in the range of 90%-110%. Recovery data states that the proposed method is accurate and reproducible. These results concluded that the proposed method is better in comparison to previously reported methods.

VII. CONCLUSION

A novel HPLC method proves to be simple, linear, precise, accurate, robust, rugged, and specific. The total runtime was 20 min. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is solution stability-indicating and can be used for quantitative determination of Cis-Bromobenzoate in Itraconazole in the Parma industry. The adopted HPLC method can also be useful for the estimation of Cis-Bromobenzoate in tablets also.

ACKNOWLEDGEMENT

We are thankful to the Dr.K.Surenrababu and Principal, for providing us necessary facilities to carry out the research work.

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