Section A-Research paper
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Identification and quantification of potential genotoxic impurities from Rilpivirine hydrochloride by HPTLC technique

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Abstract

Objective: A rapid, accurate, selective, and sensitive HPTLC method has been developed and validated for estimation of potential genotoxic impurity A, Impurity B, Impurity C and Impurity D from the standard Rilpivirine hydrochloride. **Method:** The chromatographic separation was achieved on Automatic Camag Automatic TLC Sampler 4 (ATS4) with Win CATS Planar Chromatography Manager Software Version- 1.4.4, Spray gas: Inert gas (N2) Micro litre syringe (20 µL, 100 µL): Hamilton**.** Stationary phase was Aluminium plates precoated with silica gel 60 F_{254}. The compound was separated using mobile phase Chloroform: Toluene: Ethanol in the ratio of (4: 4: 1 v/v/v**)**. The mode of application was band with band size 6mm and band distance 17 mm. Densitometric evaluation was performed at 282 nm. **Results:** Rilpivirine HCl, Impurity A, Impurity B, Impurity C and Impurity D were satisfactorily resolved with obtained Rf values 0.50, 0.58, 0.52, 0.50 and 0.71 respectively. The accuracy and reliability of the method was assessed by evaluation of linearity for Rilpivirine and all four impurities which obtained as 100-350 ng/spot for both drug and impurities with the regression coefficient of 0.9993 and 0.9995, respectively. Precision study found to be intra-day % RSD (1.02–1.08) and inter-day % RSD (1.05 –1.56) for Rilpivirine and all four impurities. The detection and quantification limits were found to be 60.96 and 129.5 ng/spot for both drug and impurities. **Conclusion:** The proposed method was validated in terms of Linearity, Range, Accuracy, Precision, Specificity and Robustness in accordance with ICH guidelines. The method was successfully applied to the estimation of Rilpivirinehydrochloride and four impurities in standard Rilpivirine hydrochloride.

Keywords: HPTLC, ICH guidelines, Method development, Quantification, Validation.

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INTRODUCTION

Rilpivirine hydrochloride is 4-{[4-({4-[(E)-2-cyanoethenyl]- 2, 6-dimethylphenyl}amino)2-pyrimidinyl]amino} benzonitrile (Fig.1)[1]. It is White crystalline solid having molecular formula $C_{22}H_{19}Cl_1N_6$. It is a di-amino pyrimidine derivative[2]. It acts by binding to reverse transcriptase which results in block in RNA and DNA dependent DNA polymerase activities. One such activity is HIV-1 replication[3]. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) play an important role in the treatment and prevention of HIV infections. NNRTIs bind and block HIV reverse transcriptase. HIV uses reverse transcriptase to convert its RNA into DNA (reverse transcription)[4,5]. Blocking reverse transcriptase and reverse transcription prevents HIV from replicating. Rilpivirine is second generation non-nucleoside reverse transcriptase inhibitor (NNRTI), it had been developed for treatment of HIV-I infected patients to have better safety[6]. Impurity profiling is a general term including structure elucidation/ identification as well as determination of the impurities of a chemical substance[7]. In case of genotoxic impurities,

i.e. substances which are suspected to potentially damage DNA even at very low level of exposure and may contribute to tumor development. Therefore the analytical limits for these substances are in the lower ppm level in pharmaceutical substances[8]

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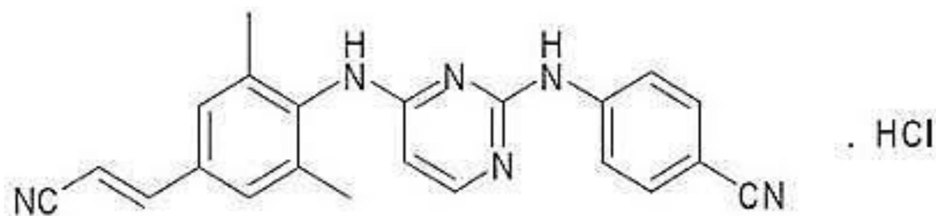

Fig. 1: Structure of Rilpivirine hydrochloride

Impurities:

1) Impurity A (2-Amino benzonitrile)

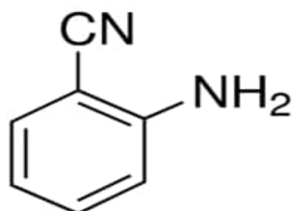

Fig.2: Structure of 2-Amino benzonitrile

2) ImpurityB (3-Amino benzonitrile)

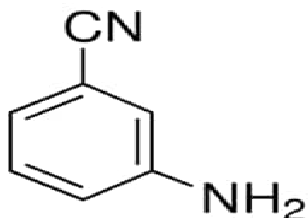

Fig.3: Structure of3-Amino benzonitrile

3) Impurity C (4-Amino benzonitrile)

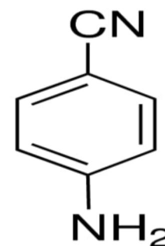

Fig.4: Structure of 4-Aminobenzonitrile

4) Impurity D (3-iodo,2-6 dimethyl aniline)

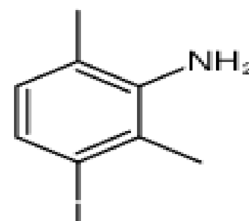

Fig. 5:Structure of 3-iodo,2-6 dimethyl aniline

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A wide range of reactive reagents are used in the multistep synthesis process of drug substance, i.e. Active Pharmaceutical ingredient (API). The use of these reagents may result in the generation of potentially genotoxic, cytotoxic, and mutagenic impurities in addition to API. Starting materials, process related drug substance or intermediate impurities, catalysts, organic or inorganic reagents, enantiomer impurities, heavy metals, drug substance degradation, organic degradation products, and residual solvents are well known sources of these impurities. Furthermore, impurities alter the properties of certain compounds and bind with human deoxyribonucleic acid (DNA) to cause oncological disease, which can have an impact on product risk assessment. The residual solvents change the physicochemical properties of the drug substances, such as the crystal nature of the bulk drug, which can affect solubility, odour, and colour changes in final products. As a result, even in low concentrations, these genotoxic impurities (GTIs) have a significant effect on the DNA sequence and structure[9,10]. The European Medicines Agency (EMA) was an integral controller in developing the fundamental standards for assessing genotoxic impurities in medications. The FDA issued draft rules for drug makers and industries on genotoxic and cancer-causing impurities in drug substances and drug products at the end of 2008[11]. In compared to the FDA and EMA guidelines, the ICH M7 guidelines were relevant not only for the assessment of drug impurities that were accounted for during registration and clinical trials, but also for reconsideration of requirements for effectively enrolled drugs, for example, process change for a drug substance or medication generation and utilization of advanced analytical techniques[12,13]. RPV nitrile and RPV chloro undergo reaction in presence of dimethyl acetamide and acetic acid to manufacture crude RPV. This crude converted to RPV base, purified and converted to pure RPV hydrochloride (Figure 1) form[14,15]. During this process there is a possibility of generation of 2 Amino Benzonitrile, 3 Amino Benzonitrile, 4 amino Benzonitrile and 3-iodo-2,6 dimethyl aniline showed in Fig. 2, Fig 3, Fig 4 and Fig 5 respectively which are potentially genotoxic. According to the ICH M7 guideline for genotoxic impurities, a threshold of toxicological concern (TTC) based acceptable intake of a mutagenic impurity of 1.5g/g is associated with a minor risk and can be used as a default for most pharmaceuticals to derive an acceptable limit for intake[16]. The maximum daily dose of RPV is approximately 50 mg. According to TTC, the limit of these four genotoxic impurities for intake is 30 ppm. A comprehensive survey of the literature on RPV hydrochloride demonstrated only a few analytical methods using spectrophotometry[17], high performance liquid chromatography (HPLC)[18], and high- performance thin layer chromatography (HPTLC)[19]. HPTLC has rapidly become a standard analytical technique due to its low operating costs, high sample throughput, and minimal sample preparation requirements. The main advantages of HPTLC over HPLC are that several samples can be run simultaneously using a small amount of mobile phase, reducing analysis time and cost per analysis. It enables automated application and scanning in real time. For the simultaneous determination of 2ABN, 3ABN, 4ABN, and 3 iodo 2,6 dimethyl aniline. a simple, accurate, and precise high performance thin layer chromatography method can be developed.

Though, an intensive literature search revealed to the best of our knowledge that methods are not available for the quantitative determination of 2 ABN, 3ABN, 4ABN and 3 iodo-2,6 Dimethyl aniline with desire limit in RPV drug substances as well as drug products. Thus, present study was a successful effort to develop a scientific HPTLC method for quantitative estimation of RPV and 2 ABN, 3ABN, 4ABN and 3-Iodo-2,6 Dimethyl aniline in marketed formulation as per ICH guidelines.

MATERIALS AND METHODS

Chemicals and reagents:

The pharmaceutical grade working standards of Rilpivirine HCl and impurities 2-ABN, 3-ABN, 4ABN and 3-iodo 2, 6-dimethyl aniline were obtained from Cipla Ltd in Mumbai Central, Mumbai. Chloroform, ethanol, methanol, formic acid and all other chemicals and reagents of analytical grade were purchased from Merck Chemicals, Mumbai, India.

Instrumentation:

The samples were spotted in the form of bands of width 6 mm with a Camag 100µL sample syringe (Hamilton, Bonaduz, Switzerland) syringe on precoated silica gel aluminium plate G 60 F254 (20×10) with 250 µm thickness; (E. Merck, Darmstadt, Germany) using a Camag Linomat IV (Switzerland) as a sample applicator. A constant application rate of 0.1 µg/ spot was employed and space between two bands was 5mm. The plates were prewashed with methanol and activated at 110^0 C for 5 min prior to chromatography. The mobile phase consisted of Chloroform: Toluene: Ethanol in the ratio of (4: 4: 1 v/v/v). Linear ascending development was carried out in a 12 cm × 4.7 cm × 12.5 cm twin through glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 15 min at room temperature (25 ± 2) at relative humidity of 60 % ± 5. The length of chromatogram run was 8cm and approximately 10 min saturation time was kept for each chromatographic run. Following the development, the TLC plates were dried in a stream of air with the help of an air dryer in a wooden chamber with adequate ventilation. Densitometric scanning was performed using a Camag TLC scanner III in the reflectance absorbance mode at 281 nm and operated by Win CATS software (Version 1.4.4.). The source of radiation used was deuterium lamp emitting a continuous UV spectrum between 200 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of the light. Evaluation was performed by linear regression of peak areas determined by UV absorption as a function of sample amounts.

Preparation of Standard Stock Solutions

10 mg of Rilpivirine HCl and 10 mg of ImpurityA, Impurity B, Impurity C, Impurity D in individual 10ml volumetric flask and was dissolved in methanol to obtain 1000 µg/mL as a standard stock solution. From the standard stock solutions, diluted mixed standard solutions were prepared containing 100 µg/mL for Rilpivirine HCl and 100 µg/mL for respective impurities respectively, by diluting 1 ml of standard stock solution in 10 ml methanol. The stock solution was stored properly.

Chromatographic conditions

The HPTLC procedure was optimized with a view to develop a quantification method for Rilpivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D respectively. The mixed standard stock solution (10 µg/mL of Rilpivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D) was taken and sample was spotted on to HPTLC plates and run-on different solvent systems. Finally mobile phase composed of Chloroform: Toluene: Ethanol in the ratio of (4: 4: 1 v/v/v). was found optimum. In order to decrease the neckless effect, HPTLC chamber was saturated for 15 min using saturation pads. The mobile phase was run which takes around 10 min for complete development of the TLC plate.

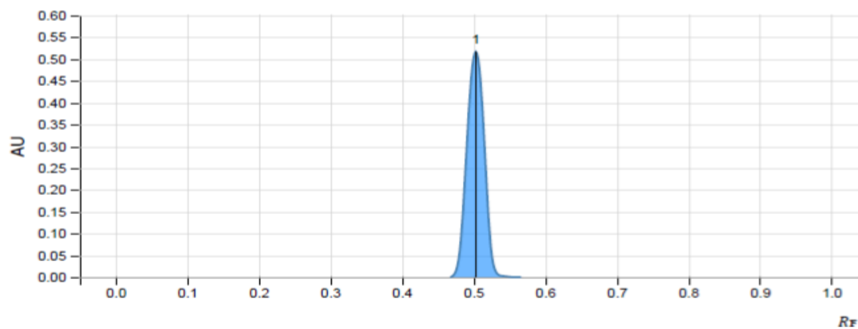

Fig No.6 Chromatographic peak of Rilpivirine Hydrochloride

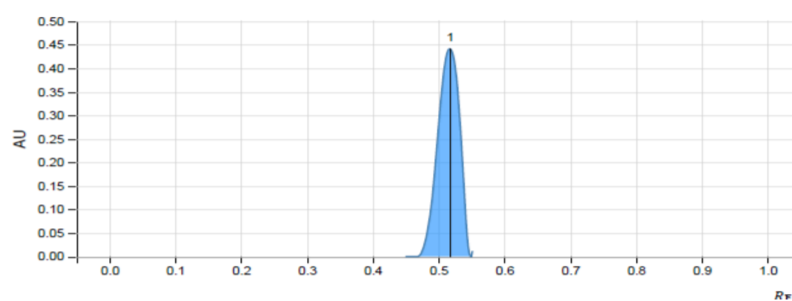

Fig No.7 Chromatographic peak of 2ABN

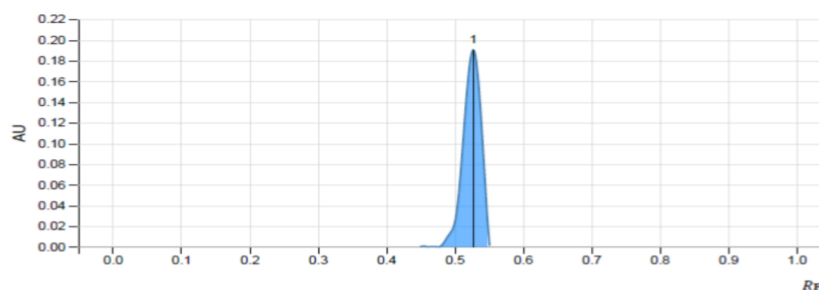

Fig. no.7 Chromatographic peak of 3 ABN

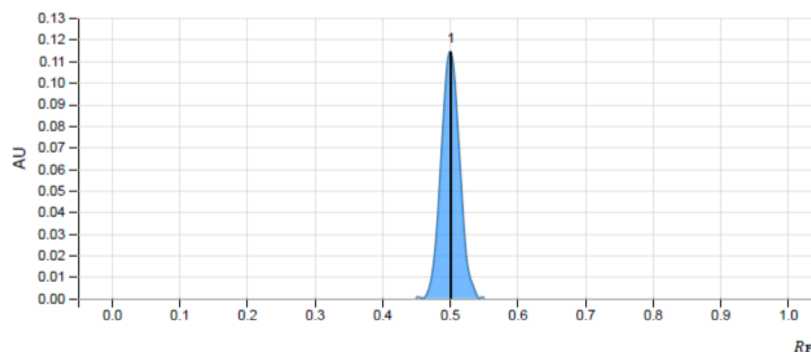

Fig.no.8 Chromatographic peak of 4ABN

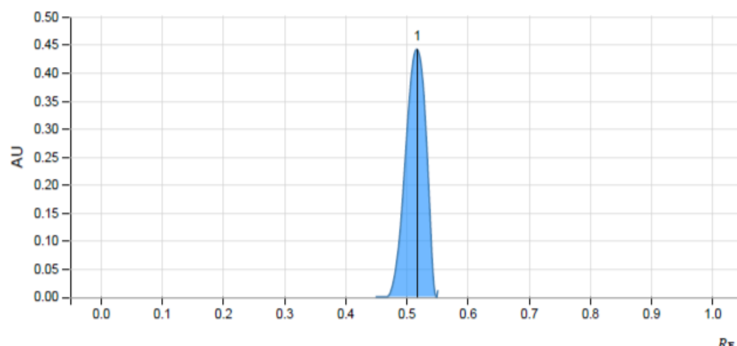

Fig.no.9 Chromatographic peak of 3iodo 2,6 dimethyl aniline

Validation of the method

Validation of the optimized HPTLC method was carried out with respect to the evaluating parameters linearity, precision, Limit of detection, limit of quantification, recovery studies in accordance to ICH guidelines.

Linearity and range

From the mixed standard stock solution, 100 µg/mL of Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D 100 µg/mL of was taken, solution was spotted on TLC plate to obtain final concentration 100- 350 ng/spot for Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D. Linearity of the method was studied by applying six concentrations of the drug, each concentration was applied three times to the TLC plates. The plate was then developed using the previously described mobile phase and the peak areas were plotted against the corresponding concentrations to obtain the calibration curves. Calibration coefficients, slopes, and intercept were analysed from coefficient equation.

Precision

The precision of this method was verified by repeatability and intermediate precision studies. Repeatability studies were carried out by analysis of three different concentrations (100 ng/spot, 200 ng/spot and 300 ng/spot for both the drug Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D respectively. six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days.

Limit of detection and limit of quantitation

Limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by spotting a blank and calculating the signal-to-noise ratio for drug Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D respectively by spotting a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. To determine the LOD and LOQ, sequential dilutions of mixed standard solution of drug Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D respectively were prepared from the standard stock solution. The samples were applied to HPTLC plate and the chromatograms were run and measured signal from the samples was compared with those of blank.

Recovery studies

Accuracy of the method was carried out by applying the method to drug sample drug Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D respectively to which known amount of drug Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D respectively standard powder corresponding to 80, 100 and 120 % of label claim had been added (standard addition method), mixed and the powder was extracted and analysed by running chromatogram in optimized mobile phase.

RESULTS AND DISCUSSION

Chromatographic method optimization for the

densitometric measurements During optimization several mobile phase compositions were tried using mixture of various polar and relatively non-polar solvents. Among several compositions of mobile phase, Chloroform: Toluene: Ethanol in the ratio of (4: 4: 1 v/v/v) gave better resolution and peak shape with the acceptable Rf values 0.50, 0.58, 0.52, 0.50 and 0.71 respectively for drug Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D is represented in Fig.no.10.

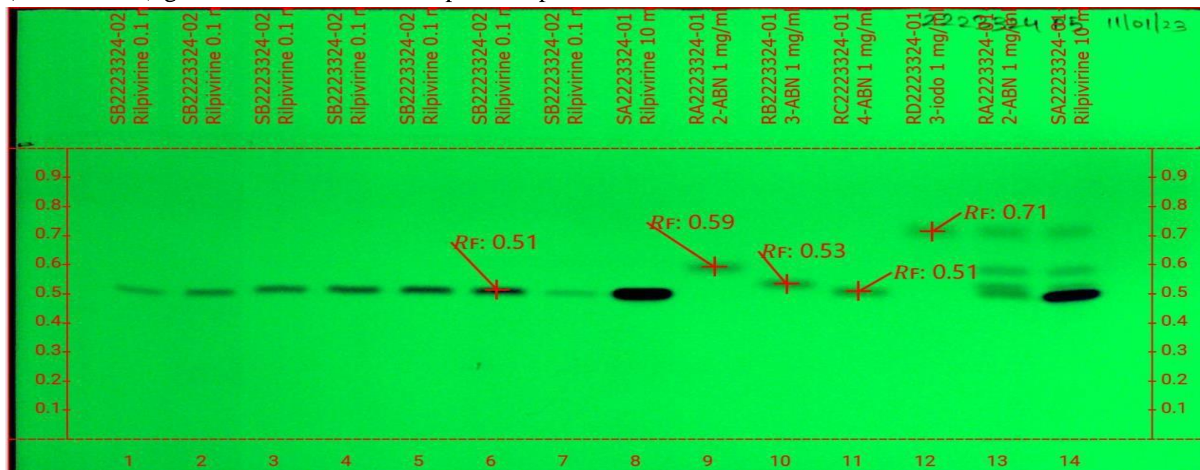

Fig.no.10 Representing Resolution of R_f of drug Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D

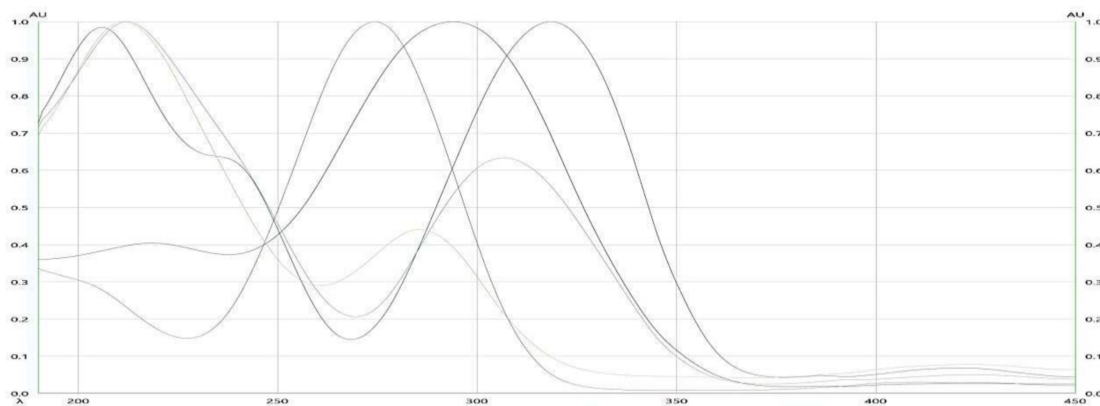

Fig no.11 Spectrum data of Rilpivirine and the given impurities

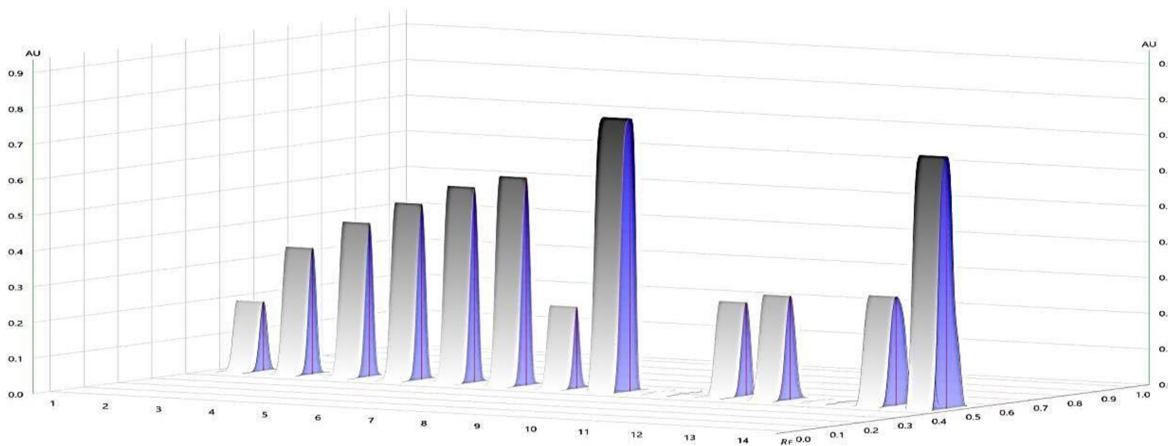

Fig. No. 12 3D Profile of Rilpivirine with respect to given impurities

Linearity

The drug response was found to be linear (regression coefficient were 0.994428 for Rilpivirine HCl and 0.999534 for impurities over the concentration range between 100-350 ng/spot for both Rilpivirine HCl and impurities. The regression equations show slopes (m) and intercept(c) value is 4.06 x 10^{-8} and 5.294 x 10^{-3}respectively (Figure 13). Where indicates the peak area and x indicates concentration in ng/ml.

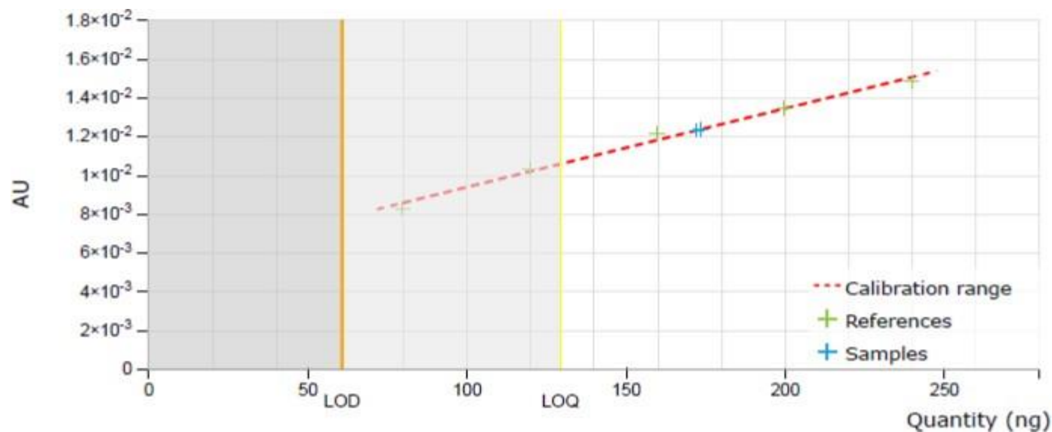

Fig.no 13 Linearity of References and samples

Precision

Precision was determined by studying the intermediate precision and repeatability over the concentration range of 100 ng/spot to 300 ng/spot for Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D. results of the repeatability and intermediate precision experiments are shown in Table 1. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2%, as recommended by ICH guidelines.

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Table No. 1: Precision Study

Intraday study			Inter Day study		
Drug	**Concentration in ng/spot**	**% RSD**	**Drug**	**Concentration in ng/spot**	**% RSD**
Rilpivirine HCl	100	1.02	Rilpivirine HCl	100	1.05
	200	1.03		200	1.04
	300	1.07		300	1.08
2ABN	100	0.89	2ABN	100	0.88
	200	1.02		200	1.08
	300	1.09		300	1.06
3ABN	100	1.15	3ABN	100	1.12
	200	1.05		200	1.09
	300	1.35		300	1.45
4ABN	100	0.85	4ABN	100	1,78
	200	0.95		200	1.97
	300	1.02		300	1.07
3iodo 2,6dimethyl aniline	100	1.08	3iodo 2,6dimethyl aniline	100	1.87
	200	1.57		200	1.88
	300	1.08		300	1.56

LOD and LOQ

The LOD and LOQ were found to be 60.96 ng and 129.5 ng for Rilpivirine HCl, 63.23ng and 173.7ng for 2ABN, 57.98 ng and 172.5 ng for 3 ABN, 55.46 ng and 123ng for 4 ABN, 74.35ng and 134.56 ng3iodo 2,6 dimethyl aniline respectively. Signal-to-noise ratio for LOD and LOQ found to be 3:1 and 10:1 respectively.

Robustness of the method

The standard deviation of peak areas was calculated for each parameter and the % RSD was found to be less than 2. The obtained values of % RSD, as shown in Table 2, indicated the robustness of the method.

Recovery studies

As shown from the data in Table II good recoveries of the Rilpivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D. in the range from 99.63 % w/w to 100.11 % w/w were obtained at various added concentrations. The study showed that the results within acceptable limit of above 99 % and below 101% and lower values of RSD indicate the proposed method is accurate. The percentage recovery shows the method is free from interference of theexcipients used in the formulations.

Table No 2: Recovery studies

Level of Recovery	Drug	Amount of Drug applied ng/spot	% Recovery	S. D.	% RSD
80%	Rilpivirine HCl	80	101.1%	± 0.94	0.98
	2ABN	80	99.88%	± 0.65	0.96
	3ABN	80	99.45%	± 0.58	0.68
	4ABN	80	99.65%	± 0.63	0.74
	3iodo 2,6dimethyl aniline	80	99.77%	± 0.96	1.01
100%	Rilpivirine HCl	100	103%	± 0.98	1.02
	2ABN	100	99.66%	± 0.45	0.66
	3ABN	100	99.40%	± 0.98	1.15
	4ABN	100	99.60%	± 0.94	1.10
	3iodo 2,6dimethyl aniline	100	99.80%	± 0.90	0.95
120%	Rilpivirine HCl	120	102.1%	± 0.95	0.99
	2ABN	120	99.55%	± 0.93	1.37
	3ABN	120	99.50%	± 0.99	1.16
	4ABN	120	99.77%	± 0.98	1.15
	3iodo 2,6dimethyl aniline	120	99.90%	± 0.97	1.02

Repeatability studies

Repeatability was assessed by spotting) concentration of drug for Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D 2μL each solution 5 times on a TLC plate followed by development of plate and recording peaks area for 5 spots (Table 3).

Table 3: Repeatability Studies

Drug	Applied Amount (μL)	No. of scan	% RSD of sample application	% RSD of measurements
Rilpivirine HCl	2	1	0.98	-
2ABN	2	1	0.96	-
3ABN	2	1	0.68	-
4ABN	2	1	0.74	-
3iodo 2,6dimethyl aniline	2	1	1.01	-
Rilpivirine HCl	2	5	-	0.97
2ABN	2	5	-	0.92
3ABN	2	5	-	0.60
4ABN	2	5	-	0.77
3iodo 2,6dimethyl aniline	2	5	-	1.05

CONCLUSION

A simple, sensitive and selective validated HPTLC method has been developed as per ICH guidelines for estimation of for Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D in bulk drug and pharmaceutical formulation. The validation study proved that the developed method is precise, specific and accurate. This is cost effective method of quantification of potential genotoxic impurities. Specificity study proved that the method is suitable for the analysis of for Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D as bulk drug. The suggested method was found to be less time consuming and cost effective and may be more advantageous for routine analysis of drug in marketed formulation. It may be extended to study the degradation kinetics of for Ripivirine HCl and Impurity A, Impurity B, Impurity C, Impurity D also for its estimation in plasma and other biological fluids.

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