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VALIDATED HPTLC METHOD FOR SIMULTANEOUS ESTIMATION OF RIFAMPICIN, ISONIAZID AND PYRIDOXINE HYDROCHLORIDE IN COMBINED TABLET DOSAGE FORM

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ABSTRACT

This present study reports for the first time simultaneous quantification of Rifampicin, Isoniazid and Pyridoxine Hydrochloride in tablet dosage form by employing High Performance Thin Layer Chromatographic method. Chromatographic separation of the drugs were performed on aluminium plates precoated with silica gel 60 G F_{254} as the stationary phase and the mobile phase used was a mixture of Ethyl acetate: Methanol: Acetone: Acetic acid (5.5: 2.0: 2.0: 0.5, v/v). Densitometric evaluation of the separated zones was performed using a UV detector at 254 nm in absorbance mode. The R_f values for Rifampicin, Isoniazid and Pyridoxine Hydrochloride were found to be 0.27±0.01, 0.47 ±0.01 and 0.75±0.01 respectively. The calibration Curve was found to be linear between 200 to 1000 ng/spot for each drug. The method was specific because no chromatographic interferences from the tablet excipients were found. The accuracy and reliability of the method was assessed by linearity, precision (intraday % RSD and interday % RSD of Rifampicin, Isoniazid and Pyridoxine Hydrochloride) and specificity in accordance with ICH guidelines. The limits of detection and quantification were found to be 75.28 ng/spot and 88.83 ng/spot for RIF, and 80.20 ng/spot and 102.49 ng/spot for INH, and 118.46 ng/spot and 132.30 ng/spot for PDX, respectively. The developed method can be successfully employed for the simultaneous determination of RIF, INH and PDX in marketed tablet formulation.

KEYWORDS: Simultaneous estimation, Rifampicin (RIF), Isoniazid (INH), Pyridoxine Hydrochloride (PDX), High Performance Thin Layer Chromatography (HPTLC), Densitometry, Validation.

1. INTRODUCTON

Rifampicin (RIF) is chemically, (7*S*, 9*E*, 11*S*, 12*R*, 13*S*, 14*R*, 15*R*, 16*R*, 17*S*, 18*S*, 19*E*, 21*Z*) - 2, 15, 17, 27, 29 - pentahydroxy-11-methoxy-3, 7, 12, 14, 16, 18, 22-heptamethyl-26-{(E) - [(4 methylpiperazin1yl) imino] methyl}-6,23-dioxo-8,30-dioxa-24 azatetracyclo [23.3.1.14, 7.05, 28] triaconta 1 (28), 2, 4, 9, 19, 21, 25 (29), 26 – octane – 13 - yl acetate. It is a semisynthetic derivative of Rifamycin B, obtained from *Streptomyces mediterranei*, used for the treatment of tuberculosis and other infectious diseases [1]. It is an antibiotic that inhibits DNA- dependent RNA polymerase activity in susceptible cells leading to a suppression of RNA synthesis and cell death [2]. It is freely soluble in chloroform, soluble in methanol and slightly soluble in water [1, 3].

Isoniazid (INH) is chemically, Pyridine -4-carbohydrazide. It is the most reliable and most commonly used medication for tuberculosis [4]. Because of the frequency of antibacterial resistance, it is always used in combination with other agents. It inhibits mycolic acid synthesis, an essential component of mycobacterial cell walls. It is soluble in water, methanol and chloroform [5]. Pyridoxine Hydrochloride (PDX) is chemically, 5-Hydroxy-6-methyl-3,4-pyridinedimethanol hydrochloride. It is a water soluble vitamin, also called as vitamin B_6. It is given to the patients taking Isoniazid (INH) to combat the toxic side effects of the drug [6]. It is administered at a dose of 10-50 mg/day to the patients in order to prevent peripheral neuropathy and CNS effects that are associated with the use of INH. It is freely soluble in water and soluble in methanol [7].

A combination of the above drugs: RIF (450 mg), INH (300 mg) and PDX (10 mg) is commercially available and used in the treatment of tuberculosis. The structures of these three drugs are shown in **Fig. 1, Fig. 2, and Fig. 3.**

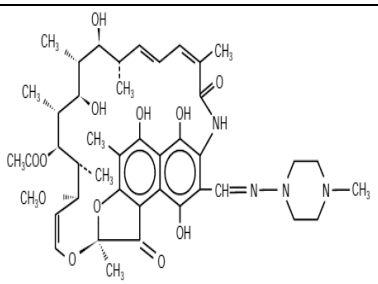

Fig.1: Structure of Rifampicin

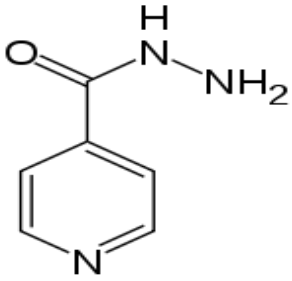

Fig. 2: Structure of Isoniazid

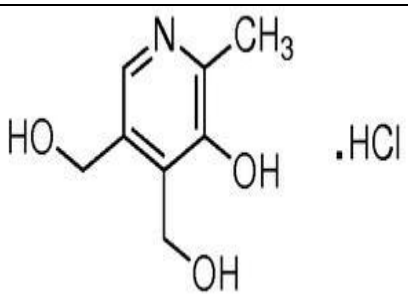

Fig. 3: Structure of Pyridoxine

RIF, INH and PDX are officially approved drugs in the Indian, British and US Pharmacopoeias. Numerous different analytical methods have been developed for their

quantitative determination of individual drugs in pure, pharmaceutical dosage forms and/or biological fluids. These methods are liquid chromatography [8,9,10,11,12], thin layer chromatography [13], voltammetric assay [14,15], chemometry [16,17,18], spectrophotometry [19,20,21,22,23,24,25], polarography [26], spectrofluorimetry [27] and chemiluminescence [28]. No HPTLC studies on Rifampicin, Isoniazid and Pyridoxine in combined dosage form in pharmaceutical preparations have been found in recent literature survey.

2. MATERIALS AND METHODS

2.1. Materials

Gift samples of Rifampicin, Isoniazid and Pyridoxine Hydrochloride reference standard (Lupin Pharma Pvt.Ltd, New Delhi). HPLC grade solvents; Methanol (S D Fine - Chemicals Limited, Mumbai), Toluene (Ranbaxy Fine-Chemicals Limited, New Delhi), Isopropyl Alcohol (Ranbaxy Fine- Chemicals Limited, New Delhi), Acetone (Ranbaxy Fine- Chemicals Limited, New Delhi), Ethyl acetate (Thomas Baker (Chemicals) Limited, Mumbai), Acetic acid (HiMedia Laboratories Pvt.Ltd, Mumbai). Commercially available tablets (Rifa-i-6, containing 450 mg RIF, 300 mg INH and 10 mg PDX**,** Concept Pharmaceuticals Ltd, Mumbai) were obtained from local pharmacy.

2.2. Instrument and Software

Camag HPTLC system comprising of Linomat V automatic sample applicator, Camag Hamilton microlitre syringe (100 µl), Camag TLC scanner III, Camag wincats software with stationary phase precoated silica gel 60 G $F_{254,}$ Shimadzu analytical balance and Ultrasonicator were used.

2.3. Preparation of Standard Stock Solution

Standard stock solution of RIF, INH and PDX were prepared by accurate weighing of 50.00 mg of each drug in the separate 50 ml volumetric flasks and dissolving in methanol and then made upto mark with methanol to obtain standard solution having concentration of RIF (1000 µg/ml), INH (1000 µg/ml) and PDX (1000 µg/ml). Accurately measured 1 ml of the three solutions were transferred to three 10 ml volumetric flasks and diluted to the mark with methanol to obtain solution having concentration 100 µg/ml of RIF, INH and PDX.

2.4. Preparation of Standard Mixture

50.00 mg of RIF, INH and PDX reference standard were accurately weighed and transferred into 50 ml volumetric flask, mixed and dissolved in methanol and then made upto mark with

methanol. 1 ml of the solution was transferred into 10 ml volumetric flask and diluted to the mark with methanol to obtain solution having concentration 100 µg/ml of RIF, INH and PDX.

2.5. Preparation of Sample Solution

Twenty tablets (Rifa - i - 6, labelled to contain 450 mg of RIF, 300 mg of INH and 10 mg of PDX per tablet, Concept Pharmaceuticals Ltd, Mumbai) were weighed and crushed to fine powder. An accurately weighed powder sample equivalent to 50 mg of RIF was weighed, transferred to stoppered volumetric flask containing 20 ml of methanol, sonicated for 30 min and the solution was transferred to a 50 ml volumetric flask through Whatman No. 1 filter paper. The residue was further extracted twice with 10 ml each of methanol and passed through the same filter paper and diluted to the mark with methanol. The resulting solution had RIF (1000 µg/ml), INH (666.6 µg/ml) and PDX (22 µg/ml). Accurately pipetted out 1.0 ml of solution into 10 ml volumetric flask (pure INH and PDX were spiked to the solution to bring the concentration in the linearity range) and diluted to the mark with methanol to obtain solution having concentration 100 µg/ml of RIF, INH and PDX.

2.6. Chromatographic Condition

Stationary Phase: Precoated silica gel 60 G F_{254} aluminium sheet (20 × 10 cm)

Mobile Phase: Ethyl acetate: Methanol: Acetone: Acetic acid (5.5: 2.0: 2.0: 0.5, v/v).

Chamber Saturation Time: 30 min.

Development Distance: 8 cm

Temperature: Ambient Temperature

Wavelength: 254 nm

Bandwidth: 4 mm

Quantity of Mobile Phase: 20 ml

Scanning speed: 10 mm/sec.

Slit Dimension: 5 mm × 0.45 mm

Application Rate: 0.1µl/sec.

Detection: Densitometrically using a UV detector at 254 nm.

2.7. Selection of Mobile Phase

For HPTLC analysis, initially various mobile phases were tried in attempts to obtain the best separation and resolution between RIF, INH and PDX. The mobile phase consisting of Ethyl acetate: Methanol: Acetone: Acetic acid (5.5: 2.0: 2.0: 0.5, v/v) was selected, that gave satisfactory

separation and gave three well resolved peaks for RIF, INH and PDX which is shown in Fig. 9 and Fig. 10. The R_f value for RIF, INH and PDX was 0.27±0.01, 0.47±0.01 and 0.75±0.01 respectively.

2.8. Selection of Wavelength for Measurement

After chromatographic development, the spots were scanned over the range of 200 - 400 nm and the spectra were overlain. It was observed that, the three drugs showed considerable absorbance at 254 nm. So, 254 nm was selected as the wavelength for detection.

3. VALIDATION OF METHOD

The method was validated according to ICH Q2 (R1) guidelines [29]. The following parameters were used for validation of the proposed method.

3.1. Linearity

Linearity was checked by preparing standard solutions of each RIF, INH and PDX at five different concentration levels. The calibration curves for RIF, INH and PDX were drawn in the concentration range of 200-1000 ng/spot. The calibration curves were constructed by plotting peak area versus concentration (Fig. 11, Fig. 12 and Fig. 13). Each reading was the average of three determinations. The correlation coefficient (r^2) for calibration curve of RIF, INH and PDX was 0.9987, 0.9983 and 0.9961 respectively (Table 1).

3.2. Accuracy

The accuracy of the method was determined by calculating recoveries of RIF, INH and PDX by the standard addition method. For that, known amounts of standard solutions at 50, 100 and 150 % level were added and analyzed by the proposed method, in triplicate (Table 2).

3.3. Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations (400 ng/spot, 600 ng/spot and 800 ng/spot) for RIF, INH and PDX six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days (Table 3).

3.4. Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were calculated from the data obtained from the linearity studies. The slope of the linearity plot was determined. For each of the six replicate determinations, y intercept was

calculated and the standard deviation of the y intercept was computed. From these values, Limit of detection (LOD) and Limit of quantitation (LOQ) were determined on the basis of response and slope of the regression equation. LOD and LOQ were calculated using following equation as per ICH guidelines. LOD = 3.3 × σ/S, LOQ = 10 × σ/S, where σ is the standard deviation of y intercepts of regression lines and S is the slope of calibration curves (Table 1).

3.5. Robustness

In order to establish the robustness of the method, small deliberate changes were made in the experimental conditions and chromatographic parameters like mobile phase composition (± 0.1 ml for each component), the plate activation time, chamber saturation time (± 10 % change from set time), volume of mobile phase (± 10 % change from set volume) and the development distance (± 10 % change from set distance). The time from spotting to development (0, 10, 20, 30 min) and from development to scanning (0, 10, 20, 30 min) was also varied. In the above changed conditions, stock solution was analyzed and results of robustness studies were expressed in term of % RSD of peak areas in each changed condition and were compared with similar results obtained in unchanged experimental conditions.

4. RESULTS AND DISCUSSION

A simple, accurate and precise HPTLC method was developed and validated for the simultaneous estimation of Rifampicin, Isoniazid and Pyridoxine Hydrochloride. The proposed HPTLC method was optimized with several solvent systems. The mobile phase consisting of Ethyl acetate: Methanol: Acetone: Acetic acid (5.5: 2.0: 2.0: 0.5, v/v) gave sharp and symmetrical peaks with the R_f values of 0.27, 0.47 and 0.75 for RIF, INH and PDX respectively. In order to reduce the broadening and tailing of peak, 0.5 ml of glacial acetic acid was added. Resolution of the peaks for mixture of standard drugs with clear baseline separation was obtained (Fig. 9) and for individual drugs (Fig. 6, Fig. 7 and Fig. 8). Well defined spots were obtained when the chamber was saturated with mobile phase for 30 min at room temperature. A 3-D chromatogram showing peaks of RIF, INH and PDX in different concentrations at 254 nm is depicted in Fig. 5. The calibration curves for RIF, INH and PDX were constructed by plotting peak area and concentration which is shown in Fig. 11, Fig. 12 and Fig. 13. The proposed HPTLC method was validated in terms of linearity, accuracy, precision, LOD, LOQ and robustness. The calibration curve was linear over the concentration range 200 to 1000 ng/spot for RIF, INH and PDX with a correlation coefficient of 0.9987,

0.9983 and 0.9961 for RIF, INH and PDX respectively. The method was found to be accurate with % recovery 97.96 % - 98.33 % for RIF, 97.95 % - 98.12 % for INH and 98.39 % - 98.86 % for PDX. The method was found to be precise with % RSD 0.14 - 0.56 (for RIF), 0.36 - 0.79 (for INH) and 0.25 - 1.08 (for PDX) for repeatability (n = 6), and 0.41 - 1.04 (for RIF), 0.29 - 1.20 (for INH) and 0.23 - 0.90 (for PDX) for intraday (n = 3), and 0.30 - 0.51 (for RIF), 0.36 - 1.21 (for INH) and 0.23 - 0.49 (for PDX) for interday (n = 3) precision studies. The limits of detection and quantification were found to be 75.28 ng/spot and 88.83 ng/spot for RIF, and 80.20 ng/spot and 102.49 ng/spot for INH, and 118.46 ng/spot and 132.30 ng/spot for PDX, respectively. Summary of validation parameters is shown in Table 1. The proposed method was applied for the estimation of marketed formulations and assay results shown in Table 4.

Table 1: Summary of validation parameters.

Parameters		**HPTLC method**		
		RIF	INH	PDX
Linearity (ng/spot)		200 - 1000	200 - 1000	200 - 1000
Regression equation (y = mx+c)		y =10.473+516.27	y =10.711x+1605.3	y = 7.6716x+945.1
Slope		10.473	10.711	7.6716
Intercept		516.27	1605.3	945.1
Correlation coefficient (r^2)		0.9987	0.9983	0.9961
Accuracy (%recovery)	**Level I (50%)**	98.33	98.12	98.57
	Level II (100%)	98.21	98.08	98.86
	Level III (150%)	97.96	97.95	98.39
Repeatability (% RSD, n = 6)		0.14 - 0.56	0.36 - 0.79	0.25 - 1.08
Intraday (% RSD, n=3)		0.41 - 1.04	0.29 - 1.20	0.23 - 0.90
Interday (% RSD, n=3)		0.30 - 0.51	0.36 - 1.21	0.23 - 0.49
LOD (ng/spot)		75.28	80.20	118.46
LOQ (ng/spot)		88.83	102.49	132.30

Table 2: Results of Recovery studies.

% Level	**Amount of sample taken (µg/ml)**			**Amount of standard added (µg/ml)**			**Amount found (µg/ml)**		
	RIF	**INH**	**PDX**	**RIF**	**INH**	**PDX**	**RIF**	**INH**	**PDX**
50	200	200	200	100	100	100	295.01	294.36	295.71
100	200	200	200	200	200	200	392.84	392.32	395.44
150	200	200	200	300	300	300	489.80	489.80	491.95

% Level	**% Recovery**			**% RSD**		
	RIF	**INH**	**PDX**	**RIF**	**INH**	**PDX**
50	98.33	98.12	98.57	0.23	0.34	0.27
100	98.21	98.08	98.86	0.34	0.48	0.44
150	97.96	97.95	98.39	0.89	0.30	0.72

Table 3: Results of Precision studies.

Drug	Amount of drug applied (ng/spot)	Repeatability (n=6)		Intermediate precision (n=3)			
				Intraday precision		Interday precision	
		Amount of drug found (ng/spot)	% RSD	Amount of drug found (ng/spot)	% RSD	Amount of drug found (ng/spot)	% RSD
RIF	400	397.23	0.56	396.86	1.04	395.49	0.38
	600	596.98	0.29	595.91	0.67	596.01	0.51
	800	802.37	0.14	803.16	0.41	794.95	0.30
INH	400	398.04	0.36	398.97	0.56	396.30	0.89
	600	597.43	0.79	596.79	1.20	595.76	1.21
	800	795.73	0.54	793.71	0.29	794.80	0.36
PDX	400	395.62	0.25	397.67	0.23	393.63	0.49
	600	596.62	1.08	594.20	0.39	595.32	0.27
	800	794.90	0.92	796.28	0.90	794.03	0.23

Table 4: Assay results for the combined dosage form using the proposed HPTLC method.

Formulation	Labelled amount(mg)			Amount found(mg)			Amount found(%)		
	RIF	INH	PDX	RIF	INH	PDX	RIF	INH	PDX
Rifa i-6	450	300	10	446.71	303.57	10.03	99.27±0.29	101.19±0.95	100.30±0.38

Fig. 4: Photograph of Developed HPTLC Plate

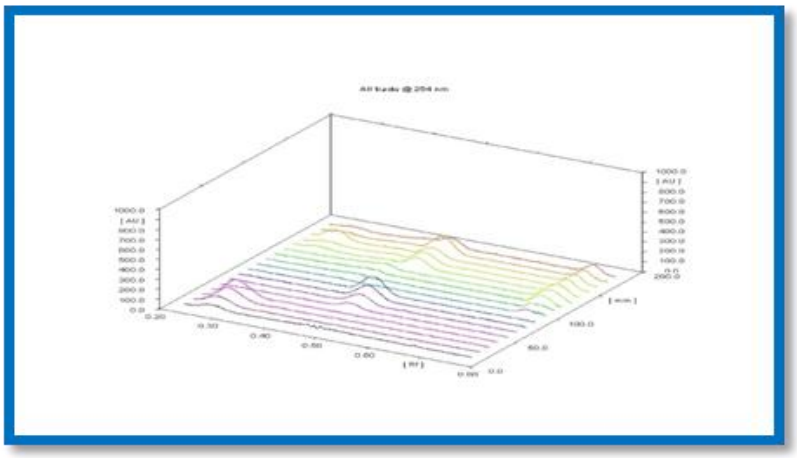

Fig. 5: 3D Overlain spectra of RIF, INH and PDX

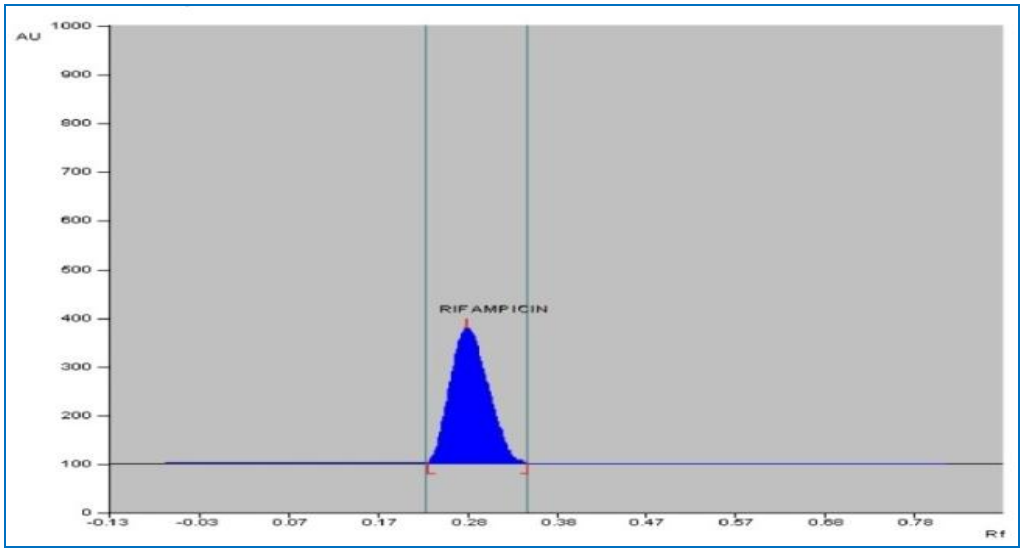

Fig. 6: Densitogram of Rifampicin.

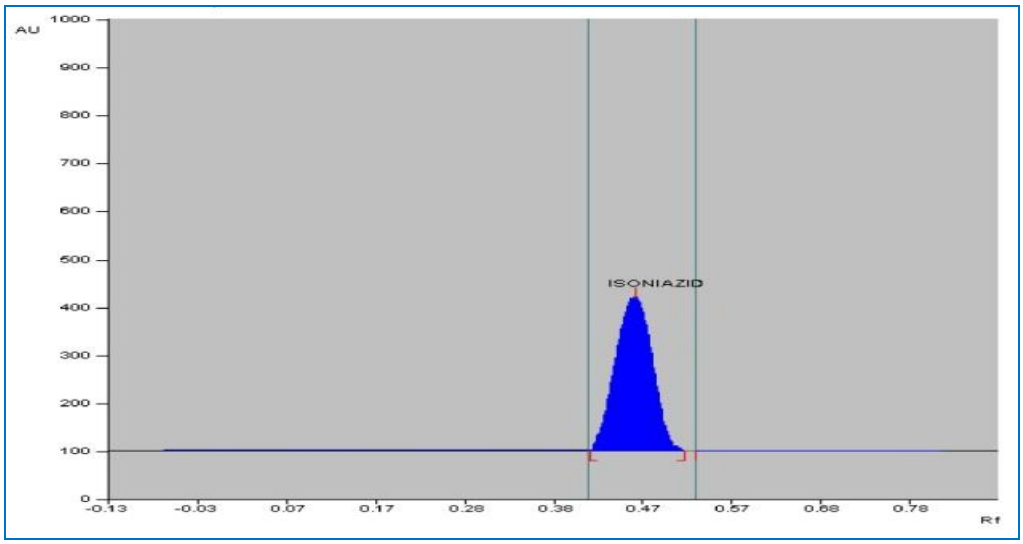

Fig. 7: Densitogram of Isoniazid

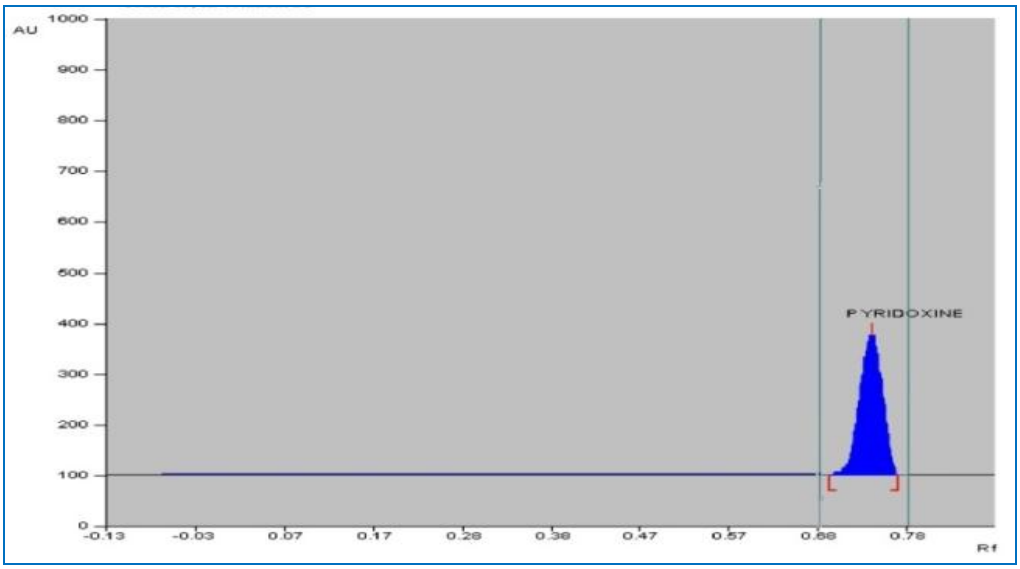

Fig. 8: Densitogram of Pyridoxine

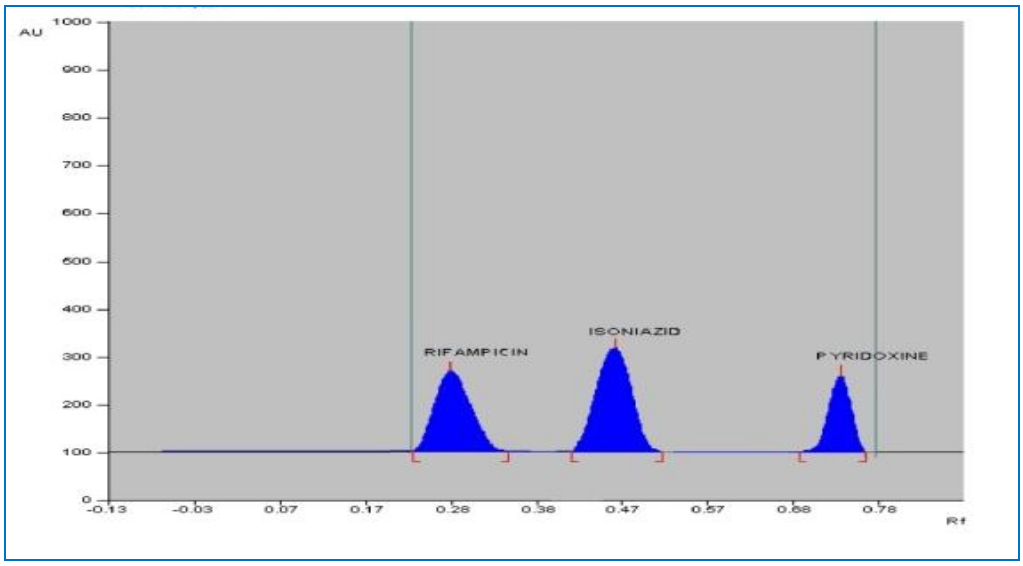

Fig. 9: Densitogram of standard mixture of RIF, INH and PDX

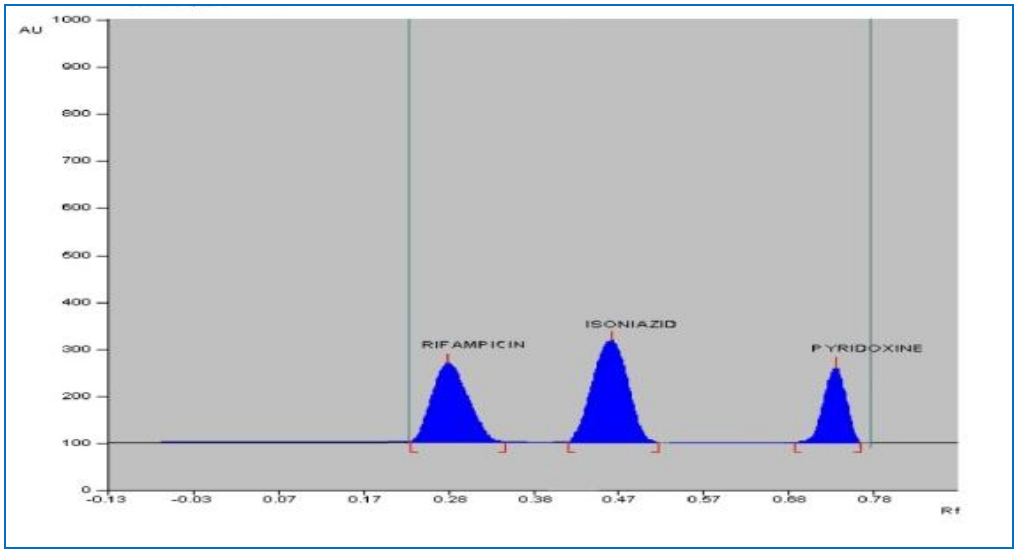

Fig. 10: Densitogram of Marketed formulation.

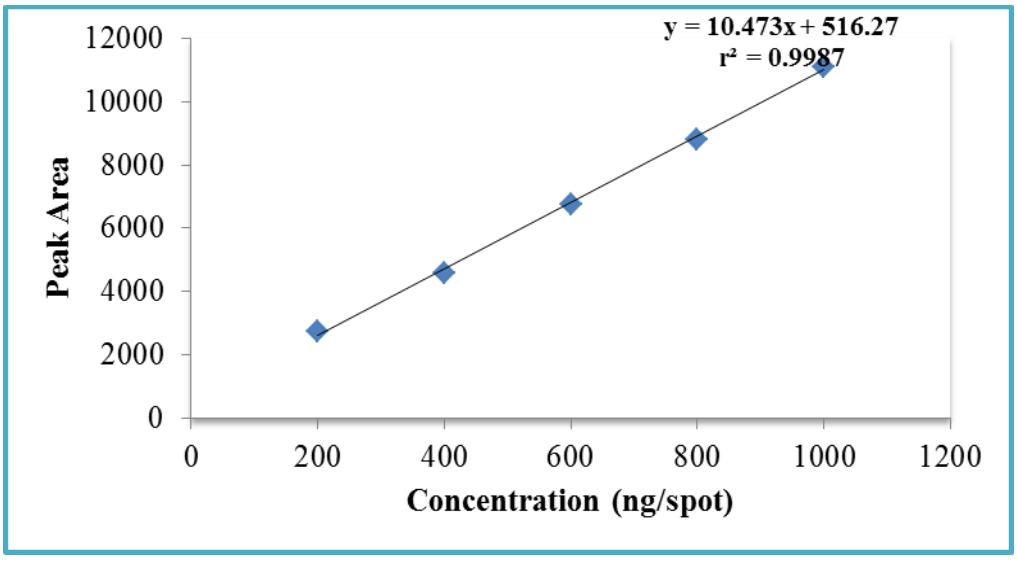

Fig. 11: Calibration curve of RIF

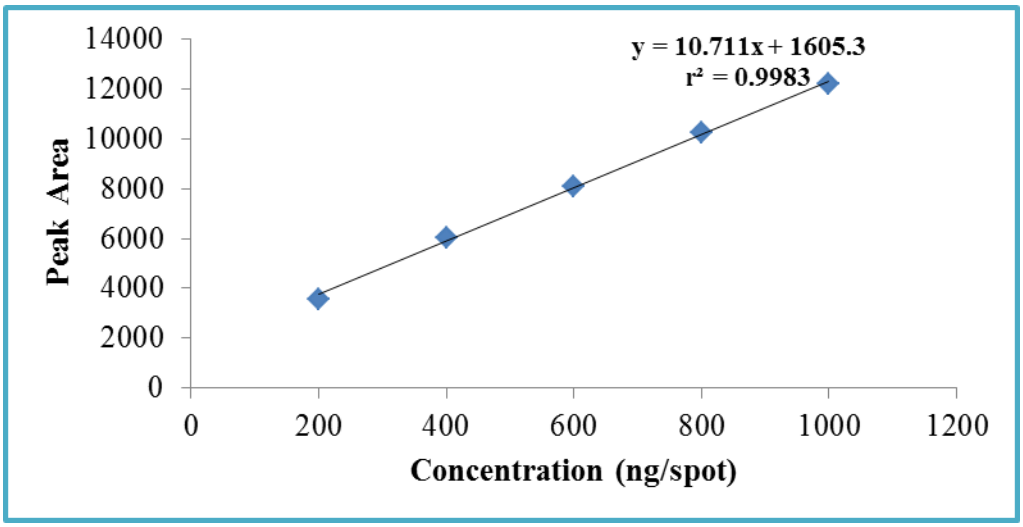

Fig. 12: Calibration curve of INH

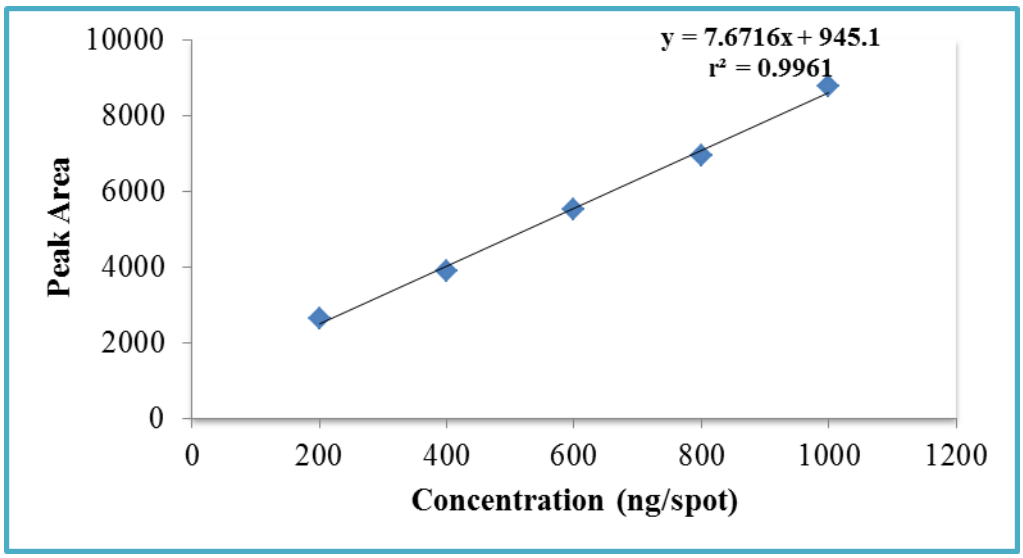

Fig. 13: Calibration curve of PDX

5. CONCLUSION

The results of analysis of pharmaceutical dosage form by the proposed method are highly reproducible, reliable and are in good agreement with label claim of the drug. The proposed method does not suffer from any interference due to common excipients. It indicates that method is accurate. Therefore the proposed method could be successfully applied to estimate commercial pharmaceutical products containing Rifampicin, Isoniazid and Pyridoxine Hydrochloride.

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