5. Analytical method development

Analytical method development and validation are the main components of any formulation development. These analytical methods are aimed to provide the physical characteristics, purity, identity and potency of the drugs. These methods were developed to support the drug testing against the various specifications during manufacturing, evaluation and as well as during their stability studies.

Before starting the method development, the researcher has to have knowledge about the information of the drug, number of compounds present, chemical structure, molecular weight, pKa, solubility and UV spectrum of the compounds.

5.1. Analytical method development of rifampicin by UV spectrophotometer

UV spectroscopic method was used in the present study for the estimation of rifampicin and isoniazid in formulations. The λ_{max} was selected for rifampicin and isoniazid in a particular medium and utilized for further quantitative analysis.

5.1.1. Rifampicin

Drug was weighed accurately for 10 mg and dissolved in 10 ml simulated intestinal fluid. 25 µg/ml of the rifampicin solution was prepared from the primary stock solution in the same buffer solution. This was scanned between 800 nm and 200 nm (UV spectrophotometer, UV-1601, Shimadzu, Kyoto, Japan). The same method was repeated with simulated gastric fluid as medium.

5.1.2. Isoniazid

Drug was weighed accurately for 10 mg and dissolved in 10 ml simulated intestinal fluid. 25 µg/ml of the isoniazid solution was prepared from the primary stock solution in the same buffer solution. This was scanned between 400 nm and 200 nm (UV spectrophotometer, UV-1601, Shimadzu, Kyoto, Japan). The same method was repeated with simulated gastric fluid.

5.2. Method validation (UV) (ICH Q2 R1 guidelines)

5.2.1. Rifampicin

5.2.1.1. Standard plot of rifampicin

Primary stock solution (1 mg/ml) was prepared in both simulated intestinal fluid and simulated gastric fluid. These primary stock solutions were further diluted with their respective media to get stock solution (100 µg/ml) in both the media.

Different concentrations in the range of 5 to 40 µg/ml were prepared from stock solution in both the media. The absorbance was measured against the respective blank solutions at 335 nm in simulated intestinal fluid and at 336 nm in simulated gastric fluid.

5.2.1.2. Accuracy

Accuracy was determined by replicate analysis of samples containing known concentrations of the analyte. The sample for recovery experiments were prepared by spiking the standard stock solutions 8, 10 and 12 µg/ml of rifampicin to the pre-analysed sample solutions with concentration of 10 µg/ml. Absorbance was measured in six replicates for all the samples.

5.2.1.3. Repeatability and intermediate precision

The concentration of 10 µg/ml rifampicin was measured for six times in the inter-day and also intra-day to determine the precision of the method.

5.2.2. Isoniazid

5.2.2.1. Standard plot of isoniazid

Primary stock solution (1 mg/ml) was prepared in both simulated intestinal fluid and simulated gastric fluid. These primary stock solutions were further diluted with their respective media to get stock solution (100 µg/ml) in both the media.

Various concentrations ranging from 5 to 35 µg/ml were prepared from stock solution in simulated intestinal fluid and concentrations from 2.5 to 25 µg/ml were prepared from stock solution in simulated gastric fluid. The absorbance was measured against their respective blank solutions at 262 nm in simulated intestinal fluid and at 265 nm in simulated gastric fluid.

5.2.2.2. Accuracy

Accuracy was mainly estimated by the replicate analysis of samples containing known amounts of the analyte. The sample for recovery experiments were prepared by spiking the standard stock solutions 4, 5 and 6 µg/ml of isoniazid to the pre-analysed sample solutions with concentration of 5 µg/ml. Absorbance was measured in six replicates for all the samples.

5.2.2.3. Repeatability and intermediate precision

The concentration of 5 µg/ml isoniazid was measured for six times in the inter-day and also intra-day to determine the precision of the method.

5.3. Analytical method development by RP-HPLC

5.3.1. Instrument and software

The analysis was carried out by Shimadzu LC-2010CHT (Shimadzu Corporation, Kyoto, Japan) which is equipped with low pressure quarternary gradient pump along with dual wavelength UV detector, column oven, auto sampler and LC solution 1.24SPI software. Grace Vydac C_{18} column (250 mm × 4.6 mm, 5 µm) was used for drug separation. The analyte was monitored at 254 nm wave length (Glass et al.). A glass vacuum filtration set fitted with 0.22 µm membrane filter was used to filter mobile phase. Ultrasonic bath was used to remove entrapped air and dissolved gases in the mobile phase.

5.3.2. Selection of chromatographic method

Proper selection of the method depends on the various physicochemical properties of the drug like molecular weight, dissociation constant, polarity and solubility (Snyder et al.). The RP-HPLC was selected because of its simplicity, suitability, ruggedness and as it is most widely used technique.

5.3.3. Optimization of chromatographic conditions

The mobile phase selected to elute rifampicin from the stationary phase contains acetonitrile because of its favourable transmittance and low back pressure. Disodium hydrogen phosphate buffer was used as aqueous mobile phase based on available literature to elute rifampicin. The standard stock solution of rifampicin (20 µg/ml) was prepared using MilliQ water as diluent, injected into HPLC system and run for 10 min. The separations were mainly affected by the mobile phase composition and conditions. So before selecting the conditions, various preliminary trials were conducted with buffer at various buffer pH, molarity, compositions and flow rate to check their effect on retention time, tailing, fronting and other parameters of rifampicin peak like tailing factor. The effects of these factors were estimated by using the system suitability parameters such as theoretical plates, retention time and asymmetry.

5.4. Analytical method validation for RP-HPLC (ICH Q2 R1 guidelines)

5.4.1. Specificity

Specificity experiment was conducted to ensure that no other analyte or impurity is interfering with rifampicin's retention time. Six replicates of rifampicin along with isoniazid and other excipients were injected. The eluent was monitored by UV detector. Peak purity for individual test injection was calculated.

5.4.2. Linearity

It is the ability to get the test results which are linearly related to the concentration of analyte. A series of solutions ranging from concentrations of 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µg/ml of rifampicin were prepared in MilliQ water and 20 µl of the each solution was injected. Then the chromatograms were obtained under the developed chromatographic conditions. Chromatogram peak area of rifampicin was plotted against the concentration to get the regression equation and its coefficient determination.

5.4.3. Accuracy

The accuracy of an analytical procedure states the closeness of agreement between the value which is accepted as true or reference value. The sample for recovery experiments were prepared by spiking the standard stock solutions 8, 10 and 12 µg/ml of rifampicin in triplicate to the pre-analysed sample solutions with concentration of 10 µg/ml. the accuracy of the method was performed by recovery studies.

5.4.4. Precision

Repeatability or precision states the reproducibility under the same operating conditions over a period of time. It is also known as intra-assay precision. 10 µg/ml solution of rifampicin was analysed at different time intervals and the percentage relative standard deviation was calculated. Intermediate precision is carried out by analysing 10 µg/ml of rifampicin in between days and by different analysts.

5.4.5. Robustness

Ability of a method to remain unaffected by small intentional variations in method parameters is known as robustness. Changes in the mobile phase composition (±5%), flow rate (±0.1 ml/min), pH of the mobile phase (±0.1) and injection volume (±10 µl) were used as the variations to test the robustness.

5.4.6. LOD and LOQ

LOD is the capability to detect the smallest concentration of the analyte, whereas LOQ is the lowest concentration of the analyte that can be accurately and precisely quantified. It was calculated based on the standard deviation of the response and the slope as per ICH guidelines. These parameters were determined by the following equations.

$$LOD = 3.3[SD/S]$$

$$LOQ = 10[SD/S]$$

Where SD is the standard deviation of the response and S is the slope of calibration curve.

5.5. Results and discussion

5.5.1. UV spectroscopy

The maximum absorbance for rifampicin in simulated intestinal fluid was observed at wavelength 335 nm whereas in simulated gastric fluid it was observed at 336 nm (Fig. 5.1).

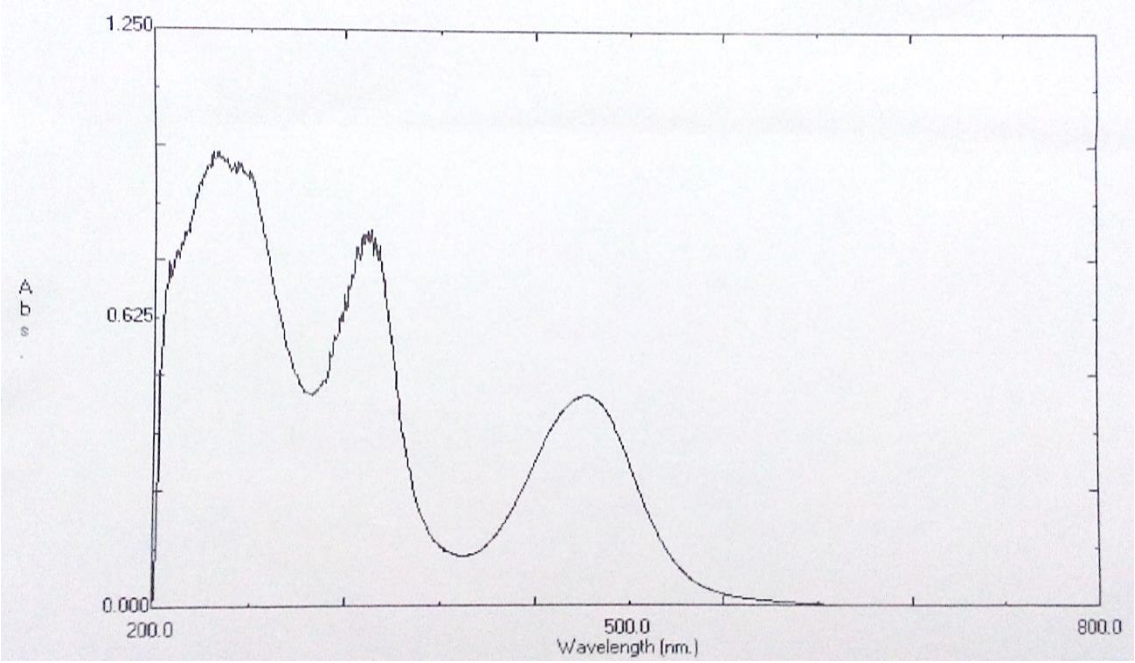

Fig. 5.1. UV spectrum of rifampicin in simulated gastric fluid

The maximum absorbance for isoniazid in simulated intestinal fluid was observed at wavelength 262 nm whereas in simulated gastric fluid it was observed at 265 nm (Fig. 5.2).

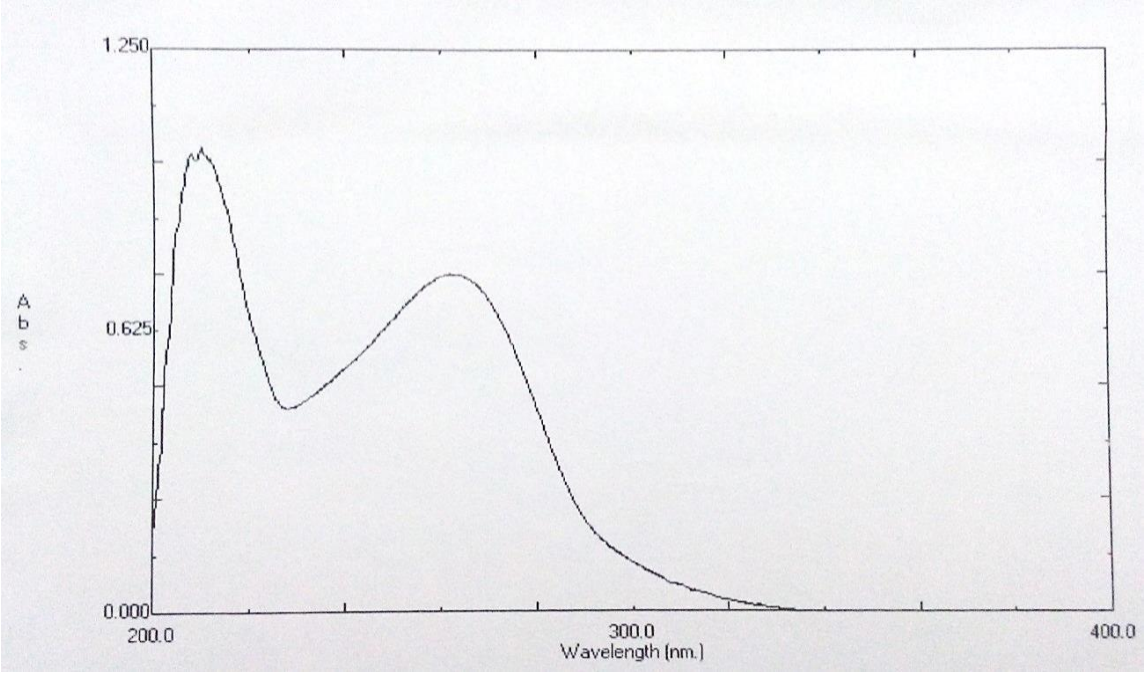

Fig. 5.2. UV spectrum of isoniazid in simulated intestinal fluid

5.5.2. Method validation (UV)

The absorbance of rifampicin standard solution was measured against their respective blank solutions at 335 nm in simulated intestinal fluid and at 336 nm in simulated gastric fluid (Fig. 5.3 and 5.4).

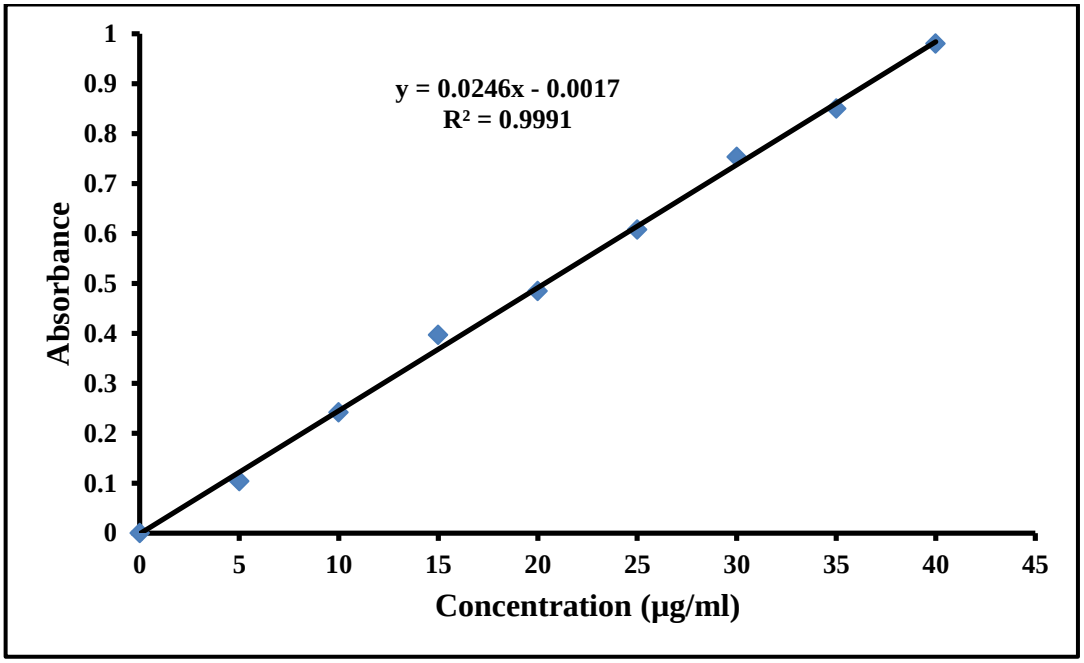

Fig. 5.3. Calibration curve of rifampicin in simulated intestinal fluid

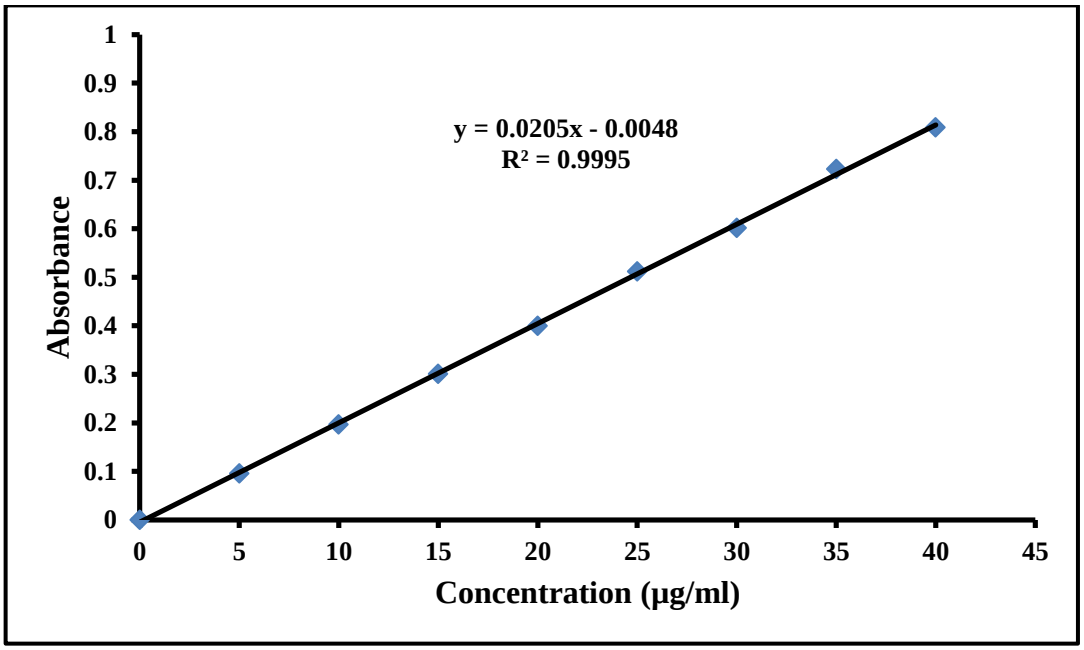

Fig. 5.4. Calibration curve of rifampicin in simulated gastric fluid

The absorbance was measured against the respective blank solutions at 262 nm in simulated intestinal fluid and at 265 nm in simulated gastric fluid (Fig. 5.5 and 5.6). Results of method validation are summarised in Table 5.1.

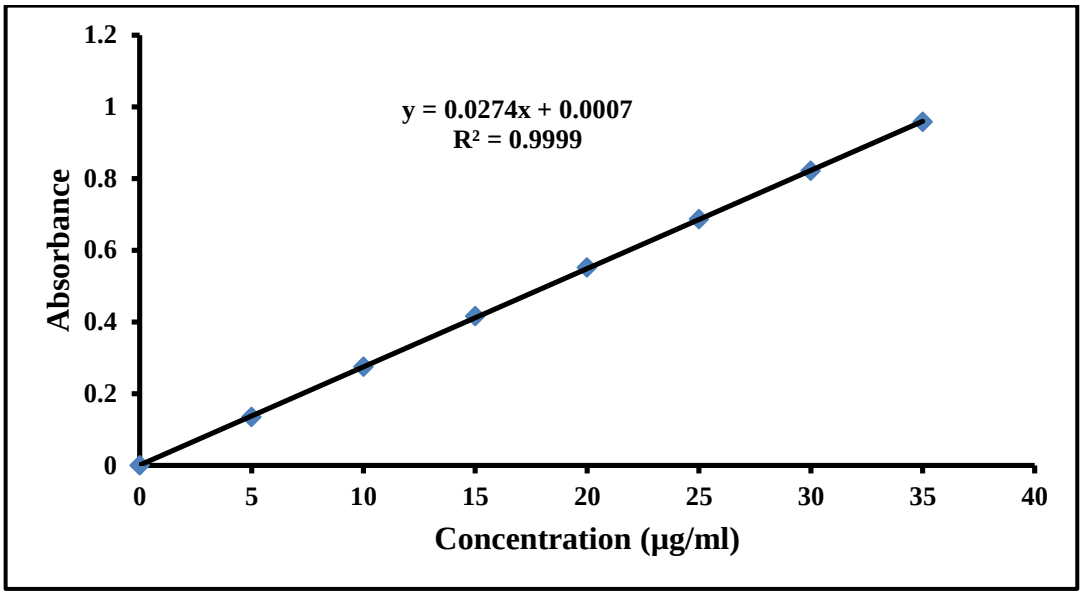

Fig. 5.5. Calibration curve of isoniazid in simulated intestinal fluid

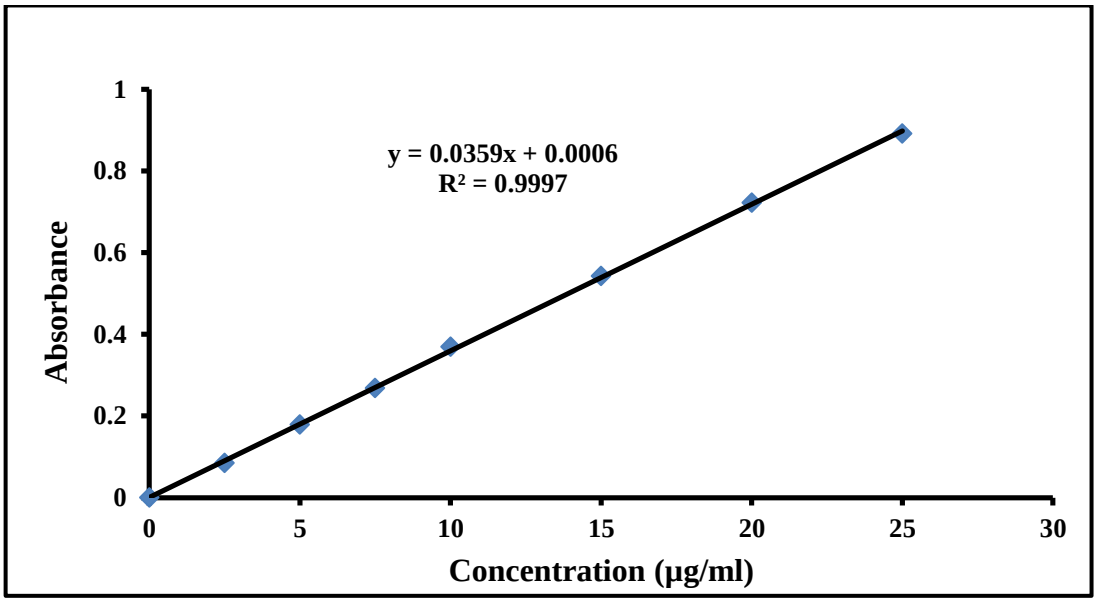

Fig. 5.6. Calibration curve of isoniazid in simulated gastric fluid

Table 5.1. Summary of analytical method validation by UV

Validation parameters	Validation results		Acceptance criteria
	Rifampicin	Isoniazid	
Accuracy (% Mean±SD)	99.18±0.94	100.32±0.87	98-102
Repeatability precision (% RSD)	0.71±0.16	0.56±0.26	<1
Intermediate precision (% RSD)	1.43±0.21	1.12±0.34	<2
Linearity (R^2)			
simulated gastric fluid	0.9995	0.9997	>0.999
Simulated intestinal fluid	0.9991	0.9999	

5.5.2. Analytical method development by RP-HPLC

5.5.2.1. Optimization of chromatographic conditions

Due to inherent lipophilicity associated with rifampicin, reversed phase was selected for method development. Rifampicin is a weak base having two pKa values; the pKa_1 is 1.7 which is due to ionization of 4-hydroxy moiety while pKa_2 is around 7.9 due to 3-piperazine nitrogen. This means that rifampicin is prone to ionization at any pH below 1.7 and at the same time it ionizes at any pH above 7.9. To improve its retention and chromatographic properties, pH in the range of 3 to 4 was ideal as it is unionized. After optimization pH of 3.1 was selected as this ensured that the drug would predominantly remain in the unionized form. Disodium hydrogen phosphate buffer was selected and its pH was adjusted to 3.1 using orthophosphoric acid. In the present study 50 mM disodium hydrogen phosphate buffer of pH was selected as the elution was rapid with adequate system suitability and a sharp peak shape with asymmetric factor (<1.5) was obtained. Mobile phase composition of 80:20 v/v of Acetonitrile: disodium hydrogen phosphate buffer, pH 3.1 (Glass et al.) was selected. When percentage of organic phase was increased, decrease in the retention time was observed. This might be due to the higher strength of acetonitrile. Based on the initial optimization procedure, following chromatographic conditions were selected for the final validation of rifampicin.

Optimized chromatographic conditions:

Stationary phase: Grace C_{18} column (250 mm × 4.6 mm, 5 µm)

Mobile phase: Acetonitrile: 50 mM disodium hydrogen phosphate buffer pH 3.1 (80:20) (Glass et al.)

Detection wave length: 254 nm (Glass et al.)

Flow rate: 0.6 ml/min

Injection volume: 20.0 µl

Column oven temperature: 25 ^{o}C

Auto sampler temperature: 4 ^{o}C

Run time: 10 min

The representative standard chromatogram of rifampicin is shown in the Fig. 5.7. Retention time of rifampicin was found to be 5.67 min.

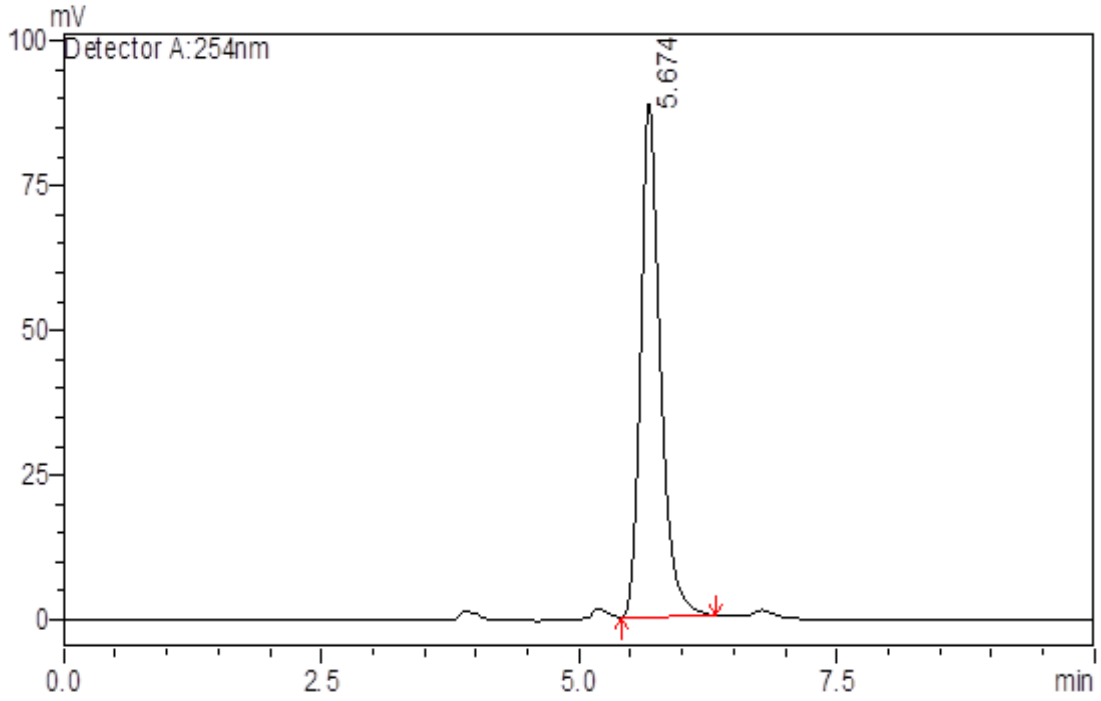

Fig. 5.7. Representative standard chromatogram of rifampicin

5.5.3. Validation of analytical method

HPLC method validation is used to confirm that the HPLC method developed for a specific test is suitable for its intended use. The results obtained from this validation process can be used to judge the reliability, quality and consistency of HPLC results.

5.5.3.1. Specificity

There was no interference of other ingredients at the retention time of rifampicin which confirms the method is specific.

5.5.3.2. Accuracy

The recovery values at these 3 different concentrations were observed to be in the range of 98 to 102%. This is as per ICH guidelines requirement. Average percentage recovery was found to be 100.08±1.25.

5.5.3.3. Precision

The repeatability precision (% RSD) of the proposed method was found to be 0.23 % RSD and the intermediate precision of the proposed method was found to be 1.44 % RSD. The acceptance criteria for the repeatability and intermediate precision are <1 and <2 % RSD respectively. The results of the proposed method indicate that method was precise and reproducible.

5.5.3.4. Linearity

The proposed method was linear in the range from 1-20 µg/ml. The slope and intercepts were used for the determination of unknown drug present in various formulations. Linearity is generally reported by the coefficient of determination (R^2). The present method was found to be >0.999 which indicated the proposed method was linear. Regression equation was y = 60664x – 11858. Calibration curve is shown in the Fig. 5.8.

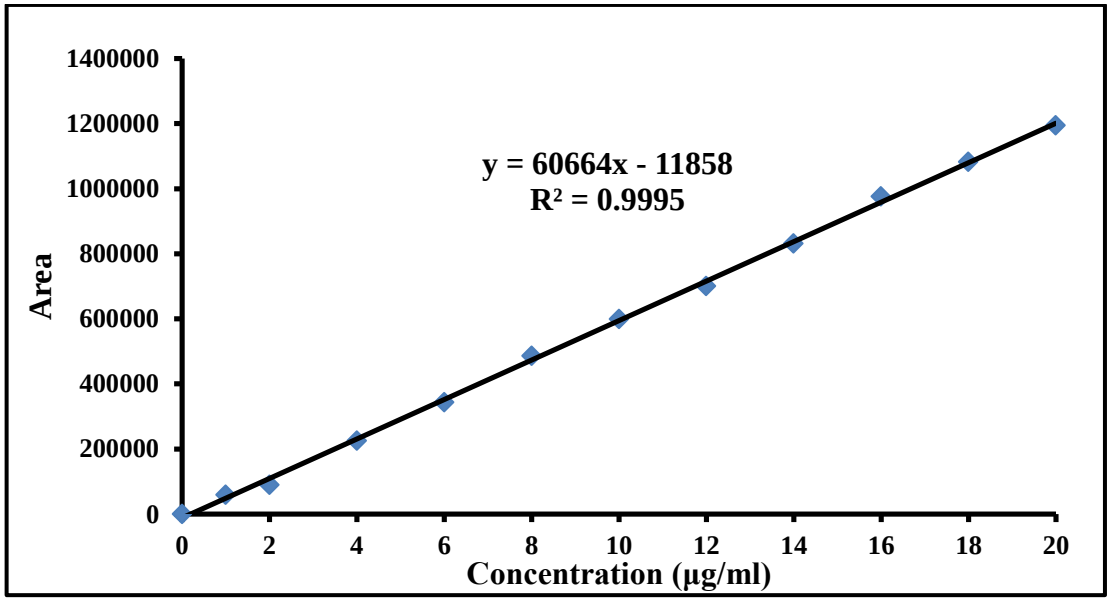

Fig. 5.8. Calibration curve of rifampicin by RP-HPLC method

5.5.3.5. Robustness

The robustness of a method was evaluated by varying method parameters such as percentage organic solvent, pH of the buffer, injection volume and flow rate of the mobile phase. The % RSD in these various parameters was found to be within 2%. The results shown in the Table 5.2 indicate that the method is robust.

5.5.3.6. LOD and LOQ

The present method LOD and LOQ was calculated based on the standard deviation of the responses and the slope. The present method LOD and LOQ was found to be 14.2 ng/ml and 43.1 ng/ml respectively. This indicates that the developed method was sensitive for the quantification of rifampicin.

5.5.3.7. System suitability

The overall system suitability was evaluated by the percentage asymmetry of rifampicin peak was 1.2 which indicated that the peak shape was symmetrical. The high counts of theoretical plates of 5032 revealed the column efficiency. The % RSD is 0.26 which is well

below 1% (6 injections) and tailing factor is 1.038 which is below 1.5. These results indicate that the proposed method passes the system suitability test. Summary of analytical method validation of rifampicin by HPLC method results are given in the Table 5.2.

Table 5.2. Summary of analytical method validation of rifampicin by HPLC

Validation parameter	**Acceptance criteria**	**Result**	
Linearity	The correlation coefficient should be NLT 0.999	0.9995	
Precision			
Repeatability	% RSD of 6 injections should be NMT 1%	0.2321	
Intermediate precision	% RSD of 6 injections should be NMT 2%	1.4362	
Accuracy			
Accuracy	% Recovery at each level should be between 98-102%	80%	99.2837
		100%	99.4173
		120%	101.5362
Robustness			
Change in mobile phase ratio	% RSD of 6 injections should be NMT 2%	15:85	1.0514
		25:75	1.1256
Change in pH of the buffer		pH 2.80	1.2397
		pH 3.40	1.1624
Change in flow rate		0.5 ml/min	0.1230
		0.7 ml/min	0.2421
Change in injection volume		10 µl	0.1642
		30 µl	0.1982
LOD and LOQ		14.2 ng/ml and 43.1 ng/ml	
System suitability			
Percentage asymmetry		1.2	
Retention factor		5.674 min	
Tailing factor	NMT 1.5	1.038	
No. of theoretical plates		5032	
Precision	% RSD of 6 injections should be NMT 2%	0.26	