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Research Article

NANO FORMULATION ANALYSIS: ANALYTICAL METHOD DEVELOPMENT OF ISONIAZID AND SIMULTANEOUS ESTIMATION OF ANTITUBERCULAR DRUGS ISONIAZID AND RIFAMPICIN BY REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

SUSHRUTA S HAKKIMANE[1], BHARATH RAJA GURU[1,2]*

[1]Department of Biotechnology, Manipal Institute of Technology (MIT), Manipal University, Manipal, India. [2]Department of Member, Manipal McGill Center for Infectious Diseases, Manipal, India. Email: bharath.guru@manipal.edu

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ABSTRACT

Objective: The objective of the study was to develop and validate a simple and reproducible reverse phase high pressure liquid chromatography (RP-HPLC) method for hydrophilic drug isoniazid (INH) to apply for the analysis of the INH in nanoparticle drug formulations. Furthermore, to estimate simultaneously rifampicin (RIF) and INH in combined form.

Methods: Isocratic elution with 10 minutes runtime on a C-18 Luna, 5 µ, 100Å, 150 mm column, methanol, and water as mobile phase with detection wavelength at 268 nm was used. INH nanoformulations were prepared by double emulsion solvent evaporation technique. Quantitative analysis of encapsulated drug was estimated via developed RP-HPLC method. Simultaneous estimation for the two drugs was carried out by gradient elution. All chromatographic separation and estimations were obtained on Shimadzu HPLC system.

Results: INH eluted with a short retention time (RT) of 4.06 minutes. Method showed good linearity in the range of concentrations 0.01-100 µg/ml. The limit of detection (LOD) and quantification (LOQ) for INH was 0.03 and 0.12 µg/mL, respectively, and developed method has been successfully applied for the analysis of drugs in nanoparticle formulations. Simultaneous estimation of antitubercular drugs INH and RIF showed two separate peaks within specified runtime.

Conclusion: Developed method showed good resolved peaks. Since the RT is short, in a shorter duration more samples could be completed and developed method will be easy for analyzing greater number of samples. Analysis of nanoformulation results have shown that this method is simple, reliable, reproducible, hence can be applied for drug delivery analysis.

Keywords: Antitubercular drugs, Reverse phase high performance liquid chromatography, Analytical method development.

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INTRODUCTION

An important step in nanoparticle drug delivery is the development of a simple and reproducible analytical method to analyze the drug concentrations. At present, reverse phase high pressure liquid chromatography (RP-HPLC) is the most effective and widely used technique for analyzing drugs in dosage forms and drug concentrations. In HPLC method development phase, all the parameters related to the method should be considered. Here, these parameters include choice of column, mobile phase, detectors, and method of quantitation. While developing new method best parameters should be selected [1]. In some cases, preferred elution or separation occurs with minimal experimentation. Some may require more number of experimental trials. Selection of best mobile phase, choice of column, detectors, stationary phase, column length, and column internal diameter plays important characters in HPLC method development steps. So process contains consideration of all these parameters pertaining to such method. In Reverse phase chromatography, common stationary phases in columns are the hydrophobic support that consists mainly of porous particles of silica gel in various shapes of various diameters and pore sizes. These particles surface is covered with many chemical entities, such as various hydrocarbons (C1, C6, C4, C8, C18, etc.) [2]. Compound which is more hydrophobic will be retained longer on the column than lesser hydrophobic one, based on the hydrophobicity they get separated. Organic and aqueous phases are the two components which are used as mobile phase [3]. As per "World Health Organization" reservation, INH is one the front line agent for the treatment of tuberculosis and INH pertains to the category of nucleoside reverse transcriptase inhibitor [4], with aqueous solubility of 140 mg/ml [5]. To determine polar compounds various analytical methods exists specifically for INH in literature [6]. Various other methods also have been described for determination and quantification of INH, such as H-point standard addition method [7], selective adsorption using a piezoelectric sensor [8], voltammetric method [9], amperometric method [10], chromatographic methods [11-14] (HPLC, GC and HPTLC), titrimetric methods [15], and chemiluminescence [16]. In addition, several (HPLC) methods for the simultaneous determination of rifampicin (RIF), INH, and pyrazinamide (PZA) in a fixed-dose combination [17] simultaneous analysis of RIF, INH and PZA in 0.1 M hydrochloric acid dissolution medium and in simulated gastric fluid [18], quantitative determination of INH in plasma, brain, liver and kidney samples and in solid lipid nanoparticles [19]. RIF and INH in combined dosage form estimation by UV spectrophotometric method [20,21] stability-indicating high performance liquid chromatographic simultaneous determination of RIF, INH and PZA in human plasma [22] Most of the methods are time-consuming (long retention times [RT]) along with gradient methods. Simple isocratic RP-HPLC method is not available for determining quantitatively INH entrapped in polymer nanoparticles. TB treatment involves an initial 2 months' therapy with combination of 4 drugs and subsequent 4 months with combination of both RIF and INH [23]. Since our work aimed to formulate this combination of INH and RIF (Fig. 1) [24] in nanoparticles, a simultaneous estimation of these two drugs becomes very important. However, there exist practical limitations, especially with regard to drug stability of RIF when estimated along with INH. Research to overcome these challenges in nanoformulation is being carried out.

Here, a simple, precise, accurate, and reproducible RP-HPLC method has been developed and validated for the estimation and of hydrophilic drug INH and also simultaneous estimation of RIF and INH in combined form is shown in Fig. 1.

METHODS

Instrumentation

SHIMADZU Model No LC-20AD RP-HPLC system attached with Autoinjector SPD-20A and UV detector, LC solution software was used to record and integrate chromatograms, and the stationary phase column (phenomenex Luna C18, 4.6×150 mm, 5 µm, 100A°) was used for the analysis. Q Sonica - Sonicator Q -700 model, electronic balance (SHIMADZU AUW 220D), UV-1700-Visible Spectrophotometer Pharma Spec, Ultra Bath Sonicator from Grant Instruments UK, Magnetic stirrer (SCHOTT Instrument) Millipore Water purification system.

Materials and reagents

Poly(lactic-co-glycolic acid) (PLGA) 50:50 MW: 1,00,000-1,20,000 Da was procured from Duret corporation AL USA, INH purchased from Himedia Ltd., Mumbai, Methanol (MERK Millipore Mumbai), Trifluroacetic acid (Loba Chemie, Mumbai, India) and all other reagents and chemicals were of HPLC grade. Polyvinyl alcohol (PVA) (87-89% hydrolyzed, MW: 1,20,000) procured from Thomas baker.

Experimental methods

Preparation of mobile phase

To establish a relevant HPLC method, couple of different mobile phases were attempted. The selection of mobile phase was decided based on the readily available solvents, short run time, results and sensitivity of assay. Mobile phases concluded here were aqueous solvent, i.e., MilliQ water and an organic solvent methanol. Solvents were filtered separately through 0.45 µm membrane filter and degassed by sonication.

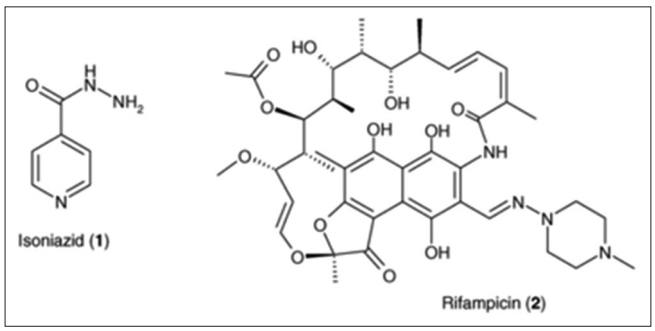

Fig. 1: Chemical structure of (1) isoniazid (2) rifampicin

Preparation of standard stock solution

About 10 mg of INH was weighed exactly and dissolved in 10 ml of water to make final concentration of 1 mg/ml.

Validation of the method

Method validation for developed RP-HPLC method was checked for linearity, specificity, precision, accuracy, and system suitability, LOD and LOQ as per the ICH guidelines [25].

Statistical analysis

Using Excel program, the method of least squares was calculated for the value of %relative standard deviation (RSD) and the linear regression analysis.

Analysis of isoniazid in nano formulations

Double emulsion solvent evaporation method was used to prepare INH nanoformulation. In brief, INH is dissolved in water and PLGA in dichloromethane. Primary emulsion was mixed with aqueous PVA solution to form a water-in-oil-in-water emulsion. Sonicated at 60% to form emulsion followed by solvent evaporation step. Repeated centrifugation was done to wash away the PVA at 13000 rpm. Pellet was re-dispersed in water, frozen overnight at −80°C and subjected to lyophilisation for 48 hrs to obtain powder form of the nanoparticles. We performed HPLC analysis using above developed method to know the amount of drug encapsulated in prepared nanoparticle formulation batches, 1 mg each INH loaded nanoparticles were weighed in triplicates. 1 ml methanol was added to each vial and placed on rocker shaker for 2d. Supernatant was collected after 2d. By centrifuging them at 10000 rpm for 20 minutes. Analyzed by developed RP-HPLC method and calculated for drug loading amount.

RESULTS AND DISCUSSION

Optimization and method development for isoniazid

INH was determined using RP-HPLC method, during the method development, many parameters with different combinations were tried, and different columns were assessed for RT, peak shape, and resolution of INH, after a number of trials, the method resulted with sharp clear peak with minimum tailing factor and short runtime was selected. INH eluted from the column at 4.06 minutes (Fig. 2) with flow rate of 1 ml/minutes using mobile phases, i.e., methanol and water (10:90). Analysis of INH was done with reverse phase phenomenex® Luna C18, 5 µ, 100A° 150 mm column in an isocratic mode with an injection volume 20 µl. Run time was set to 10 minutes, eluent seen at wavelength 268 nm. The method has been successfully applied for the analysis of the drug in nanoparticle drug formulations.

Linearity

It is an ability to attain test results that directly proportionate to the concentration of drug molecules present in the sample [26,27].

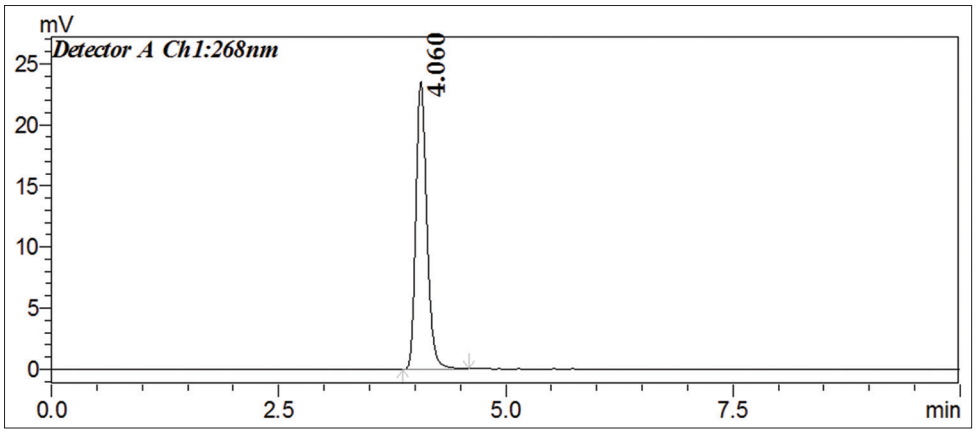

Fig. 2: Chromatogram showing isoniazid peak at 4.06 retention time

It is necessary to analyze within a suitable range for which the instrument's response must be proportional to the drug concentration. Normally, value of co-relation coefficient (R^2) >0.998 is acceptable [28].

A series of calibration standards were made by diluting the stock solution appropriately using HPLC grade water to make solutions consisting INH in the concentration range of 0.01-100 µg/ml. Calibration graph was plotted by considering peak area versus concentration of the compound.

The linearity was evaluated by using linear regression analysis, which was calculated based on the least square regression method. Calibration curve with concentrations in tested range, found to be linear for INH. Peaks eluted at same RT 4.06 minutes and the high value correlation coefficient (R^2) signified a good linearity since it was 1 (Fig. 3), where the correlation between the area of the peak and concentration is at good level. Lowest detection was found to be 0.03 µg/ml (Fig. 4) in a given range of concentrations.

Precision

With two levels (i.e., repeatability and intermediate) precision was determined, as mentioned in ICH recommendations. Sample application of repeatability was determined as intraday variation and of intermediate precision was determined as interday variation (Table 1).

Specificity

Ability to separate and to measure accurately the peak of interest indicates its specificity. This was checked by injecting replicates of INH standards and mixtures of INH and RIF.

There were clear separation and no additional peak in the chromatograms (Fig. 5), the % RSD for RT <0.5% indicate that the method developed is reproducible and has precision (Table 2).

System suitability

The system suitability test is used to ensure that the HPLC system and procedure are adequate for the analysis. For any method, the parameters of system suitability with their respective acceptance criteria should be a prerequisite. This will give an additional level of certainty that mobile phase, temperature, flow rate, and column used were proper and will ensure better system performance. This mostly includes least, a condition for injecting precision, sensitivity, and RT of the target analyte [29] HPLC system was stabilized for 30 minutes, with 3 blanks followed by 6 replicates of a single concentration of INH standard solution, injected to check the suitability of the system in this method. Parameters of this test were column efficiency (number of theoretical plates) asymmetry of chromatographic peak and reproducibility as RSD of the peak area of 6 injections of standard solutions. The peak area of %RSD and the drug RT were within 1% for entire samples (Table 3 indicates the system suitability).

LOD and LOQ

In a sample, lowest concentration of an analyte which can be detected but quantification is not necessary under a given conditions of the experiment (LOD). LOQ in a sample is lowest concentration that can be analyzed with acceptable accuracy and precision under a given conditions of the analysis.

To calculate LOD and LOQ for validating the developed RP-HPLC method, equation applied were LOD = 3.3 σ/s and LOQ = 10 σ/s [30,31].

Also when standard solutions of INH was analyzed in range of 0.01-100 µg/ml concentration the lowest detectable concentration was found to be 30 ng (Fig. 4).

Drug loading analysis in isoniazid polymeric nano formulations

Dry form of PLGA INH nanoparticles (67 mg) was obtained after lyophilization. The amount of drug encapsulated per mg of nanoparticles was estimated using calibration curve substituting the area obtained via HPLC analysis (Table 4).

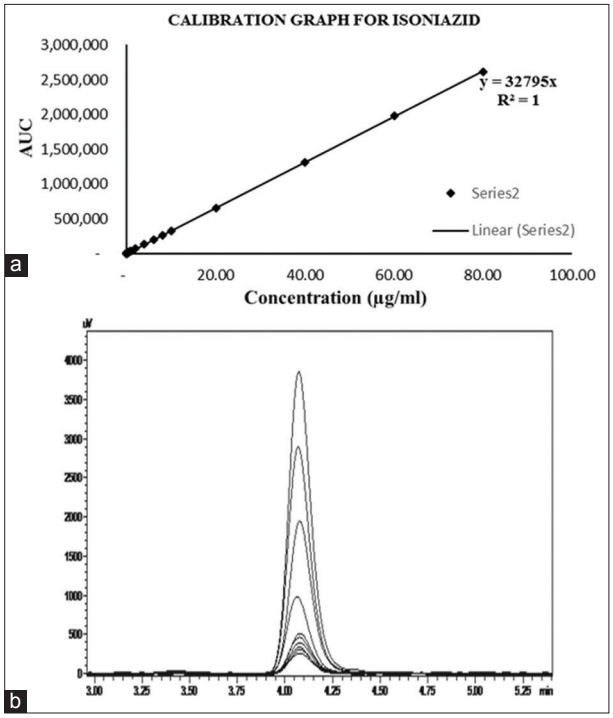

Fig. 3: (a) Calibration graph for isoniazid, (b) standard solutions of isoniazid peaks at same RT at 4.06 minutes

Table 1: Intra- and inter-day precision of RP-HPLC method for Isoniazid

Actual value (µg mL^{-1})	INH		
	40	60	80
Intraday repeatability			
Mean concentration found (µg mL^{-1})	1,167,427	1,761,028	2,334,372
Number of replicates*	3	3	3
SD	465	3795	10302
%RSD#	0.04	0.22	0.44
Interday repeatability			
Mean concentration found (µg mL^{-1})	1,177,441	1,702,516	2,370,618
Number of replicates*	3	3	3
SD	2763	1555	5912
%RSD#	0.23	0.93	0.25

RP-HPLC: Reverse phase high pressure liquid chromatography, *Average of 3 determinations; #RSD: Relative standard deviation

Table 2: Calculation for specificity

Samples*_INH ug/ml	RT
10_1	4.062
10_2	4.059
10_3	4.062
10_4	4.061
10_5	4.067
10_6	4.062
Mean±SD	4.06±0.0026
RSD	0.0006
%RSD	0.06

RT: Retention time, SD: Standard deviation, RSD: Relative standard deviation, *Analysis of 6 determinations

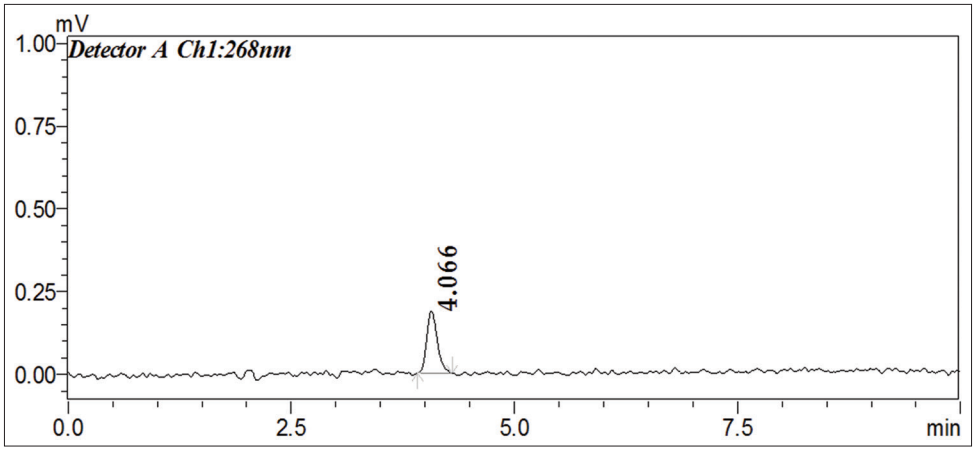

Fig. 4: Chromatogram showing peak of lowest detection 0.03 µg/ml

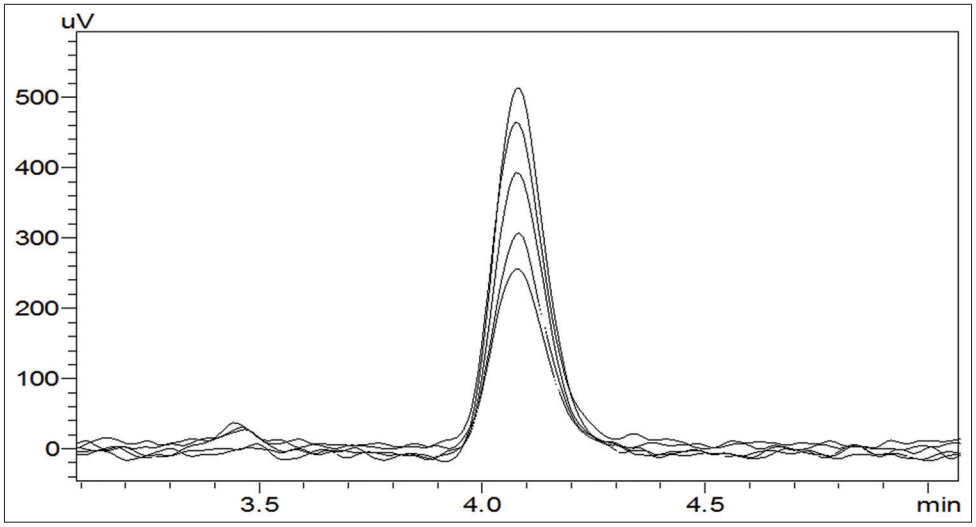

Fig. 5: Replicates of isoniazid standards showing specificity

Table 3: Results of System suitability

Samples*	INH µg/ml
INH 10 µg/ml_01.lcd 4.06	3,28,053.00
INH 10 µg/ml_02.lcd 4.05	3,27,679.00
INH 10 µg/ml_03.lcd 4.06	3,28,836.00
INH 10 µg/ml_04.lcd 4.06	3,27,945.00
INH 10 µg/ml_05.lcd 4.06	3,28,454.00
INH 10 µg/ml_06.lcd 4.06	3,28,152.00
Mean	3,28,186.50
SD#	407.01
RSD	0.0012
%RSD	0.12
Slope	32795.00
LOD	0.0372
LOQ	0.1241

SD: Standard deviation, LOD: Limit of detection, LOQ: Limit of quantification, RSD: Relative standard deviation, *Concentration and Area of 6 replicates of Isoniazid, #Analysis of 6 determinations

Table 4: Drug loading measurement of Isoniazid loaded PLGA nano formulation batch results

Batch	Area (units)	Encapsulation (amount of drug per mg of nanoparticle) (in µg)
1	278372	7.6
2	364582	10.05

Average=8.8±1.2 µg/mg, PLGA: Poly (lactic-co-glycolic acid)

Similarly, method has been successfully applied for the analysis of drugs in different batches of nanoparticle drug formulations.

Simultaneous estimation of anti-tubercular drugs

Analysis of RIF and INH were done with reverse phase phenomenex® Luna C18,5 µ, 100A° column in a gradient method. INH is a hydrophilic drug so initially when aqueous phase is more it elutes first with RT of 3.5 minutes and slowly when organic mobile phase was increased RIF elutes at 15.5 minutes, this method shows separate 2 clear peaks with different RT. The eluents were seen at wavelength 290 nm for RIF and 268 nm for INH (Fig. 6).

Calibration graph for simultaneous estimation of RIF and isoniazid

INH solution was done by diluting INH in water and RIF solution was made by diluting RIF in methanol to make primary solutions initially. Mixed the solutions by transferring 1 ml each from both the stock and vortexed to make final concentration of 1 mg/ml. Different standard solutions were prepared in the range of 10-100 µg/ml concentrations. Samples were vortexed and loaded to the autosampler and analyzed.

Equation of the curve was 79517x (R^2 = 0.9982) for RIF and 42551x (R^2 = 0.9996) for INH (Fig. 7), shows standard curves were found to be linear in the tested concentration range for RIF and INH. The R^2 values specified the linearity was in the studied range.

From initial concentrations to last run when chromatograms were observed, we could able to see the extra peaks indicating degradation of RIF in simultaneous estimation (Fig. 8).

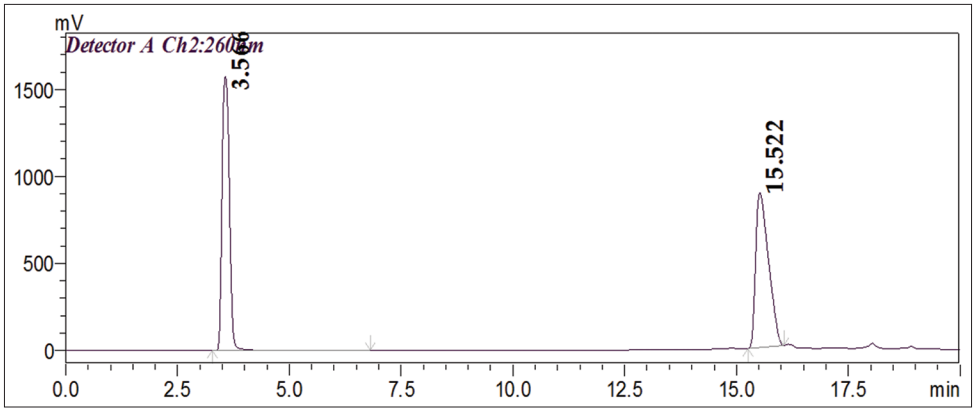

Fig. 6: Chromatogram showing 2 peaks at 2 different retention time

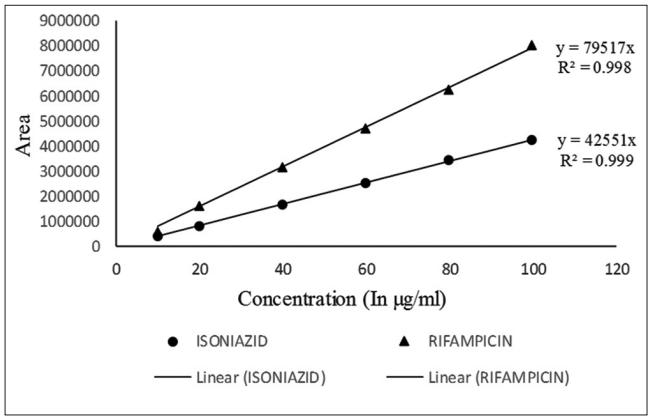

Fig. 7: Calibration graph for combination of drugs

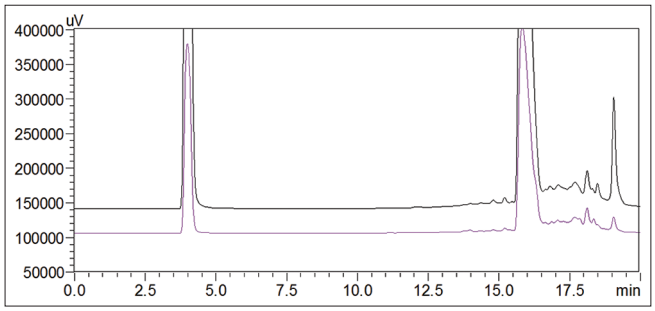

Fig. 8: Chromatogram showing extra peaks in simultaneous estimation of INH and RIF

This data would be very useful in our further combination studies to build in quality, efficacy and safety in drug formulations are being carried out.

CONCLUSION

The analysis method developed for INH has shown good resolved peaks. Since the RT is short, it indicates that in a shorter duration more samples could be completed and developed method will be easy for analyzing larger samples. The values of LOD and LOQ for these both drugs were significantly low; hence, it can be concluded that this method is appropriate for detecting and quantifying the fairly low concentrations of these drugs. Results of statistical analysis, lower %RSD (i.e., <2.0%) values confirm the ability of the analytical assay. We have used it for Nanoparticle Encapsulation efficiency and release studies. Analysis of nanoformulation results have shown that this method is simple, reliable, precised, accurate, linear and reproducible, hence can be applied for drug delivery analysis. This method could be even suitable in active pharmaceutical preparations for quality control analysis.

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