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Analytical method development and validation for estimation of rifampicin and isoniazide in bulk and tablet dosage form by high performance liquid chromatography

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ABSTRACT

A new, simple and accurate, precise HPLC method was developed for simultaneous determination of Rifampicin and Isoniazidein bulk and in combined pharmaceutical dosage form. The separation of Rifampicin and Isoniazidewas achieved within 8 minutes on an Agilent Zorbax (C18) (150mm x 4.6mm, 5µm) column using Methanol: Acetate Buffer pH-3.8 (24:76v/v)as the mobilephase.Detection was carried out using wavelength at 263nm. The method showed adequate sensitivity concerning linearity, accuracy and precision over the range 100-500µg/ml and 30-70µg/ml for Rifampicin and Isoniazide, respectively. Careful validation proved advantages of high sensitivity, accuracy, precision, selectivity, robust and suitability for quality control laboratories. The developed method was robust as the %RSD was within the range and without effecting system suitability parameters. The proposed method is suitable for simultaneous determination of Rifampicin and Isoniazidein bulk and pharmaceutical dosage form.

Keywords: Rifampicin and Isoniazide,HPLC, Validation, Precision, Robustness, ICH Guidelines.

INTRODUCTION

Chromatography [2]

Chromatography is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the *mobile phase*, which carries it through a structure holding another material called the *stationary phase*. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation. [1]

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for later use, and is thus a form of purification. Analytical chromatography is done normally with smaller amounts of material and is for establishing the presence or measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive. [2]

Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights [1,2]. Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into mobile phase, and leave the system faster [3].

Based on this approach three components form the basis of the chromatography technique.

- Stationary phase: This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support".

- Mobile phase: This phase is always composed of "liquid" or a "gaseous component."
- Separated molecules

The type of interaction between stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on separation of molecules from each other. Chromatography methods based on partition are very effective on separation, and identification of small molecules as amino acids, carbohydrates, and fatty acids. However, affinity chromatographies (ie. ion-exchange chromatography) are more effective in the separation of macromolecules as nucleic acids, and proteins. Paper chromatography is used in the separation of proteins, and in studies related to protein synthesis; gas-liquid chromatography is utilized in the separation of alcohol, esther, lipid, and amino groups, and observation of enzymatic interactions, while molecular-sieve chromatography is employed especially for the determination of molecular weights of proteins. Agarose-gel chromatography is used for the purification of RNA, DNA particles, and viruses.

Stationary phase in chromatography, is a solid phase or a liquid phase coated on the surface of a solid phase. Mobile phase flowing over the stationary phase is a gaseous or liquid phase. If mobile phase is liquid it is termed as liquid chromatography (LC), and if it is gas then it is called gas chromatography (GC). Gas chromatography is applied for gases, and mixtures of volatile liquids, and solid material. Liquid chromatography is used especially for thermal unstable, and non-volatile samples.

The purpose of applying chromatography which is used as a method of quantitative analysis apart from its separation, is to achive a satisfactory separation within a suitable timeinterval. Various chromatography methods have been developed to that end. Some of them include column chromatography, thin-layer chromatography (TLC), paper chromatography, gas chromatography, ion exchange chromatography, gel permeation chromatography, high-pressure liquid chromatography, and affinity chromatography.

- Column chromatography
- Ion-exchange chromatography
- Gel-permeation (molecular sieve) chromatography
- Affinity chromatography
- Paper chromatography
- Thin-layer chromatography
- Gas chromatography
- Dye-ligand chromatography
- Hydrophobic interaction chromatography
- Pseudoaffinity chromatography
- High-pressure liquid chromatography (HPLC)

High-prssure liquid chromatography (HPLC)

Using this chromatography technique it is possible to perform structural, and functional analysis, and purification of many molecules within a short time, This technique yields perfect results in the separation, and identification of amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, and other biologically active molecules, In HPLC, mobile phase passes through columns under 10–400 atmospheric pressure, and with a high (0.1–5 cm//sec) flow rate. In this technique, use of small particles, and application of high presure on the rate of solvent flow increases separation power, of HPLC and the analysis is completed within a short time.

Essential components of a HPLC device are solvent depot, high- pressure pump, commercially prepared column, detector, and recorder. Duration of separation is controlled

Essential components of a HPLC device are solvent depot, high- pressure pump, commercially prepared column, detector, and recorder. Duration of separation is controlled with the aid of a computerized system, and material is accrued

Types of HPLC

There are following variants of HPLC, depending upon the phase system (stationary) in the process:

Normal Phase HPLC

This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these.

Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

Reverse Phase HPLC

The stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions hence the more nonpolar the material is, the longer it will be retained.

Size-exclusion HPLC

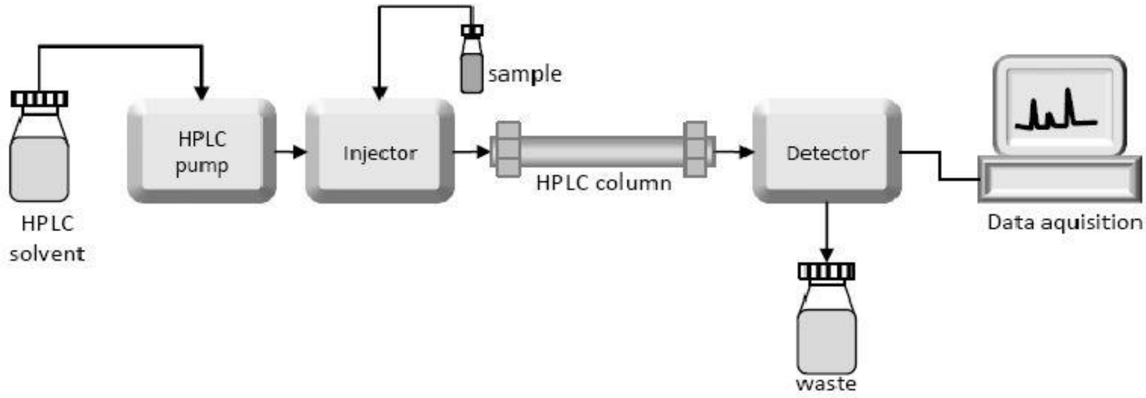
The column is filled with material having precisely controlled pore sizes, and the particles are separated according to its their molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later.

Ion-Exchange HPLC

The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples.

The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

Instrumentation of HPLC



As shown in the schematic diagram in Figure above, HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs.

Solvent Reservoir

Mobile phase contents are contained in a glass resorvoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.

Pump

A pump aspirates the mobile phase from the solvent resorvoir and forces it through the system's column and detecter. Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated.

Sample Injector

The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

Columns

Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 µm.

Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally the temperature of the mobile phase and the column should be kept constant during an analysis.

Detector

The HPLC detector, located at the end of the column detects the analytes as they elute from the chromatographic column. Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.

Data Collection Devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

MATERIALS AND METHOD

Rifampicin-Sura labs,Isoniazide-Sura labs,Water and Methanol for HPLC-LICHROSOLV (MERCK), Acetonitrile for HPLC-Merck

HPLC method development

Trails

Preparation of standard solution

Accurately weigh and transfer 10 mg of Rifampicin and Isoniazide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 2ml of Rifampicin and 2ml of Isoniazide from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization

Initially the mobile phase tried was Methanol: Water, Acetonitrile and water with varying proportions. Finally, the mobile phase was optimized to Methanol: Acetate Buffer pH-3.8 in proportion 24:76 v/v respectively.

Optimization of Column

The method was performed with various columns like C18 column, Symmetry and X-Bridge. Agilent Zorbax (C18) (150mm x 4.6mm, 5µm) column was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Optimized chromatographic conditions

Instrument used : Waters HPLC with auto sampler and PDA Detector 996 model.
Temperature : 37ºC
Column : Agilent Zorbax (C18) (150mm x 4.6mm, 5µm) column
Mobile phase : Methanol: Acetate Buffer pH-3.8 (24:76v/v)
Flow rate : 1ml/min
Wavelength : 263nm
Injection volume : 10 µl
Run time : 8 min

Method validation

Preparation of mobile phase

Accurately measured 240 ml (24%) of Methanol and 760 ml of Acetate Buffer (76%) a were mixed and degassed in digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

RESULTS AND DISCUSSION

Optimized Chromatogram

Instrument used : Waters HPLC with auto sampler and PDA Detector 996 model.
Temperature : 37ºC
Column : Agilent Zorbax (C18) (150mm x 4.6mm, 5µm) column
Mobile phase : Methanol: Acetate Buffer pH-3.8 (24:76v/v)
Flow rate : 1ml/min
Wavelength : 263nm
Injection volume : 10 µl
Run time : 6 min

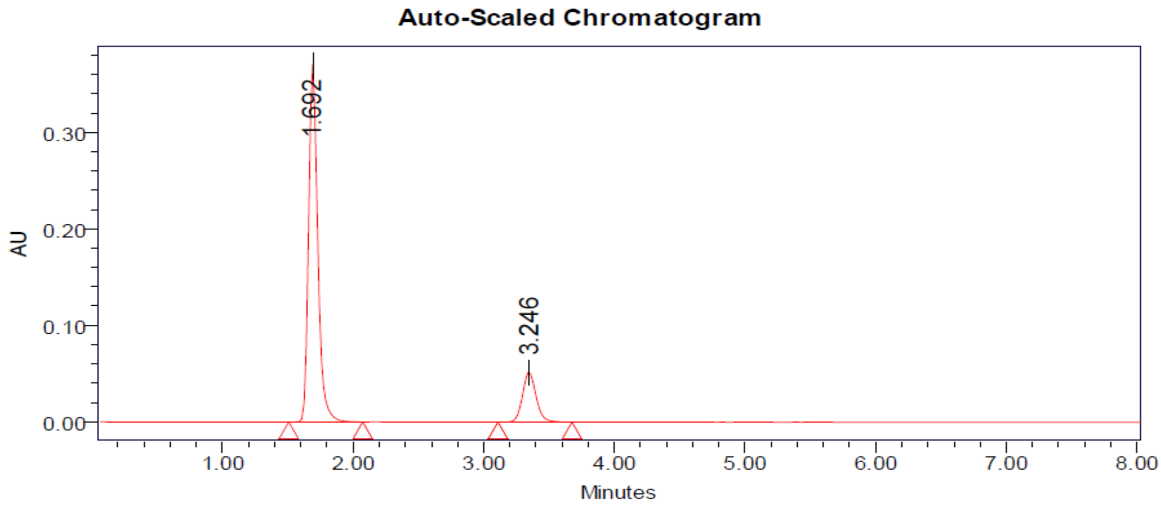


Fig 1: Optimized Chromatogram

Table 1: Observation of Optimized Chromatogram

S. No.	Peak Name	Retention Time	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Rifampicine	1.692	1658785	385669	1.69	7586	10.85
2	Isoniazide	3.246	425631	65245	1.58	6235	

Inference

1. The Retention Time is decreased observed from chromatogram by increasing flow rate.
2. The retention time was Rifampicin and Isoniazide was found to be 1.692 and 3.246 respectively.
3. The tailing is not more than two and plate count observed is more than 2500. Pass all the system suitability parameters.

The peak shapes are good with good resolution and less Retention Time and more theoretical levels, pass the system suitability parameters

Standard Chromatogram

Table 2: Observation of Standard Chromatogram-1

S.No	Peak Name	Retention Time (min)	Area	USP Tailing	USP Plate Count	USP Resolution
1	Rifampicin	1.694	1659824	1.68	7595	10.89
2	Isoniazide	3.244	426521	1.59	6274	

Table 3: Observation of standard Chromatogram-2

S.No	Peak Name	Retention Time (min)	Area	USP Plate Count	USP Tailing	Resolution
1	Rifampicin	1.689	1658598	7599	1.68	10.86
2	Isoniazide	3.238	426598	6254	1.57	

Table 4: Observation of standard Chromatogram-3

S.No	Peak Name	Retention Time (min)	Area	USP Plate Count	USP Tailing
1	Rifampicin	1.692	1658985	7659	1.70
2	Isoniazide	3.246	425865	6359	1.59

Table 5: Observation of standard Chromatogram-4

S.No	Peak Name	Retention Time (min)	Area	USP Plate Count	USP Tailing
1	Rifampicin	1.688	1658579	7672	1.71
2	Isoniazide	3.265	435698	6295	1.60

Table 6: Observation of standard Chromatogram-5

S.No	Peak Name	Retention Time (min)	Area	USP Plate Count	USP Tailing
1	Rifampicin	1.688	1659852	7695	1.69
2	Isoniazide	3.265	436598	6498	1.59

Assay (Sample)

Table 7: Observation of sample Chromatogram-1

S.No	Peak Name	Retention Time (min)	Area	USP Plate Count	USP Tailing
1	Rifampicin	1.691	1669558	7695	1.70
2	Isoniazide	3.242	436589	6359	1.61

Table 8: Observation of sample Chromatogram-2

S.No	Peak Name	Retention Time (min)	Area	USP Plate Count	USP Tailing
1	Rifampicin	1.694	1678598	7698	1.72
2	Isoniazide	3.240	436985	6557	1.59

Table 9: Observation of sample Chromatogram-3

S.No	Peak Name	Retention Time (min)	Area	USP Plate Count	USP Tailing
1	Rifampicin	1.694	1668985	7659	1.72
2	Isoniazide	3.234	436598	6347	1.61

$$\%\text{ASSAY} = \frac{\textbf{Sample area}}{\textbf{Standard area}} \times \frac{\textbf{Weight of standard}}{\textbf{Dilution of standard}} \times \frac{\textbf{Dilution of sample}}{\textbf{Weight of sample}} \times \frac{\textbf{Purity}}{\textbf{100}} \times \frac{\textbf{Weight of tablet}}{\textbf{Label claim}} \times \mathbf{100}$$

Linearity
Chromatographic data for linearity study

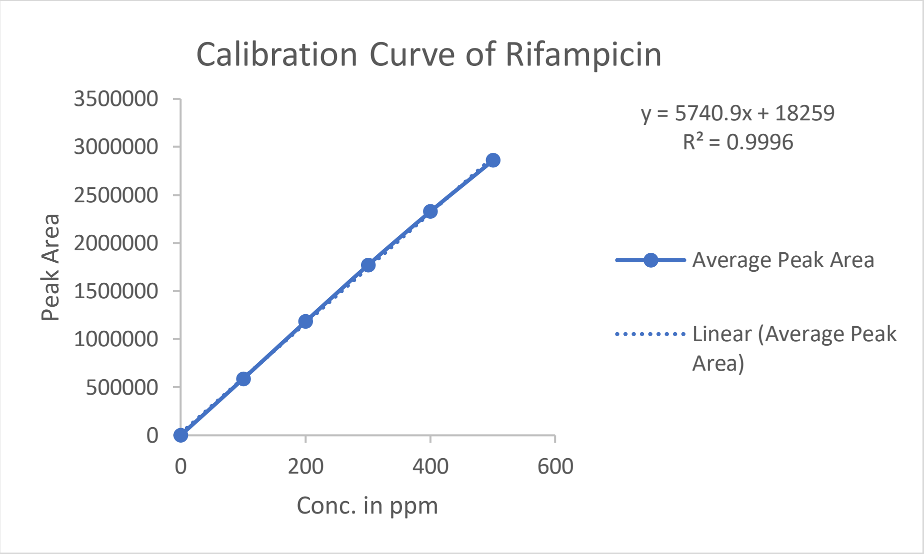


Fig 2: Calibration Curve for Rifampicin

Table 10: Linearity Observation of Rifampicin

S. No	Concentration Level (%)	Concentration µg/ml	Average Peak Area
1.	I	100	585985
2.	II	200	1182468
3.	III	300	1768785
4.	IV	400	2326852
5.	V	500	2856874
Correlation coefficient			0.999

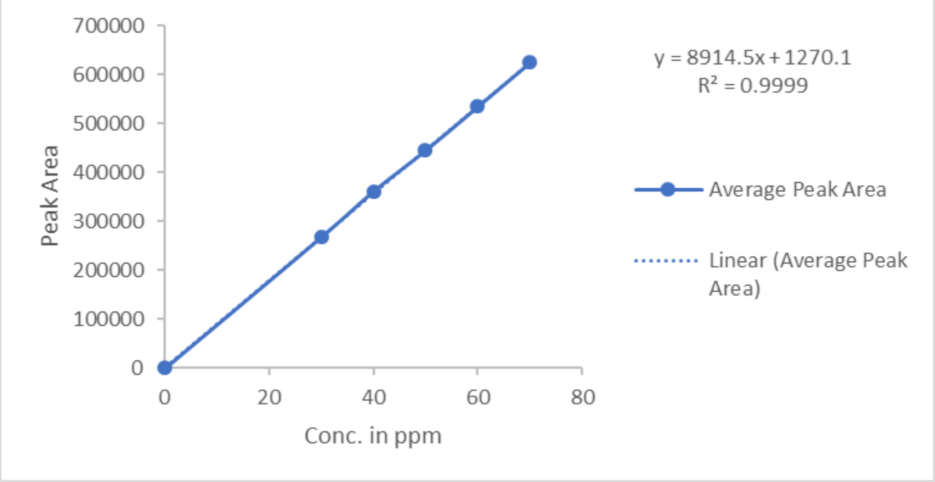


Fig 3: Calibration Curve for Isoniazide

Table 11: Linearity Observation of Isoniazide

S. No.	Concentration Level (%)	Concentration µg/ml	Average Peak Area
1	I	30	268764
2	II	40	361958

3	III	50	445631
4	IV	60	535186
5	V	70	624698
Correlation coefficient			0.999

Table 12: System suitability results Rifampicin

Organic phase		**System suitability Results**		
		USP Plate Count	**USP Tailing**	**Retention Time (min)**
Less organic phase	50:50	7269	1.61	1.868
Actual organic phase	55:45	7586	1.69	1.688
More organic phase	60:40	7496	1.64	1.675

Table 13: System suitability result Isoniazide

Organic phase		**System suitability Results**		
		USP Plate Count	**USP Tailing**	**Retention Time (min)**
Less organic phase	50:50	6182	1.54	3.621
Actual organic phase	55:45	6235	1.58	3.282
More organic phase	60:40	6322	1.56	2.302

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Accuracy

Table 14: Accuracy Observation of Rifampicin

%Concentration (at specification Level)	**Average Area**	**Amount Added (ppm)**	**Amount Found (ppm)**	**% Recovery**	**Mean Recovery**
50%	879537	150	150.048	100.032	100.112%
100%	1743252	300	300.521	100.172	
150%	2609693	450	450.598	100.132	

Isoniazide

Table 15: Accuracy Observation of Isoniazide

%Concentration (at specification Level)	**Average Area**	**Amount Added (ppm)**	**Amount Found (ppm)**	**% Recovery**	**Mean Recovery**
50%	224271	25	25.114	100.456%	100.16%
100%	445748.3	50	49.952	99.904%	
150%	670006.3	75	75.101	100.134%	

CONCLUSION

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Rifampicin and Isoniazide was done by HPLC. The separation was optimized with mobile phase consists of Methanol: acetate buffer (pH-3.8) mixed in the ratio of 24:76%v/v. An Agilent Zorbax (C18) (150mm x 4.6mm, 5µm) column or equivalent chemically bonded to porous silica particles were used as stationary phase. The solutions were chromatographed at a constant flow rate of 1.0 ml/min. The linearity range of Rifampicin and Isoniazide were found to be from 100-500µg/ml, 30-70µg/ml respectively. Linear regression coefficient was not more than 0.999, 0.999.

The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of Rifampicin and Isoniazide. LOD and LOQ were found to be within limits.

The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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