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# ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC FOR ANTIBACTERIAL AGENT

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**Abstract:**

*The present study focuses on the development and validation of a robust Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method for the accurate estimation of antibacterial agents in pharmaceutical formulations. In the era of increasing pharmaceutical competition and innovation, ensuring drug quality and safety is critical. Analytical method development is a fundamental aspect of pharmaceutical product development, especially in identifying and quantifying impurities that may pose toxicological risks. The method was optimized using suitable mobile phases and detection wavelengths, followed by rigorous validation as per ICH guidelines to assess parameters such as linearity, accuracy, precision, specificity, and robustness. This validated method is designed to effectively monitor the purity and potency of antibacterial agents, ensuring consistency in quality and compliance with regulatory standards. The study highlights the critical role of analytical testing in safeguarding public health by confirming the identity, strength, and purity of pharmaceutical substances.*

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## INTRODUCTION:

In current competitive and business world, a number of organizations have entered into pharmaceutical development and manufacturing business with multiple drugs. Manufacturing process of every drug is distinct from each other. Different kind of drug manufacturing generate different impurity profile depending with the process. These impurities are sometimes are toxic and thus may cause severe adverse effects on the human health hence theses impurities need to be accurately classified and controlled.

Pharmaceutical analytical development plays a very important role in pharmaceutical product development and manufacturing. Scientifically developed analytical testing methods ensure the quality, efficacy and adverse effects of the drug product as well as the drug substance (API). Drug substance manufactured through various chemical reactions to get the desired product which has a pharmacological activity and this drug substance further is converted into different types of drug products according to the patients need and the requirement. Drug manufacturing process different chemicals, reagents, catalyst, solvents, buffers and excipients are used. Analytical methods shall establish the quality of all these chemical substances.

## MATERIALS & METHODS:

**Materials**

The drug used for present investigation was obtained from MG labs Mumbai as a gift sample.

**Details of Pure Drug**

**Table No.01: Details of API**

| Sr. No. | Drug | Supplied by | Quantity | Purity (Assay) |
|---|---|---|---|---|
| 01 | Linezolid | MG Lab Mumbai | 10g | 99.7 |

**Marketed Preparation:**

**Table No.02: Details of Marketed preparation**

| Sr. No. | Brand name | Mfg. by | Content | Quantity |
|---|---|---|---|---|
| 01 | Linospan 600 | Cipla Ltd India | Linezolid | 600 mg |

**Reagents and Chemicals:**

All reagents and chemicals used were of AR grade and HPLC grade.

**Table No.03: List of Reagent and Chemicals used.**

| Sr. No. | Name of chemicals | Manufacturer. |
|---|---|---|
| 1. | Acetonitrile HPLC Grade | Merck Ltd., India |
| 2. | Methanol HPLC grade. | Merck Ltd., India |
| 3. | Ortho-phosphoric acid. | Merck Ltd., India |
| 4. | Water HPLC grade. | Merck Ltd., India |

**Instruments:**

**Table No.04: List of Instruments used.**

| Sr. No. | Instrument | Make | Model |
|---|---|---|---|
| 1. | UV-Visible Spectrophotometer | Shimadzu | Double beam carry-UV 1800 |
| 2. | HPLC | Waters India | UV Detector |
| 3. | PH Meter | Equip-tronich | Eq-614A |
| 4. | Analytical column | Grace | C18,(4.6 x 150 mm) |
| 5. | Balance | Citizen | CY 104 (Micro-Analytical Balance) |
| 6. | Ultrasonicator | Meta-lab | 1.5 L 50 |

**METHODS & PROCEDURE**

**Identification and characterization of drug**

Previous to commenced the experimental work it is necessary to determine the different physical and chemical property of the drug which provide information regarding the purity and nature of drug. This will help in selection of solvents and procedure for the robust and stable analytical method development.

**Selection and procurement of drug**

Linezolid (LIN) were selected as model drug candidate for method development and validation. The drugs were kindly gifted from Pharmaceutical industry India. The procured drug was analyzed for different physical properties viz. color, odor, melting point, etc.

**Physico-chemical characterization**

The physico-chemical characterization of drug molecule is important with regard to its purity, identification in development and validation of analytical method. The various tools used for characterization of drug molecules include melting point, UV spectroscopy, solubility study, etc.

**Solubility Studies**

As a first step of method development solubility of both drugs was tasted in different solvents to obtain a common solvent which can be used for simultaneous estimation of both drugs in a mixture.

**Melting point range determination**

Melting point of Linezolid (LIN) was determined by placing small amount of sample in capillary tube closed at one end and holds the capillary on melting point apparatus. The melting point was noted and readings were depicted in Table 06.

**FT-IR analysis:**

The IR absorbance spectrum of LIN was recorded using FTIR 8400S spectrometer (Shimadzu) over range of 4000 to 400 cm-1.

The IR spectroscopy theory utilizes the concept that molecules tend to absorb specific frequencies of light that are characteristic of the corresponding structure of the molecules. The energies are reliant on the shape of the molecular surfaces, the associated vibronic coupling, and the mass corresponding to the atoms.

The IR absorbance spectrum of LIN was recorded using FTIR 8400S spectrometer (Shimadzu) over range of 4000 to 400 cm-1.

**UV Spectroscopy Analysis**

The ultraviolet absorption spectrum of LIN was obtained using Shimadzu1800- UV visible spectrophotometer and 1cm quartz cells, over a wavelength range of 400 to 200 nm. The wavelength maxima (λmax) were analyzed showed in table no. 07.

**Selection of mobile phase:**

**a)Preparation of standard solutions:**

**LIN standard solution:**

Accurately weighed quantity 5 mg of LIN was dissolved in methanol and volume was made up to 25 ml mark (200 g/ml). The 1 ml of stock standard solution was diluted further with 20 ml methanol to get final concentration of about 60 g/ml of LIN.

**b)Procedure:**

The Methanol was allowed to equilibrate with stationary phase until steady baseline was obtained. The standard solution containing mixture of LIN was run and different individual solvents as well as combinations of solvents have been tried to get a good separation and stable peak. Each mobile phase was filtered through Whatman filter paper No. 42. Peak, well resolved peaks with symmetry within limits and significant Based on sample solubility & stability, various mobile phase compositions were evaluated to achieve acceptable separation using

selected chromatographic conditions. The mobile phases tried are as follows:

1) Methanol: Water (90:10)
2) Methanol: Water (80:20)
3) Methanol: Water (70:30)
4) Acetonitrile: Water (80:20)
5) Acetonitrile: Water (80:20) pH 5.5
6) Acetonitrile: Water (80:20) pH 5
7) Acetonitrile: Water (80:20) pH 4

From various mobile phases tried, mobile phase containing Acetonitrile: water (70:30) pH 4 adjust by OPA was selected, since it gives sharp reproducible retention time for LIN.

**Chromatographic conditions:**

The following chromatographic conditions were established by trial and error and were kept constant throughout method.

**Column**: Inertsil 4.6 (id) x 250 mm
**Particle size packing**: 5 □m
**Stationary phases**: C18 Inertsil
**Mobile phase**: Acetonitrile: water (70:30) pH 4 by OPA
**Detection wavelength**: 254nm
**Flow rate**: 1 ml/min.
**Temperature**: Ambient
**Sample size**: 20 □L
**Preparation of calibration curve**:

**Preparation of standard solutions:**

**LIN standard stock solution:**

Accurately weighed quantity 10 mg of LIN was dissolved in methanol and volume was made up to 100 ml mark (100 □g/ml). The stock standard solution was diluted further with mobile phase to get various concentrations.

**i) Procedure**:

The mobile phase was allowed to equilibrate with the stationary phase until steady baseline was obtained. The series of concentration from 10-100 □g/ml for LIN was injected and peak area was recorded. The graph plotted as the concentration of the drug Vs peak area depicted in Fig. No.21.

**1. System suitability test:**

System suitability is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The tests were performed by collecting data from five replicate injections of standard solutions.

**Preparation of standard drug solution. LIN standard solution:**

Accurately weighed quantity 10 mg of LIN was dissolved in mobile phase and volume was made up to 100 ml mark. The stock standard solution was diluted further with mobile phase to get final concentration of about 60 □g/ml of LIN.

**B) Procedure:**

filtered mobile phase was allowed to equilibrate with stationary phase until steady baseline Was obtained. A 20 □L std. drug solution was injected which was made in five replicates and the system suitability parameters were recorded as shown in Table No. 10.

**Application of proposed method for estimation of LIN Laboratory Sample: Preparation of laboratory mixture (standard):**

Accurately weighed quantity of LIN 10 mg was transferred to 100 ml volumetric flask, shaken vigorously for five minutes and volume was made up to mark with mobile phase. The standard solution of LIN was mixed and diluted with mobile phase properly to obtain laboratory Sample containing a concentration 60 □g/ml of LIN.

**Preparation of laboratory mixture (sample)**

Five different laboratory mixtures of LIN were prepared by appropriately weighing the quantities of drug samples so as to get the concentration of 60 □g/ml of LIN.

The peak area of standard laboratory mixture and sample laboratory mixture was compared to obtain the concentration.

The amount of each drug estimated in laboratory mixture was calculated using following formula –

$$\% \text{ Estimation} = \frac{A_t}{A_s} \times \frac{D_s}{D_t} \times \frac{W_s}{W_t} \times 100$$

| Where- | | |
|---|---|---|
| At | = | Area count for sample solution. |
| As | = | Area count for standard solution. |
| Ds | = | Dilution factor for standard. |
| Dt | = | Dilution factor for sample. |
| Ws | = | Weight of standard (mg) |
| Wt | = | Weight of sample (mg) |

**Application of proposed method for estimation of LIN in formulation: Standard stock solution:**

Accurately weighed quantity of LIN 10 mg was transferred to 100 ml volumetric flask, shaken vigorously for five minutes and volume was made up to mark with mobile phase. The standard solution of LIN was mixed and diluted with mobile phase properly to obtain laboratory mixtures containing a

concentration 60 □g/ml of LIN.

**Sample solution preparation:**

The twenty tablets were weighed, and then average weight was determined and finely grounded. The weight of the powdered tablet equivalent to 10 mg of LIN was transferred into a 100 mL standard volumetric flask. Added 50 mL of solvent sonicated for 10 min and diluted to 100 mL with the same solvent and then filtered through Whatmann filter paper No: 41.

**Procedure:**

Equal volume (20□L) of standard and sample solution was injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response i.e. peak area of major peaks were measured. The content of LIN was calculated by comparing a sample peak with that of standard.

Amount of drug in tablet was calculated using following formula

$$\text{Assay (mg/ml)} = \frac{A_t}{A_s} \times \frac{D_s}{D_t} \times \frac{W_s}{W_t} \times \frac{p}{100}$$

$$\% \text{ Label claim} = \frac{\text{Assay (mg/ml)} \times 100}{\text{Label claim in mg/ml}}$$

Where -

At = Area count for sample solution. As = Area count for standard solution. Ds = Dilution factor for standard.

Dt = Dilution factor for sample. P = Potency of drug.

**Validation parameters:**

a) **Accuracy:**

It was ascertained on the basis of recovery studies performed by standard addition method. The results of recovery studies and statistical data are recorded in Table No. 13 **Preparation of standard solution:**

An accurately weighed quantity of preanalysed 1ml formulation was taken in 10 ml volumetric flask; to it standard solution of LIN was added in different proportions. Then volume was adjusted up to the mark with the mobile phase. The solution was diluted with mobile phase to get final concentration & filtered through whatman filter paper No.41. The amount of drug contributed by the formulation was deduced from the total amount of respective drugs estimated and the resultant quantities were assumed to be recovered from the added pure drugs. The content of drug was calculated using same formula as in the marketed formulation.

The % Recovery was then calculated by using formula

$$\% \text{ Recovery} = \frac{A}{B+C} \times 100$$

Where-

A= Total amount of drug estimated.

B= Amount of drug found on preanalysed basis.

C= Amount of pure drug added.

Results are shown in the Table No. 13

**b)Precision:**

Precision of an analytical method is expressed as S.D or R.S.D of series of measurements. It was ascertained by replicate estimation of the drugs by proposed method.

~~wt mg/ml of test sample~~

**c) Ruggedness:**

Ruggedness is the ability of an analytical method to remain unaffected by small changes in experimental conditions, such as those that might occur during normal use.

The studies of ruggedness were carried out under two different conditions-

1) Days
2) Analyst.

**i) Interday (Different days):**

Same procedure was performed as under marketed formulation analysis on different days. The % label claim was calculated. Data obtained for day 1, day 2, and day 3 is shown in Table No. 15

**ii) Intraday:**

It was performed by using same procedure as under marketed formulation analysis and absorbance recorded at 3 hrs. interval within a day. The percent label claim was calculated using formula, Result and statistical data are shown in Table No. 16

**iii) Different analyst:**

The sample solution was prepared by two different analysts and same procedure was followed as described earlier. The % label claim was calculated

as done in marketed formulation estimation.

**d) Specificity:**

Specificity was measured as ability of the proposed method to obtain well separated peak for LIN without any interference from component of matrix.

**Mean retention time for – LIN – 3.923**

The values obtained were very close to that in standard laboratory mixture indicates no interference from the component of matrix.

Typical chromatogram is shown in the Fig. No. 23

**e) Linearity and range:**

According to USP tablet powder equivalent to 80, 90, 100, 110, 120 % of label claim was taken and dissolved & diluted appropriately with mobile phase to obtain a concentration in the range of 80%-120% of the test concentration. The chromatograms of the resulting solutions were recorded. LIN marketed formulation was found to be linear in the range ± 20% of the test concentration of the respective drug.

The plot showing linearity and range study for LIN is shown in the Fig. No.24.

**f) Robustness**

Robustness and ruggedness - the ability of an analytical method to remain unaffected by small variations in method parameters and influential environmental factors and characterize its reliability during normal usage. No change of the detected amount of the analyte in a certain sample in spite of the variation of the method parameter.

The robustness study indicated that the factors selected remained unaffected by small variation of organic composition of mobile phase, wavelength and the flow rate. The system suitability results should lie within the limit. Hence the method was robust. The results are shown in table no 19.

**f) Limit of Detection (LOD) and Limit of Quantitation (LOQ):**

Limit of detection is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Limit of quantitation is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision accuracy.

As per ICH guideline both LOD & LOQ were performed on the basis of standard deviation of the response and slope and expressed by following formula.

$$LOD = \frac{3.3\,\sigma}{S}$$

$$LOQ = \frac{10\,\sigma}{S}$$

Where,

σ = The standard deviation of the response S = The slope of the calibration curve

The results of LOD and LOQ are shown in table 20

Now a day's drugs are commonly used clinically and analyst is required to develop suitable method for their analysis. A fixed dose containing Linezolid (LIN) is recently available in market as tablet dosage form.

Percent purity of above mentioned drugs were reported by Supplier Company as follows-

1) Linezolid - 99.7 %

Primary this was not analysed in our study and the % purity stated by the suppliers was taken as standard for comparison studies

The physico-chemical characterization of drug molecule is important with regard to its purity, identification in development and validation of analytical method. The various tools used for characterization of drug molecules include melting point, UV spectroscopy, solubility study, etc. The solubility study, melting point analysis, UV spectroscopy of the drug was done.

**Melting point range determination**

Melting point of drug was determined by placing small amount of sample in capillary tube closed at one end and holds the capillary on melting point apparatus. The melting point was noted and readings were depicted in Table 06.

**Table No 05: Melting point range analysis result**

| Sr. No. | Name of Drug | Melting point |
|---|---|---|
| 1 | LIN | 198 ºC |

**FT-IR analysis:**

**a) FT-IR of Linezolid**

The IR absorbance spectrum of Linezolid was recorded using FTIR 8400S spectrometer (Shimadzu) over range of 4000 to 400 cm-1.

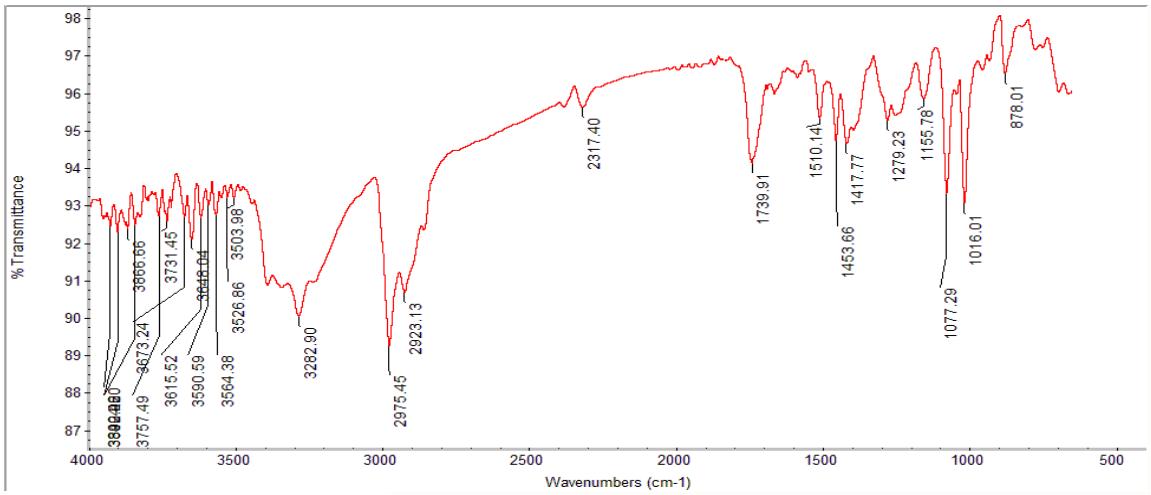


**Fig No 1: FT-IR Spectra of LIN**

The IR spectroscopy theory utilizes the concept that molecules tend to absorb specific frequencies of light that are characteristic of the corresponding structure of the molecules. The energies are reliant on the shape of the molecular surfaces, the associated vibronic coupling, and the mass corresponding to the atoms.

The FTIR spectra of a sample of pure LIN is shown in figure above. The FTIR spectrum of Linezolid showed a distinct peak at 3282.90 $cm^{-1}$ (N-H Stretching), 2975.45 $cm^{-1}$ (C-H stretching), 1739.91 $cm^{-1}$ (C=O Stretching), 1510.14 $cm^{-1}$ (N-H Bending), 1279.23 $cm^{-1}$ (stretch vibrations –C-N) and 1417.77 $cm^{-1}$ (O-H Bending) This FTIR spectra confirmed the drug.

**UV Spectroscopy Analysis**

The ultraviolet absorption spectrum of LIN was obtained using Shimadzu1800- UV visible spectrophotometer and 1cm quartz cells, over a wavelength range of 400 to 200 nm. The wavelength maxima (λ max) were analyzed showed in table no. 07

**Table no 02: Drug wavelength maxima (λ max)**

| Sr. No. | Name of Drug | Observed value ( λ max )nm |
|---|---|---|
| 1 | LIN | 254 |

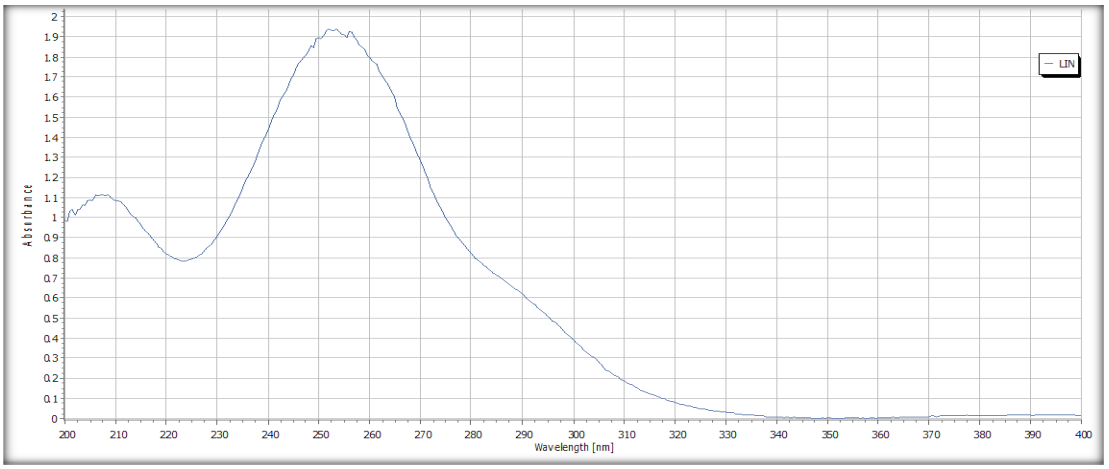


**Fig. No. 03: - UV Spectra of LIN**

**Selection of mobile phase:**

Each mobile phase was filtered through Whatman filter paper No. 42. Peak, well resolved peaks with symmetry within limits and significant Based on sample solubility & stability, various mobile phase compositions were evaluated to achieve acceptable separation using selected chromatographic conditions. The mobile phases tried are as follows:

**Table no 07: List of mobile phase tried**

| Sr. No. | List of mobile phase tried |
|---|---|
| 1 | Methanol: Water (90:10) |
| 2 | Methanol: Water (80:20) |
| 3 | Methanol: Water (70:30) |
| 4 | Acetonitrile: water (90:10) pH |
| 5 | Acetonitrile: water (80:20) pH 5 |
| 6 | Acetonitrile: water (75:25) pH 4.5 |
| 7 | Acetonitrile: water (70:30) pH 4 |

From various mobile phases tried, mobile phase containing Acetonitrile: water (70:30) pH 4 adjust by OPA was selected, since it gives sharp reproducible retention time for LIN.

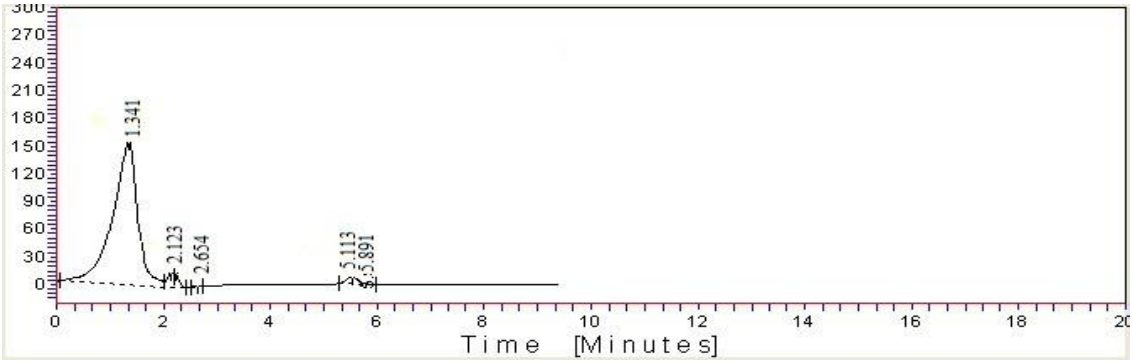


**Fig. No.04: Trial Chromatogram obtained by using Methanol: Water (90:10) as mobile phase**

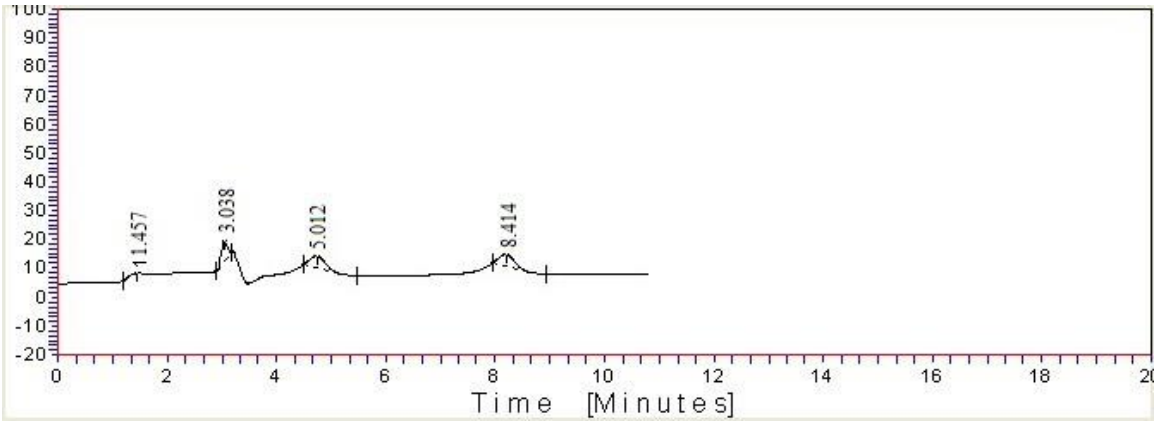


**Fig. No.05: Trial Chromatogram obtained by using Methanol: Water (80:20) as mobile phase**

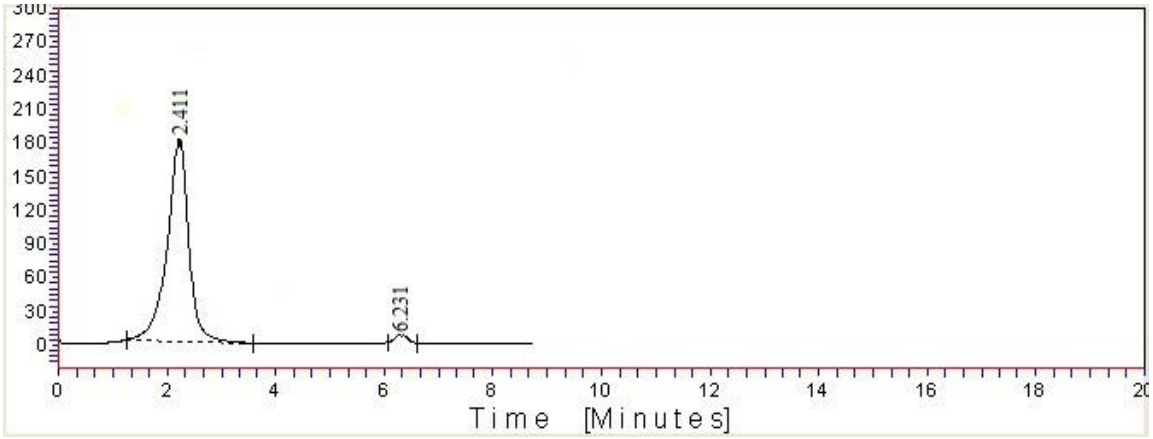


**Fig. No.06: Trial Chromatogram obtained by using Acetonitrile: water (80:20) as mobile phase.**

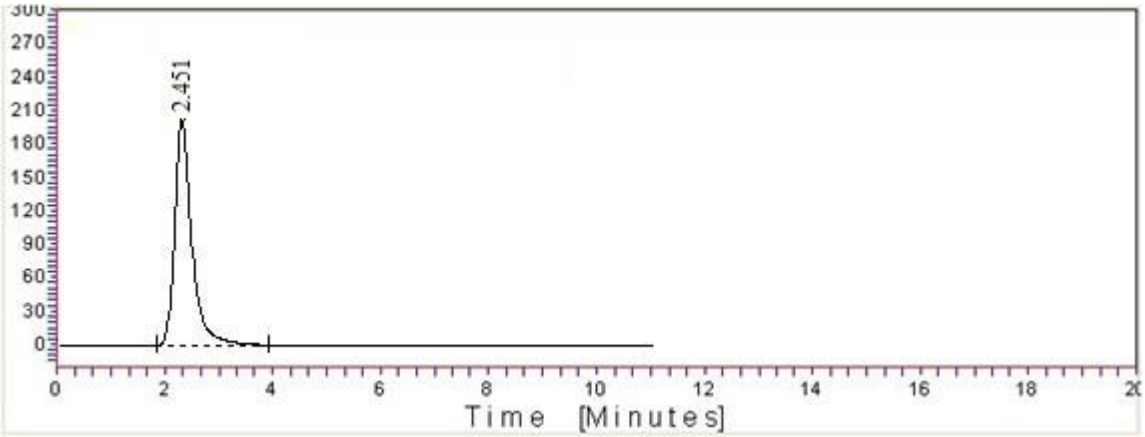


**Fig. No.07: Trial Chromatogram obtained by using Acetonitrile: water (75:25) pH 5 as mobile phase.**

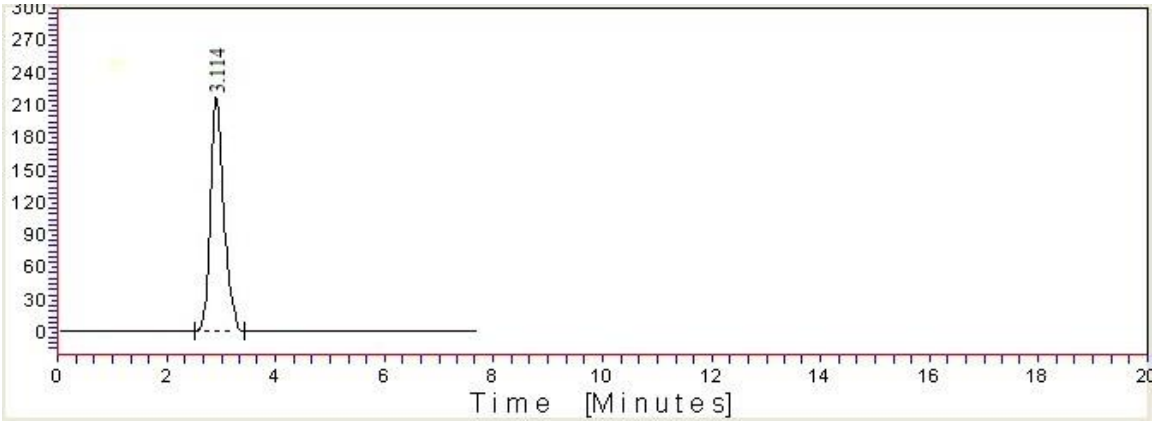


**Fig. No.08: Final Chromatogram obtained by using Acetonitrile: water (70:30) pH 4 as mobile phase of LIN.**

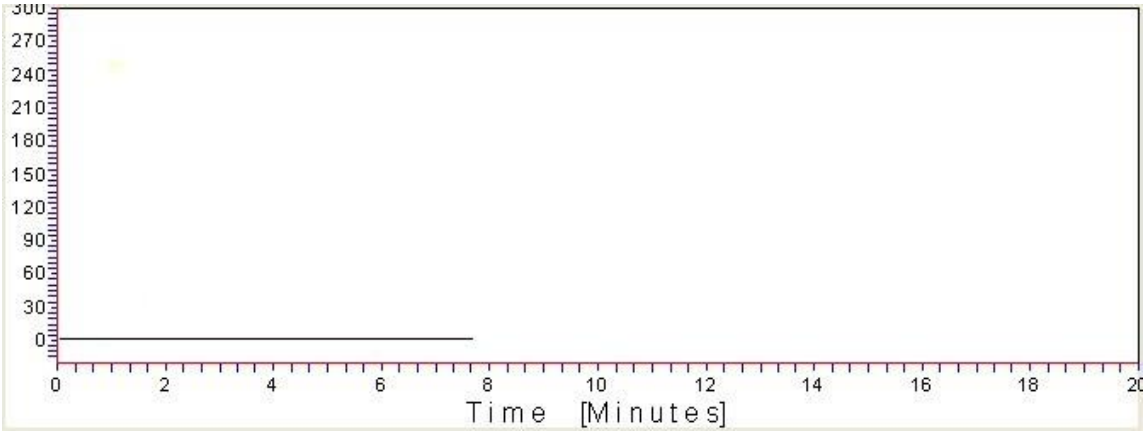


**Fig. No.09: Blank Chromatogram obtained by using Acetonitrile: Phosphate Buffer (65:35) pH 4 as mobile phase.**

**Chromatographic conditions:**

The following chromatographic conditions were established by trial and error and were kept constant throughout method.

| | |
|---|---|
| **Column** | : Inertsil 4.6 (id) x 250 mm |
| **Particle size packing** | : 5 □m |
| **Stationary phases** | : C18 Inertsil |
| **Mobile phase** | : Acetonitrile: water (70:30) pH 4 by OPA |
| **Detection wavelength** | : 254 nm |
| **Flow rate** | : 1 ml/min. |
| **Temperature** | : Ambient |
| **Sample size** | : 20 □L |

**Preparation of calibration curve**:

The mobile phase was allowed to equilibrate with the stationary phase until steady baseline was obtained. The series of concentration from 10-100 □g/ml for LIN drug solutions was injected and peak area was recorded. The graph plotted as the concentration of the drug Vs peak area depicted in Fig. No.21.

**Table No.10: Observation of standard curve of LIN**

| Sr. No. | Conc.(□g/ml) LIN | Peak Area LIN |
|---|---|---|
| 1 | 10 | 53042.7 |
| 2 | 20 | 106085.4 |
| 3 | 30 | 159128.1 |
| 4 | 40 | 212170.7 |
| 5 | 50 | 265213.4 |
| 6 | 60 | 318256.1 |
| 7 | 70 | 371298.8 |
| 8 | 80 | 424341.5 |
| 9 | 90 | 469384.2 |
| 10 | 100 | 530426.8 |

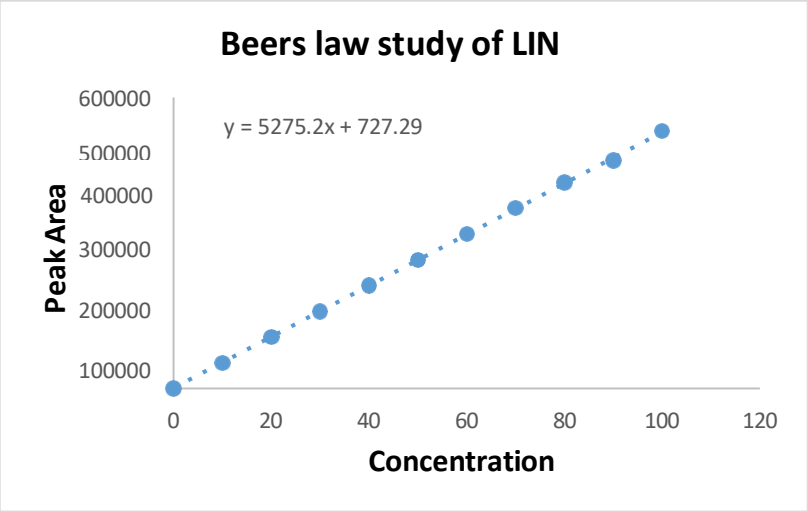


**Fig. No.11: Standard calibration curve for LIN**

**System suitability test:**

System suitability is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The tests were performed by collecting data from five replicate injections of standard solutions.

**Table No. 09: Result of System Suitability Study**

| Sr. No | Peak area | Retention Time | Asymmetry | Efficiency |
|---|---|---|---|---|
| | LIN | LIN | LIN | LIN |
| 1 | 318256.1 | 3.114 | 1.892 | 211547.1 |
| **2** | 318224.3 | 3.116 | 1.889 | 211502.5 |
| **3** | 318287.9 | 3.121 | 1.887 | 211498.9 |
| **4** | 318256.1 | 3.125 | 1.895 | 211602.3 |
| **5** | 318256.1 | 3.113 | 1.892 | 211547.1 |
| **Mean** | 318256.1 | 3.1178 | 1.891 | 211539.58 |
| **± S.D** | 22.48599564 | 0.005069517 | 0.003082207 | 42.06152636 |
| **C.V** | 0.000070654 | 0.001625992 | 0.001629935 | 0.000198835 |

**Application of proposed method for estimation of LIN Laboratory Sample:**

The standard and Sample solution of LIN was prepared and inject. The peak area of standard and sample laboratory was compared to obtain the concentration.

**Table No10: Results and statistical data for estimation of LIN in lab. Sample**

| Sr. No. | Weight of Standard (mg) | Weight of Sample (mg) | Peak Area of Standard | Peak Area of Sample | % Drug Estimation |
|---|---|---|---|---|---|
| | LIN | LIN | LIN | LIN | LIN |
| 1 | 10 | 10 | 318256.1 | 316983.1 | 99.6 |
| 2 | | 10 | | 320802.1 | 100.8 |
| 3 | | 10 | | 321120.4 | 100.9 |
| | | | | Mean | 100.43 |
| | | | | ±S.D. | 0.723 |
| | | | | C.V. | 0.007 |

***Results are mean of three replicates**

**Application of proposed method for estimation of LIN in formulation:**

Equal volume (20□L) of standard and sample solution were injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response i.e. peak area of major peaks were measured. The content LIN was calculated by comparing a sample peak with that of standard.

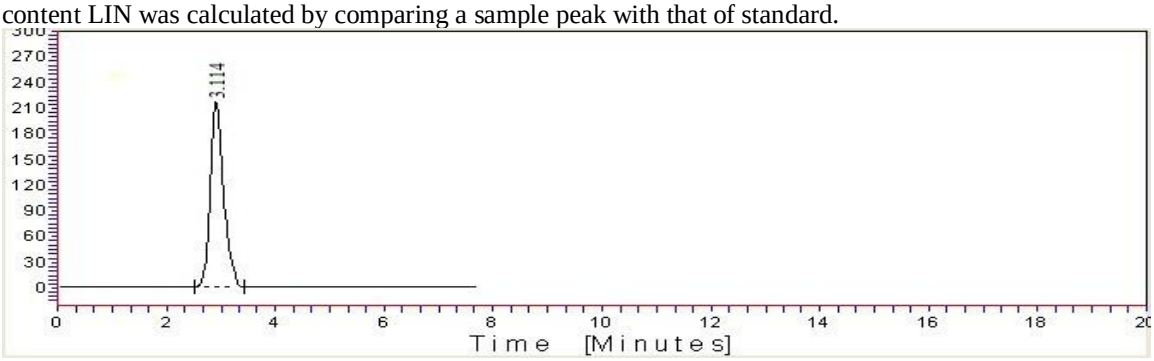


**Fig. No.12: Chromatogram obtained by formulation of LIN**

**Table No.11: Results and statistical data for estimation of LIN in marketed formulation.**

**Brand name :** Linospan **Avarg wt :** 790 mg

| Sr. No. | Weight of Standard (mg) | Weight of Sample (mg) | Peak Area of Stand. | Peak Area of Sample | % Drug Estimation |
|---|---|---|---|---|---|
| | LIN | LIN | LIN | LIN | LIN |
| 1 | 10 | 13.16 | 318256.1 | 321120.4 | 100.9 |
| 2 | | 13.2 | | 321756.9 | 101.1 |
| 3 | | 13.17 | | 323029.9 | 101.5 |
| | | | | Mean | 101.17 |
| | | | | ±S.D. | 0.306 |
| | | | | C.V. | 0.003 |

***Results are mean of three replicates**

**Validation parameters:**

**a) Accuracy:**

It was ascertained on the basis of recovery studies performed by standard addition method. The results of recovery studies and statistical data are recorded in Table No. 13

**Table No.12: Results and statistical data for Recovery study of LIN**

| Sr. No | wt. of formulation | Amount of Drug Added in (µg/ml) | Peak Area of stand. | Peak Area of sample | % Recovery |
|---|---|---|---|---|---|
| | LIN | LIN | LIN | LIN | LIN |
| 1 | 13.16 | 1 | 318256.1 | 317937.8 | 99.9 |
| 2 | | 1 | | 318574.4 | 100.1 |
| 3 | | 1 | | 316983.1 | 99.6 |
| 4 | | 2 | | 317301.3 | 99.7 |
| 5 | | 2 | | 316983.1 | 99.6 |
| 6 | | 2 | | 320802.1 | 100.8 |
| 7 | | 3 | | 321756.9 | 101.1 |
| 8 | | 3 | | 323029.9 | 101.5 |
| 9 | | 3 | | 318892.6 | 100.2 |
| | | | | **Mean** | 100.28 |
| | | | | **S.D.** | 0.696 |
| | | | | **C.V** | 0.007 |

***Results are mean of three replicates**

**b) Precision:**

Precision of an analytical method is expressed as S.D or R.S.D of series of measurements. It was ascertained by replicate estimation of the drugs by proposed method. The Results and statistical data of Precision Study of LIN is recorded and shown in following table

**Table No.13: Results and statistical data of Precision Study Brand Name:** Linospan

| Sr. No. | Weight of Standard (mg) | Weight of Sample (mg) | Peak Area of Stand. | Peak Area of Sample | % Label claim |
|---|---|---|---|---|---|
| | LIN | LIN | LIN | LIN | LIN |
| 1 | 10 | 13.16 | 318256.1 | 321120.4 | 100.9 |
| 2 | | 13.16 | | 321756.9 | 101.1 |
| 3 | | 13.19 | | 323029.9 | 101.5 |
| | | | | **Mean** | 101.17 |
| | | | | **±S.D.** | 0.306 |
| | | | | **C.V.** | 0.003 |

***Results are mean of three replicates**

**c) Ruggedness:**

The studies of ruggedness were carried out under two different conditions-

1) Days

2) Analyst.

**i) Interday (Different days):**

Same procedure was performed as under marketed formulation analysis on different days. The % label claim was calculated. Data obtained for day 1, day 2, and day 3 is shown in Table No. 15

**Table No.14: Results and statistical data of Interday Study Brand Name:** Linospan

| Sr. No. | Weight of Standard (mg) | Weight of Sample (mg) | Peak Area of Stand. | Peak Area of Sample | % Label claim |
|---|---|---|---|---|---|
| | LIN | LIN | LIN | LIN | LIN |
| 1 | 10 | 13.16 | 318256.1 | 319210.9 | 100.3 |
| 2 | | 13.16 | | 319529.1 | 100.4 |
| 3 | | 13.14 | | 316346.6 | 99.4 |
| | | | | Mean | 100.03 |
| | | | | ±S.D. | 0.551 |
| | | | | C.V. | 0.006 |

***Results are mean of three replicates**

**ii) Intraday:**

It was performed by using same procedure as under marketed formulation analysis and absorbance recorded at 3 hrs. interval within a day. The percent label claim was calculated using formula & Result and statistical data are shown in Table No. 16

**Table No.15: Results and statistical data of Intraday Study Brand Name:** Linospan

| Sr. No. | Weight of Standard (mg) | Weight of Sample (mg) | Peak Area of Stand. | Peak Area of Sample | % Label claim |
|---|---|---|---|---|---|
| | LIN | LIN | LIN | LIN | LIN |
| 1 | 10 | 13.16 | 318256.1 | 321120.4 | 100.9 |
| 2 | | 13.15 | | 321756.9 | 101.1 |
| 3 | | 13.2 | | 323029.9 | 101.5 |
| | | | | Mean | 101.17 |
| | | | | ±S.D. | 0.306 |
| | | | | C.V. | 0.003 |

***Results are mean of three replicates**

### iii) Different analyst:

The sample solution was prepared by two different analysts and same procedure was followed as described earlier. The % label claim was calculated as done in marketed formulation estimation.

**Table No.16: Result and statistical data of Different analyst study**

| Sr. No | % Label claim | |
|---|---|---|
| | ANALYST I | ANALYST II |
| | LIN | LIN |
| 1 | 99.8 | 100.3 |
| 2 | 99.7 | 100.4 |
| 3 | 99.6 | 99.4 |
| 4 | 100.8 | 99.8 |
| 5 | 100.9 | 99.7 |
| **Mean** | 100.16 | 99.92 |
| **S.D** | 0.63482281 | 0.420713679 |
| **C.V** | 0.006338087 | 0.004210505 |

***Results are mean of three replicates.**

**Specificity:**

Specificity was measured as ability of the proposed method to obtain well separated peak for LIN without any interference from component of matrix.

Mean retention time for –

**LIN – 3.114**

The values obtained were very close to that in standard laboratory mixture indicates no interference from the component of matrix.

Typical chromatogram is shown in the Fig. No. 23

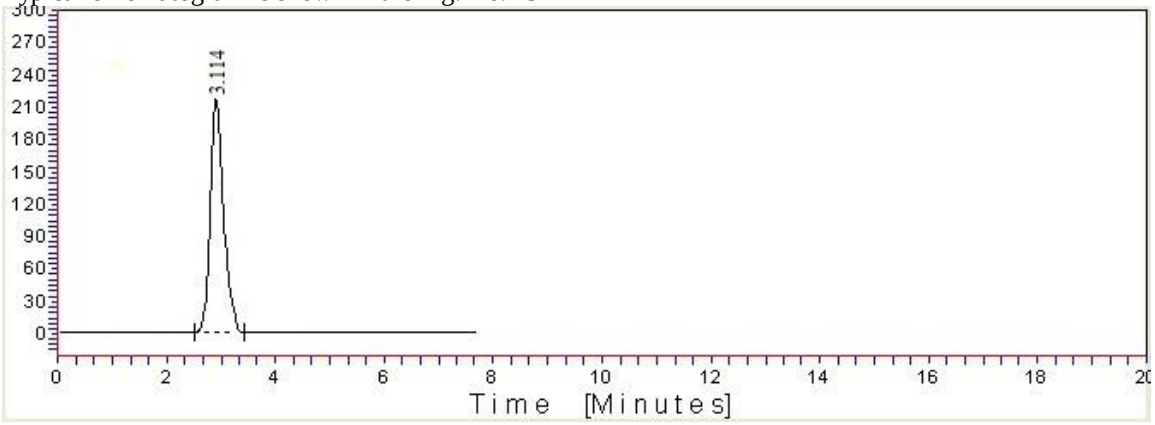


**Fig. No.23: Chromatogram obtained by formulation of LIN**

### d) Linearity and range:

According to USP tablet powder equivalent to 80, 90, 100, 110, 120 % of label claim was taken and dissolved & diluted appropriately with mobile phase to obtain a concentration in the range of 80%-120% of the test concentration. The chromatograms of the resulting solutions were recorded. LIN marketed formulation was found to be linear in the range ± 20% of the test concentration of the respective drug.

The plot showing linearity and range study for LIN is shown in the Fig. No. 24

**Table No.17: Observations of Linearity and range study for LIN.**

| Sr. No. | %Label claim | Peak area LIN |
|---|---|---|
| 1 | 80 | 254604.9 |
| 2 | 90 | 286430.5 |
| 3 | 100 | 318256.1 |
| 4 | 110 | 350081.7 |
| 5 | 120 | 376907.3 |

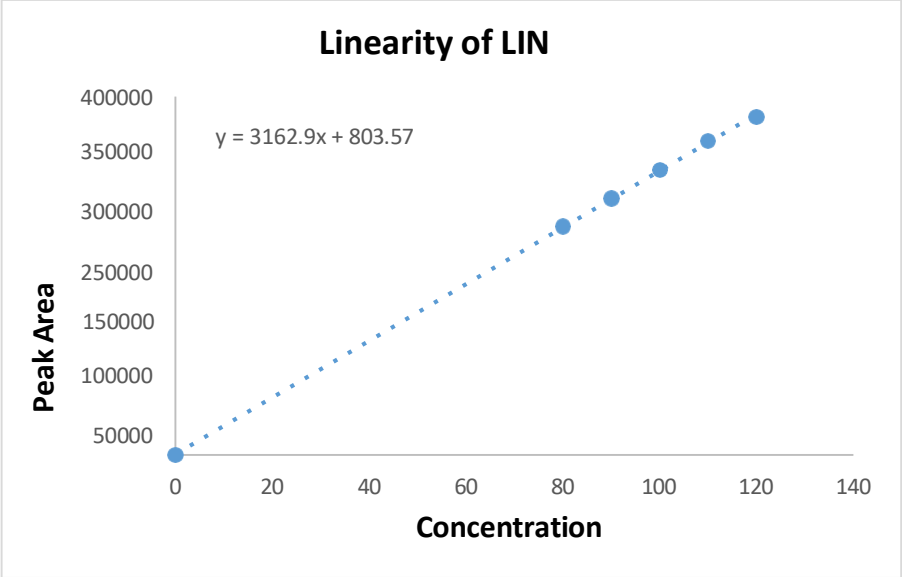


**Fig. No.14: -Plot of linearity and range study for LIN**

**e) Robustness**

The robustness study indicated that the factors selected remained unaffected by small variation of organic composition of mobile phase, wavelength and the flow rate. The system suitability results should lie within the limit. Hence the method was robust.

**Table No.18: Result of Robustness study of LIN**

| Sr. No. | Condition | Parameter | Peak Area | RT |
|---|---|---|---|---|
| 01 | Change of wavelength | 252 nm | 318224.3 | 3.116 |
| 02 | | 254 nm | 318287.9 | 3.114 |
| 03 | | 256 nm | 318256.1 | 3.117 |
| 04 | Change in Temperature | 30 ºC | 318128.8 | 3.112 |
| 05 | | 25 ºC | 318224.3 | 3.114 |
| 06 | | 20 ºC | 317874.2 | 3.118 |
| 07 | Change in Flow rate | 0.8 ml/min | 318033.3 | 3.121 |
| 08 | | 1ml/min | 318252.9 | 3.114 |
| 09 | | 1.2 ml/min | 318097 | 3.09 |
| 10 | Change in Mobile Phase | 75:25 | 318574.4 | 3.111 |
| 11 | | 70:30 | 318256.1 | 3.114 |
| 12 | | 65:35 | 318252.9 | 3.117 |

**g) Limit of Detection (LOD) and Limit of Quantitation (LOQ):**

Limit of detection is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Limit of quantitation is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision accuracy. The result shown in following table

**Table 19: LOD & LOQ of LIN**

| Sr. No. | Drug Name | LOD g/ml | LOQ g/ml |
|---|---|---|---|
| 1 | LIN | 1.092 | 2.115 |

**SUMMARY & CONCLUSION:**

HPLC has gained the valuable position in the field of analysis due to ease of performance, specificity, sensitivity and the analysis of sample of complex nature. This technique is commonly used for the quantitative estimation of the drugs from their formulation as well as for studying their metabolites of drugs and their estimation in their biological fluids. This method offers advantages of estimating the constituents for the multicomponant system without prior separation and even nano quantities can be estimated. This technique was employed in the present investigation for Method development and validation by RP-HPLC method for estimation of the antibacterial agent in the pharmaceutical dosage form.

The estimation of Linezolid (LIN) and development its stability indicating RP- HPLC method in tablet dosage form. Careful evaluation of various parameters influencing analysis is an important aspect for the development of analytical method. In order to establish RP-HPLC method the following parameters were studied.

HPLC with Inertsil 4.6 (id) x 250 mm column and UV detector was used for the study. The standard and sample solution of LIN were prepared in mobile phase. Different pure solvents of varying polarity in different proportions were tried as mobile phase for development of the chromatogram.

During selection and optimization of the mobile phase it was observed that the sharpness of the peak is achieved with increasing the proportion of methanol whereas the increased proportion of aqueous resulted in broadening of the peak.

The mobile phase that was found to be most suitable was Acetonitrile: Water (70:30) pH 4 by OPA and detection wavelength 254 nm was selected for the evaluation of the chromatogram of both drugs. The selection of the wavelength was based on the λmax obtained by scanning of standard laboratory mixture. This system gave good resolution and optimum retention time with appropriate tailing factor (<2). The mean values of system suitability test result are depicted in Table below.

**Table No. 20: Summary of system suitability test results**

| Sr. no. | Parameter | LIN |
|---|---|---|
| 1. | Peak area | 318256.1 |
| 2. | Retention time (min) | 3.1178 |
| 3. | Asymmetry | 1.891 |
| 4. | Efficiency | 211539.58 |

After establishing the chromatographic conditions, standard laboratory mixture was prepared and analysed by following procedure described under experimental and results. It gave accurate, reliable results and was extended for estimation of drugs in marketed tablet formulation.

**Table No. 21: Summary of laboratory mixture and marketed formulation analysis by RP-HPLC Method**

| Sr. no. | Sample | Statistical data | LIN | |
|---|---|---|---|---|
| | | | % Estimation | %Recovery |
| 1. | Standard Laboratory mixture | Mean | 100.43 | - |
| | | S.D. | 0.723 | - |
| | | C.V. | 0.007 | - |
| 2. | Linospan | Mean | 101.17 | 100.28 |
| | | S.D. | 0.306 | 0.696 |
| | | C.V. | 0.003 | 0.007 |

The
above results clearly indicate that RP-HPLC technique can be successfully applied for the estimation of above-mentioned drugs in their pharmaceutical formulation without prior separation.

**Validation:**
Validation of these methods was performed as per the ICH guidelines for these following parameters

**Accuracy -** Accuracy of the proposed method was ascertained from the recovery studies by standard addition method. Result are shown in the Table No. 13
**Precision -** Replicate estimation of tablet analysed by the proposed method has yielded quite consistent result indicating repeatability of method. Study showed
±S.D. <2.

**Specificity –** Studies shows that there is no interference of peak from the component of matrix.
**Ruggedness -** Studies were carried out only for the two different parameters like different time, different day and different analyst. Results of estimation by proposed method are very much similar under variety of conditions. This study signifies the ruggedness of the method under varying condition of its performance.
**Robustness**

The robustness study indicated that the factors selected remained unaffected by small variation of organic composition of mobile phase, wavelength and the flow rate. The system suitability results should lie within the limit. Hence the method was robust.

**Table No.22: Summary of results of Ruggedness by RP-HPLC method**

| Parameter | Statistical data | % Estimation |
|---|---|---|
| | | LIN |
| **Interday** | Mean | 100.03 |
| | S.D. | 0.551 |
| | C.V. | 0.006 |
| **Intraday** | Mean | 101.17 |
| | S.D. | 0.306 |
| | C.V. | 0.003 |
| **Different analyst** | Mean | 99.92 |
| | S.D. | 0.420713679 |
| | C.V. | 0.004210505 |

### 1) Linearity and Range-

LIN marketed formulation was found to be linear in the range of 80% to 120 % of test concentration with $R^2 \approx 0.9998$ at selected wavelength for RP- HPLC the methods. Same procedure as described in USP was followed.

## CONCLUSION:

From the studies it can be concluded that RP-HPLC technique can be successfully used for the estimation of the Linezolid (LIN) in their pharmaceutical dosage tablet formulations.

The method shows good reproducibility, the RP-HPLC method is accurate, precise, specific,

reproducible and sensitive. The analysis of tablet dosage formulation of Linezolid (LIN) can also be successfully performed.

No interference of additives, matrix etc. is encountered in these methods. Further studies on other pharmaceutical formulations would throw more light on these studies.

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