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RP-RPHPLC ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF TECOVIRIMAT BULK DRUG AND FORMULATION

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ABSTRACT:

Literature survey was done on Tecovirimat. There were some reports for the analysis of and Tecovirimat as a single component and multi component in dosage form.

Development and Validation of a RP-HPLC Method for Determination of Tecovirimat bulk and Pharmaceutical Dosage Forms.

Development and Validation of RP-HPLC Method for a determination of Tecovirimat in Bulk Drug and Pharmaceutical Dosage Form"

Objective of these research is as follows:

- To develop method for Anti-inflammatory drug in bulk and solid dosage form.
- To validate accuracy, precision, linearity, robustness as per ICH guidelines.
- The objective of the present study is to establish and generate inheriting Validation data for Tecovirimat used the wavelength of uv spectrophotometer.
- Method development and validation are extremely important in the development of drug material.

For many drug products, Scientists working on Investigational New Drug (IND), New Drug Application (NDA), and Abbreviated New Drug Application (ANDA) used to characterized API'S and excipients have not been sufficiently developed or v

PLAN OF WORK

The work was planned on the conventional lines of procedure in the development of analytical method for single component formulation and it included:

- Literature Survey
- Selection of drug
- Procurement of standard Tecovirimat samples and their marketed tablet formulations
- Solid State characterization of drug
- Selection of analytical techniques
    - Chromatographic method Such as Reverse Phase High Performance Liquid chromatography (RP-HPLC) was selected.
- Method Development
- UV-Spectroscopy
    - Selection of solvent
    - Preparation of stock standard solution
    - Selection of wavelengths
- Development of RP- HPLC method using following steps:

1. Selection of stationary phase and mobile phase.
2. Selection of chromatographic condition.
3. Study of system suitability parameter.
4. Determination of Tecovirimat by proposed method.
5. Application of proposed method for estimation of marketed formulation.
6. Validation of proposed method by using following parameters:
    a) Accuracy
    b) Precision
    c) Linearity and range
    d) System Suitability Parameter
    e) Robustness
    f) Limit of detection
    g) Limit of quantitation
    h) Specificity/Selectivity
7. Compilation of data.
8. Thesis Writing.

DRUG PROFILE

Tecovirimat[34-36]

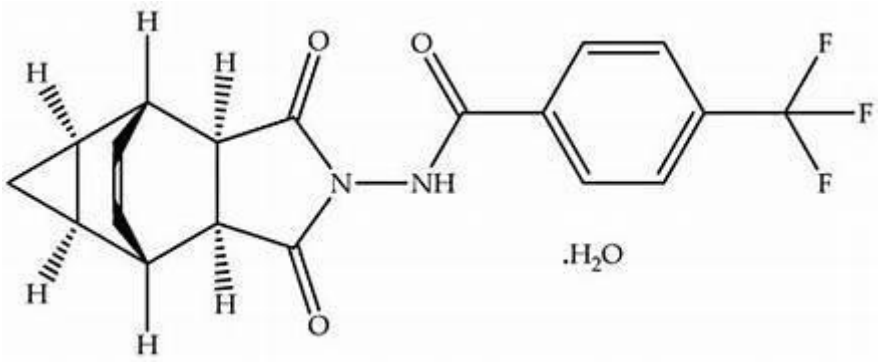

Fig.1: Structure of Tecovirima

Table No.1: Profile of Tecovirimat

Molecular Formula	$^{C}19^{H}15^{F}3^{N}2^{O}3$
IUPAC Name	*N*-{3,5-Dioxo-4-azatetracyclo[5.3.2.0$^{\{2,6\}}$.0$^{\{8,10\}}$]dodec-11-en-4- yl}-4-(trifluoromethyl) benzamide
Molecular weight	376.335 g·mol^{-1}
Appearance	White to off-white powder
Solubility	Soluble in DMSO
Category	Anti-viral agent

MATERIAL AND METHODS

Selection and Procurement of Drug

Drug sample supplier

Table 2: Drug and Drug Supplier

Name of Drug	Drug Supplier
Tecovirimat	RSITC Jalgaon.

List of reagents & chemicals used

Table 3: List of Reagents and Chemicals used

Sr. No.	Name of chemicals	Manufacturer.
1.	Acetonitrile (HPLC grade)	Merck Ltd., India
2.	Methanol (HPLC grade)	Merck Ltd., India
3.	0.1% Acetic Acid (HPLC grade)	Merck Ltd., India
4.	water (HPLC grade)	Merck Ltd., India

Selection of formulation

Table No.4: List of brand names of combined formulations of Tecovirimat

The marketed preparation was obtained from local market and is referred here after in this thesis

Sr. No	Brand name	Formulation	Available strength	Address of manufacturer
1.	Tpoxx	Capsules	Tecovirimat 200 mg	Nuary chemicals pvt Ltd

by the name as such.

Selection of Analytical Technique

HPLC was selected as analytical technique for estimation of Tecovirimat.

- **Instruments:**

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, (DAD) &Gradient Detector. Equipped with Reverse Phase (Waters) C18 column (4.6mm x 150mm; 5µm), and UV730D Absorbance detector and running chemstation 10.1 software.

❖ **Stock preparations**:

➢ Stock I : Standard Sample Preparation

Std. Tecovirimat 5 mg in 10 ml Methanol = 500 µgm/ml

➢ Stock II : cap solution Preparation:-

Take 5.625 mgs in 10 ml Methanol i.e= 500 µgm/ml

❖ **For Accuracy Solution Preparations:-**

➢ Take 10 µgm/ml CAPSULES SOLUTION FOR ACCURACY,

80 % = 0.1 ML CAPSULES SOLUTION and ADD 4 µgm/ml STD TVR AND MAKE UP VOL 10 ML WITH Mobile PHASE

100 % =0.1 ML CAPSULES SOLUTION and ADD 5 µgm/ml STD TVR AND MAKE UP VOL 10 ML WITH Mobile PHASE

120 %= 0.1 ML CAPSULES SOLUTION and ADD 6 µgm/ml STD TVR AND MAKE UP VOL 10 ML WITH Mobile PHASE

Instruments and Equipments

Table. 5: Instrument (HPLC) Details used during Method Development

	Name of Instrument	Company Name
1	HPLC Instrument	Agilent Tech. Gradient System with Auto injector (Chemstation software)
2	UV-Spectrophotometer	Analytical Technologies Limited
3	Column(C18)	Waters C18 (150mmX 4.6mm,5µm)
4	pH meter	VSI pH meter(VSI 1-B)
5	Balance	WENSAR™ High Resolution Balance.
6	Sonicator	Ultrasonics electronic instrument

EXPERIMENTAL WORK

HPLC:

Selection of Analytical Technique

- HPLC was selected as analytical technique for estimation of Tecovirimat.
- **Instruments:**

  The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, DAD Detector. Equipped with Reverse Phase C18 (Waters) with 150mm x4.6; (5µm), UV730D Absorbance detector and running chemstation 10.1 software.

a) Selection of stationary phase:

- The column used in this method C18 Waters The configuration of the column is 4.6 x 150 mm, particle size 5 µm. C18 column gives high non polar retentively, symmetric peak shape, highly reproducible and stable ideal for HPLC method

b) Solubility Studies:

- This study was carried out to find an ideal solvent in which drugs are completely soluble. Various solvents were tried for checking solubility of Tecovirimat. From solubility studies it was concluded that of Tecovirimat is freely soluble in Methanol and poorly soluble in water PH adjusted Potassium Phosphate, Buffer pH 3.1

c) Chromatographic conditions :

- ***The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.***

Table No.6: chromatographic conditions (HPLC) details used during method Development.

1.	HPLC	Agilent Tech. Gradient System with Auto injector
2.	Software	***chemstation 10.1***
3.	Column	(waters) C18 column (4.6mm x 150mm
4.	Particle size packing	***5 µm***
5.	Stationary phase	C18 (Agilent)
6.	Mobile Phase	***Methanol : 0.1% Acetic acid) 72 : 38***
7.	Detection Wavelength	***222nm***
8.	Flow rate	***1 ml/min***

9.	Temperature	***Ambient***
10.	Sample size	***20 µl***
11.	pH	***3.1***
12.	Run Time	15 min
13.	Filter paper	0.45 µm

UV-VIS Spectrophotometer:

UV-VIS Spectrophotometer was selected as analytical technique for estimation of Tecovirimat .UV absorbance range of 200-400nm.

- **Instrument** :

Analytical Technologies® Limited UV-VIS Spectrophotometer is double beam, high seed scanning spectrophotometer, The instrument needs about 1minute for initialization. The light source used is Deuterium lamp of spectrophotometer, a computer is attached which helps in data processing and manipulation Quartz curette with path length 1cm was used.

Study on the selection of uv spectrum use in uv-vis spectrometer of Tecovirimat :

Accurately weigh and transfer 5 mg Tecovirimat working standard into 100 ml volumetric flask as about dilute Methanol prepared in completely and make volume up to
the mark with the same solvent to get 100µg/ml standard (stock solution) and 15 min sonicate to dissolve it and from the resulting solution 0.2 ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with Methanol and water 1:1

Study on the chromatographic conditions of Tecovirimat:

Accurately weigh and transfer 5 mg Tecovirimat working standard into 10 ml volumetric flask as about dilute Methanol prepared in completely and make volume up to the mark with the same solvent to get 500 µg/ml standard (stock solution) and 15 min sonicate to dissolve it and from the resulting solution 0.1ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with mobile phase Methanol:(0.1% Acetic acid) Water solvent. The resulting 10 µg/ml of solution was subjected to chromatographic analyses using mobile phases of different strengths with chromatographic conditions mentioned below

- Analytical column : Waters C18 Column (150mm x 4.6mm), 5µm particle size.
- Injection volume : 20µl
- Flow rate : 1 ml/min
- Detection : 222nm
- Run Time : 10 min

- Following Mobile phase were tried:

METHOD DEVELOPMENT OF HPLC:

- **List of Mobile Phase :**

Table No.7: Selection of mobile Phase.

Sr.No.	Mobile Phase
1.	Methanol+ 0.1% OPA Ph3, (90+10),222 nm 20 Mcg, Fl. 0.7ml,
2.	Methanol+ 0.1% OPA,Ph3 (85+15),222 nm 20 Mcg, Fl. 0.7ml,
3.	Methanol + 0.1% OPA(50+50)PH3.0, 222 nm, Flow rate 0.7mL
4.	Methanol + Buffer,(50+50)PH3.0, 222 nm, Flow rate 0.8mL
5.	Methanol + 0.1% Acetic acid (60+40)PH3.0, 222 nm, Flow rate 0.7mL
6.	Methanol + 0.1% Acetic acid (60+40 %v/v)PH3.0, 222 nm, Flow rate 0.7 mL
7.	Methanol + 0.1% acetic acid (70+30 %v/v)PH3.0, 222 nm, Flow rate 1 mL sample in mob ph
8.	Methanol + 0.1% Acetic acid (72+38%v/v)PH3.1, 222 nm, Flow rate 1 mL

Analysis of standard drugs was done by following parameters:

- Melting point
- Solubility
- UV spectra and λmax
- HPLC chromatogram and retention time

Selection of wavelength by UV-Visible Spectrophotometer:- Preparation of standard stock solution:-

- **Tecovirimat standard stock solution : (Stock I)**

An accurately weighed quantity, 5 mg of Tecovirimat (TVR) was dissolved in Methanol and water in a 100ml volumetric flask and volume made up to 10.0 ml to produce a solution of 100 µg/ml.

HPLC used for chromatographic condition applies on the Preparation of standard solution:-

- **Preparation of std. Tecovirimat solution: (Stock I)**

From the freshly prepared standard stock solution (500 µg/ml), 0.1-0.5ml stock solution was pipetted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration of 5-25 µg/ml.

Selection of mobile phase:

Each mobile phase was vacuum degassed and filtered through 0.45µ membrane filter. The mobile phase was allowed to equilibrate until steady baseline was obtained. The standard solution containing of Tecovirimat was run with individual solvents as well as combinations of solvents were tried to get a good separation and stable peak. From the various mobile phases tried, mobile phase containing Methanol and Buffer with pH adjust (3.0) was selected since it gave sharp, well resolved peaks with symmetry within the limits and significant reproducible retention time for Tecovirimat.

Studies of Calibration plot:-

Optimization of Chromatographic condition:

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

- Analytical column : Waters C18 Column (150mm x 4.6mm)
- practical size : 5µm
- Injection volume : 20µl
- Flow rate : 1.0 ml/min
- Detection : 222nm
- Run Time : 15 min
- Mobile phase : Methanol : 0.1% Acetic Acid pH adjust 3.0

(72: 38)

Procedure for calibration curve of Tecovirimat:

The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. From the freshly prepared standard stock solution, pipette out 5 mg Tecovirimat in 10 ml of volumetric flask and diluted with mobile phase. From it 0.1, 0.2, 0.3, 0.4 and 0.5ml of solution were pipette out in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 5, 10,15,20,25 µg/ml of Tecovirimat. Sample were injected and peaks were recorded at 222nm as the graph plotted as concentration of drug verses peak area is depicted in respectively.

Study of system suitability parameters:

The system suitability is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test was performed by collecting data from five replicate injections of standard solution.

. Calibration Experiment:

- **RP-HPLC Method :**

a) Preparation of Calibration curve standard:

The above standard stock solution (500µg/ml) of Tecovirimat was diluted with mobile phase to yield five calibration curve (cc) standards with concentrations of 5, 10,15,20,25 µg/ml of Tecovirimat. The calibration curve of Tecovirimat is depicted in (**FigNo.23).**

b) Selection of detection Wavelength:

Standard solutions were scanned in the range of 200-400nm ,against 10 ml Methanol and volume make with water solvent system as reference Tecovirimat were showed absorbance maxima (lamda max) at 222nm **(Figure No:19**).

c) Calibration standard drug and regression equation data :

From the standard stock solution of Tecovirimat, different concentration were prepared respectively in the range of 5-25µg/ml for Tecovirimat **(Figure No:23)** and measured at 222nm. The calibration curves were plotted Regression equation data presented in **(Table No: 20).**

d) Calibration runs and regression analysis:

These calibration standard solutions were analyzed in three replicates using the under mentioned chromatographic conditions.

- Analytical column : C18 Column (150mm x 4.6mm, 5µm partical size).
- Injection volume : 20µl.
- Flow rate : 0.8 ml/min.
- Mobile phase : Methanol: 0.1% Acetic Acid (72: 38 % V/V).

- Detection : 222nm.

Validation of method for analysis of Tecovirimat:

- The developed method was validated as per ICH guidelines.

Linearity:

Linearity of an analytical method is its ability to elicit test results that are directly or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a given range, The Result are shown in;**(Table No 21).**

- **Determination:**

The linearity of the analytical method is determined by mathematical treatment of test results obtained by analysis of samples with analyte concentrations across the claimed range. Area is plotted graphically as a function of analyte concentration. **(Fig No. 24-34)** Percentage curve fittings are calculated**.** The Result is shown in; **(Table No.21 and Table No. 22).**

- **Acceptance Criteria:**

The plot should be linear passing through the origin.

Correlation Coefficient should not be less than 0.999. The Result are shown in;

- **Preparation of standard stock solution for linearity:**

Weight of sample 5 mg of Tecovirimat was weighed and transferred to 10 mL volumetric flask & diluents was added to make up the volume. Sonicated for 10 min with occasional swirling. 0.1 ml of this solution diluted up to 10 ml volumetric flask with diluents was added to make up the volume.

- **Preparation of linearity solution:**

A series of standard preparations of working standard of were prepared.

Table No.8: Table of Linearity for Rp-HPLC Method

Linearity of Tecovirimat HPLC	
Sr.No.	**Concentration (µg/mL)**
1	5
2	10
3	15
4	20
5	25

Accuracy (recovery):

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often the expressed as percent recovery by the assay of known added amounts of analyte. The accuracy of an analytical method is determined by applying the method to analyzed samples, to which known amounts of analyte have been added. The accuracy is calculated from the test results as the percentage of analyte recovered by the assay, The RP-HPLC Method Result are shown in; **(Table No:23,24).**

- **Acceptance Criteria:**

Mean recovery should be in the range of 98-102%.

The Relative Standard Deviation should not be more than 2.0%.

- **Preparation of standard stock solution:**

5 mg of Tecovirimat working standards were weighed and transferred to 10 mL volumetric flask & diluents was added to make up the volume 0.1 ml of this solution diluted upto 10 ml with diluents.

- **Application of proposed method for analysis of Capsules formulation:**

- **Accuracy**

The accuracy was determined by Tecovirimat (equivalent to 5 mg (80 %, 100 % and 120 % of the label claimed, respectively) to quantity equivalent to average weight of marketed Capsules. This powder containing 5 mg of Tecovirimat were triturated and then subjected to chromatographic analysis using the described method. The resulting was analyzed in triplicates over three days. The

% recovery of added drug was taken as a measure of accuracy**.** The Result is shown in; **(Fig No: 37,38,39)**

Table No. 9: Table of Accuracy for Rp-HPLC Method

Sample	Amount Added (mg)
	Tecovirimat
Accuracy 80%	4
Accuracy 100%	5
Accuracy 120%	6

Repeatability:

Precision of the system was determined with the sample of RP-HPLC for. Two replicates of sample solution containing 10 mg of Tecovirimat were injected and peak areas were measured and %RSD was calculated.

- **Application of proposed method for analysis of Capsules formulation:**

Weight of 5 mg of Tecovirimat was weighed and transferred to 10mL volumetric flask & diluents were added to make up the volume. Sonicated for 10 min with occasional swirling. The above solution was filtered through 0.45µm membrane filter 0.1 ml of this solution diluted up to 10 ml with diluents.

Precision:

Precision of an analytical method is the degree of agreement among Individual test results when the procedure is applied repeatedly to multiple Samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation. Also, the results obtained were subjected to one way ANOVA and within-day mean square and between-day mean square was determined and compared using F-test.

- **Result of Intraday and Inter day Precision studies on RP-HPLC method for Tecovirimat**

Intra-day precision:

Sample solutions containing 5 mg of Tecovirimat three different concentration (10µg/ml, 15µg/ml, 20µg/ml) Tecovirimat were analyzed three times on the same day and %R.S.D was calculated.

Inter-day precision:

Sample solutions containing 5 mg of Tecovirimat three different concentrations (10µg/ml, 15µg/ml, 20µg/ml) in HPLC and three different concentrations in Tecovirimat different days and % R.S.D was calculated. It is usually expressed as standard deviation or relative standard deviation.

- **Acceptance criteria:**

The Relative Standard Deviation should not be more than 2% for test

- **Preparation of standard stock solution:**

5 mg of Tecovirimat working standards were weighed and transferred to 10 mL volumetric flask & diluents was added to make up the volume. 0.1 ml of this solution diluted up to 10 ml with diluents.

Robustness:

The mobile phase composition was changed in (±1 ml/ min^{-1}**)** proportion and the flow rate was **(**of Methanol : 0.1% Acetic acid) in the mobile phase composition (±1ml/ min^{-1}**)** and the change in detection wavelength (±1 ml/ min^{-1}**)** and the effect of the results were examined**.** it was performed using 25µg/ml solution of Tecovirimat in triplicate.

Detection Limit

Based on the S.D. of the response and the slope of calibration curve, the detection limit (DL) was calculated as,

$$DL = \frac{3.3\sigma}{S}$$

Where,

σ = the S.D. of the y-intercepts of regression lines. S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

Quantitation Limit

Based on the S.D. of the response and the slope of calibration curve, the quantitation limit (QL) was calculated as,

$$QL = \frac{10\sigma}{S}$$

Where,

σ = the S.D. of the y-intercepts of regression lines. S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

Analysis of marketed formulation

To determine the content of Tecovirimat in marketed Capsules (label claim 200 mg of Tecovirimat), 20 Capsules powder weighed in 4.50 gms and average weight of powder was calculated in 225mg. Capsules were triturated and powder equivalent to weighed in 5.625 mg The drug was extracted from the Capsules powder with 10 mL Methanol . To ensure complete extraction it was sonicated for 15 min. 0.4 mL of supernatant was then diluted up to 10 mL with mobile phase. The resulting solution was injected in HPLC and drug peak area was noted**.**

Regression equation was generated using peak areas of standard solutions. Using the regression equation and peak area of the sample the amount of Tecovirimat in the sample was calculated. The amount of Tecovirimat per formulation was obtained from the regression equation of the calibration curve as described in analysis of Capsules formulation are shown in **(Table No.28)**

RESULT AND DISCUSSION:

Preliminary studies on Tecovirimat. Melting point

The procured reference standard of Tecovirimat was found to melt in the range of 196-199^{0}C.

Solubility

The drug was found to be

- Freely soluble in Acetonitrile, methanol DMSO.
- freely *soluble in organic solvents*.

UV Spectroscopy

Standard solutions were scanned in the range of 200-400nm ,against 10 ml methanol and volume make with water solvent system as reference Tecovirimat in water was found to be selected wavelength is 222 nm .

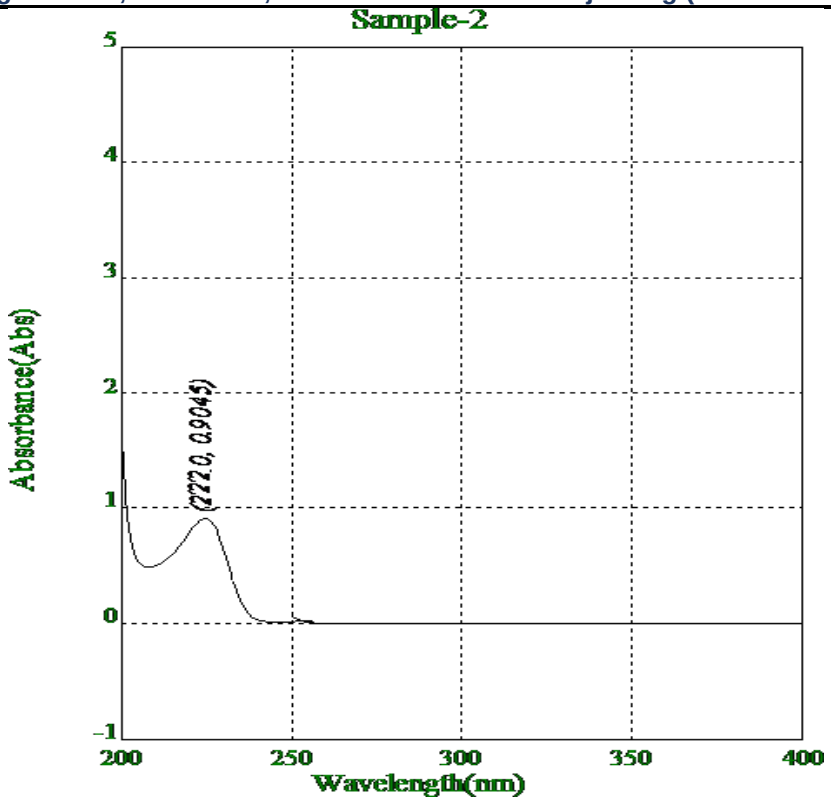

Fig No.2: UV Spectrum of Tecovirimat

TABLE NO-10: Chromatographic behavior of Tecovirimat mobile phase of various compositions.

Fig No	Column used	Mobile phase, Flow Rateand Wavelength	Inj. Vol.	Observation	Conclusion
1.	C18 (Waters) (4.6mm x 150mm), 5.0µ)	Methanol+ 0.1% OPA Ph3, (90+10),222 nm 20 Mcg, Fl. 0.7ml,	20µl	Sharp Peaks were not obtained	Hence rejected
2.	C18 (Waters) (4.6mm x 150mm), 5.0µ)	Methanol+ 0.1% OPA,Ph3 (85+15),222 nm 20 Mcg, Fl. 0.7ml,	20 µl	Sharp Peaks were not obtained	Hence rejected
3.	C18 (Waters) (4.6mm x 150mm), 5.0µ)	Methanol + 0.1% OPA(50+50)PH3.0, 222 nm, Flow rate 0.7mL	20 µl	Sharp Peaks were not obtained	Hence rejected

4.	C18 (Waters) (4.6mm x 150mm), 5.0µ)	Methanol + Buffer,(50+50)PH3.0 , 222 nm, Flow rate 0.8mL	20 µl	Sharp Peaks were not obtained	Hence rejected
5.	C18 (Waters) (4.6mm x1250mm), 5.0µ)	Methanol + 0.1% Acetic acid (60+40)PH3.0, 222 nm, Flow rate 0.7mL	20 µl	Sharp Peaks were not obtained	Hence rejected
6.	C18 (Waters) (4.6mm x 150mm), 5.0µ)	Methanol + 0.1% Acetic acid (60+40 %v/v)PH3.0, 222 nm, Flow rate 0.7 mL	20 µl	Sharp Peaks were not obtained	Hence rejected
7.	C18 (Waters) (4.6mm x 150mm), 5.0µ)	Methanol + 0.1% acetic acid (70+30 %v/v)PH3.0, 222 nm,	20 µl	Sharp Peaks were not obtained	Hence rejected
		Flow rate 1 mL sample in mob ph			
8.	C18 (Waters) (4.6mm x 150mm), 5.0µ)	Methanol + 0.1% Acetic acid (72+38%v/v)PH3.1, 222 nm, Flow rate 1 mL		Sharp and well resolved Peaks were obtained	Hence selected

Thus, from the above, it has been observed that, using mobile phase of meoh+0.1% Acetic acid,(72:38 % v/v),PH 3.1, 222 nm, Flow rate 1 ml gave adequate retention at 3.142 min with good peak shape (Theoretical plates: Tecovirimat 5989).

Chromatogram of Trial 1:

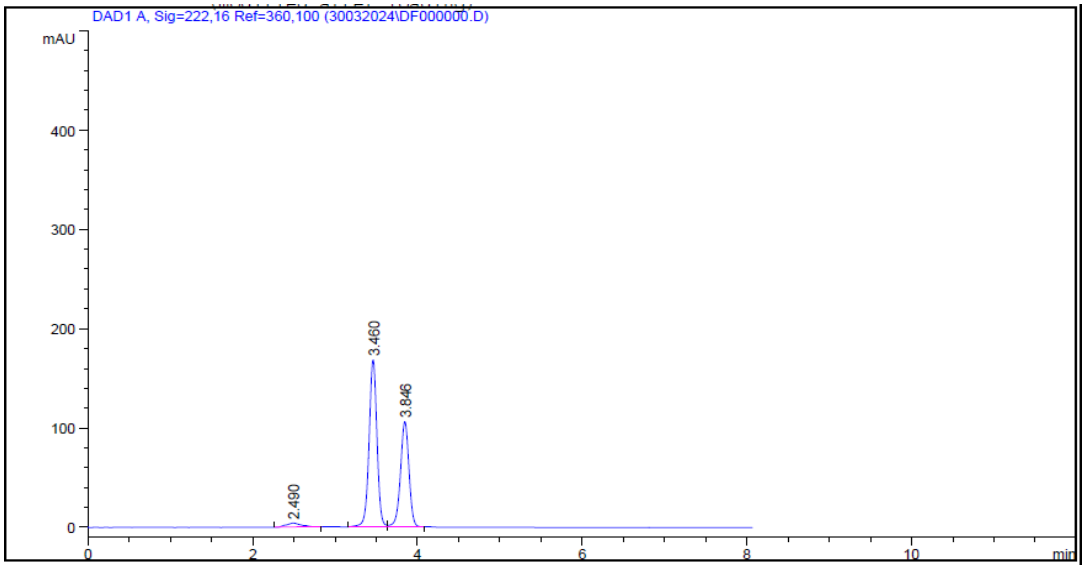

Fig No 3: Chromatogram of Trial 1 Table.No.11. Trial-1 of chromatogram of Tecovirimat

No.	RT[min]	Area[mV*s]	TP	TF
1	3.460	1120.7733	6784	1.02
2	3.846	765.4099	7026	1.04

Observation: peak splitting were observation so unsatisfactory result, so method was rejected.

Chromatogram of Trial 2:

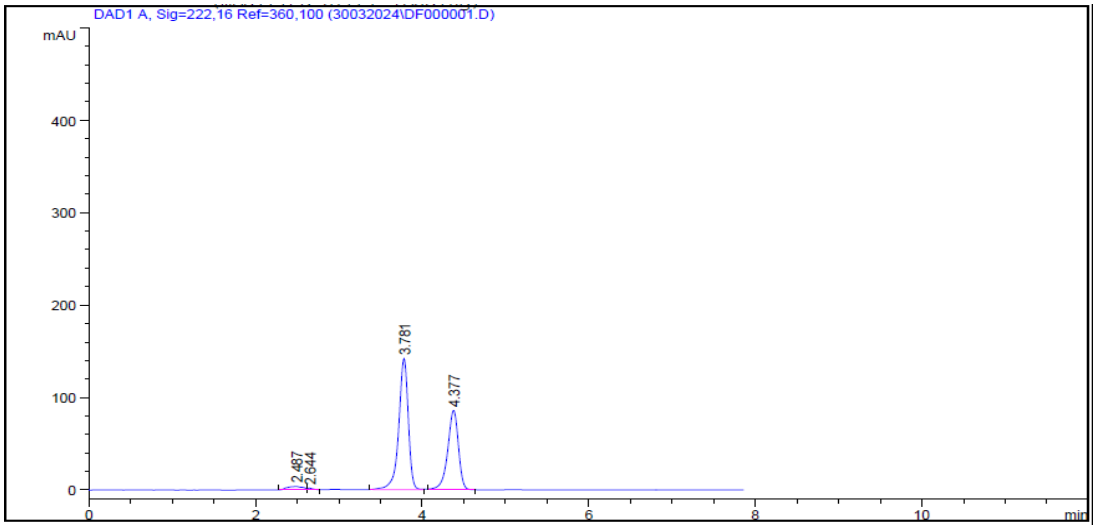

Fig No 4: Chromatogram of Trial 2

Table.No.12. Trial-2 of chromatogram of Tecovirimat

No.	RT[min]	Area[mV*s]	TP	TF
1	3.781	1135.6019	5990	1.21
2	4.377	769.4758	5970	1.22

Observation: peak splitting were observation so unsatisfactory result, so method was rejected.

Chromatogram of Trial 3:

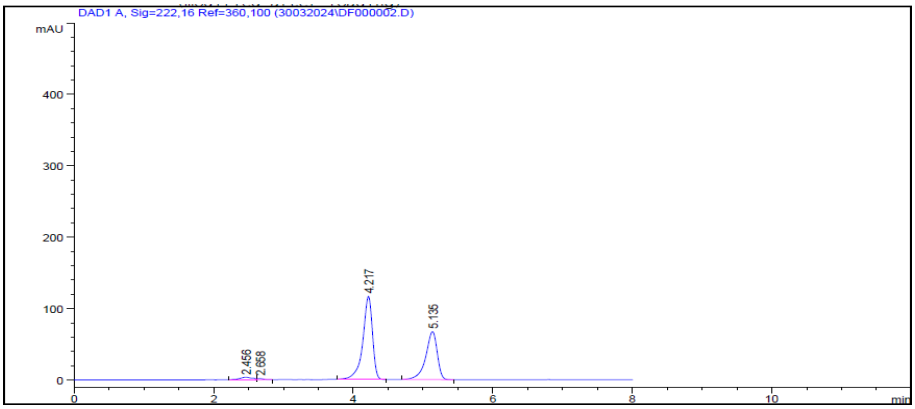

Fig No 5: Chromatogram of Trial 3 Table.No.13. Trial-3 of chromatogram of Tecovirimat

No.	RT[min]	Area[mV*s]	TP	TF
1	4.217	1126.5670	5191	1.41
2	5.135	769.7840	5259	1.42

Observation: peak splitting were observation so unsatisfactory result, so method was rejected.

Chromatogram of Trial 4:

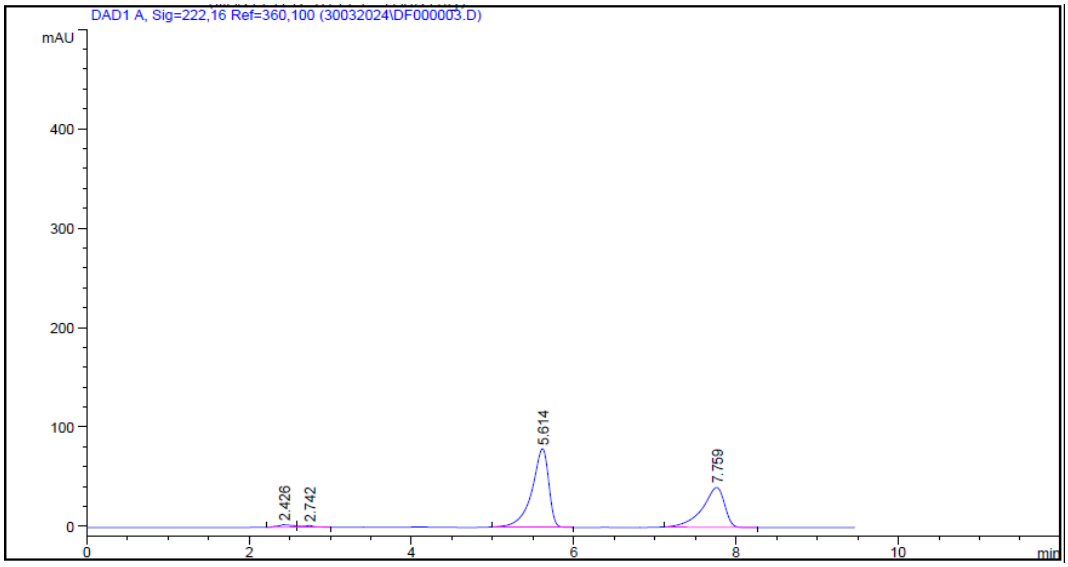

Fig No 6: Chromatogram of Trial 4

Table.No.14. Trial-4 of chromatogram of Tecovirimat

No.	RT[min]	Area[mV*s]	TP	TF
1	5.614	1149.8631	4088	1.72
2.	7.759	769.028	4464	1.83

Observation: Broad peak and peak splitting were observation so unsatisfactory result, so method was rejected.

Chromatogram of Trial 5:

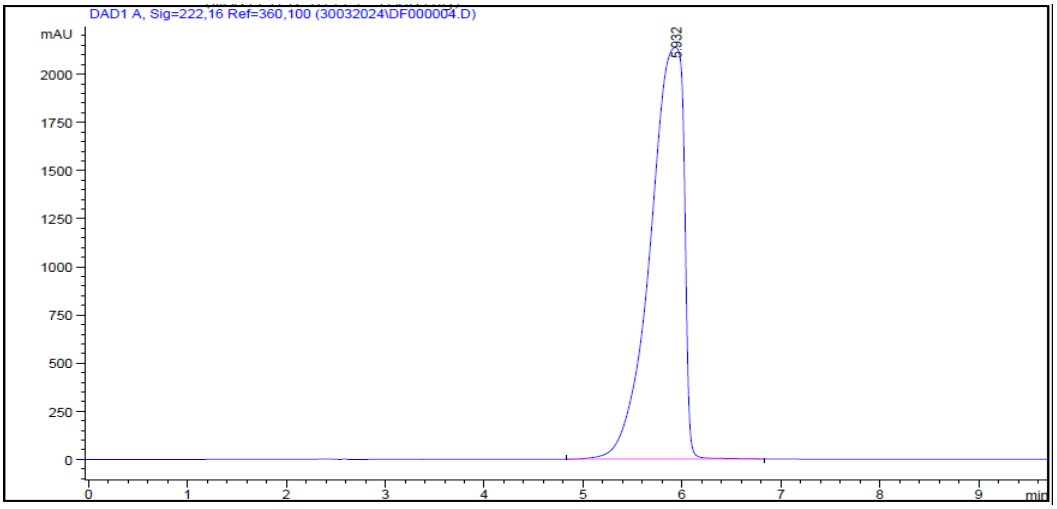

Fig No 7: Chromatogram of Trial 5 Table.No.15. Trial-5 of chromatogram of Tecovirimat

No.	RT[min]	Area[mV*s]	TP	TF
1	5.932	50710.0	1504	2.75

Observation: Broad peak were observation so unsatisfactory result, so method was rejected.

Chromatogram of Trial 6:

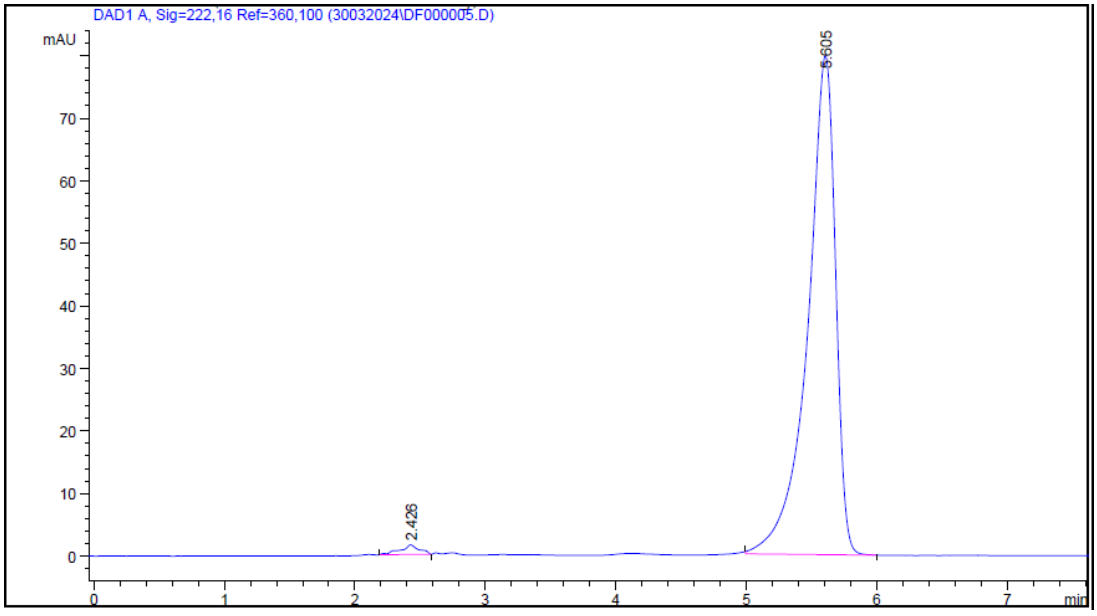

Fig No 8: Chromatogram of Trial 6 Table.No.16. Trial-6 of chromatogram of Tecovirimat

No.	RT[min]	Area[mV*s]	TP	TF
1	5.605	1180.8557	4076	1.74

Observation: peak fronting was observation so unsatisfactory result, so method was rejected.

Chromatogram of Trial 7:

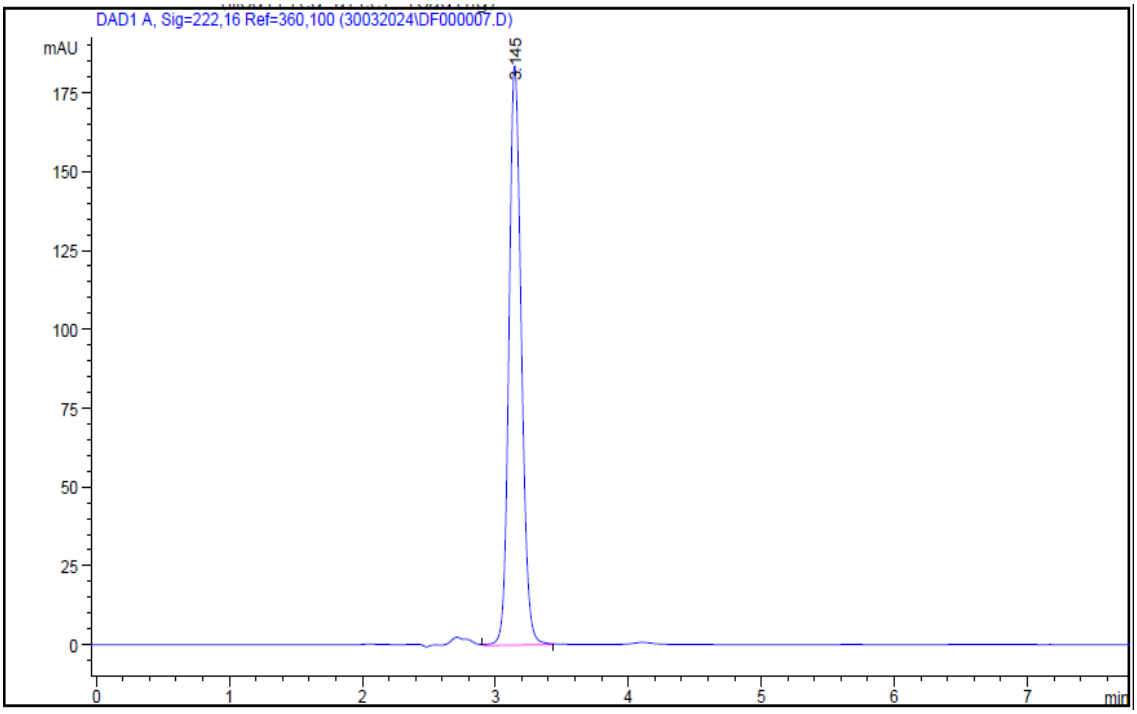

Fig No 9: Chromatogram of Trial 7 Table.No.17. Trial-7 of chromatogram of Tecovirimat

No.	RT[min]	Area[mV*s]	TP	TF
1	3.145	1190.9309	5783	0.82

Chromatogram of Final Trial 8:

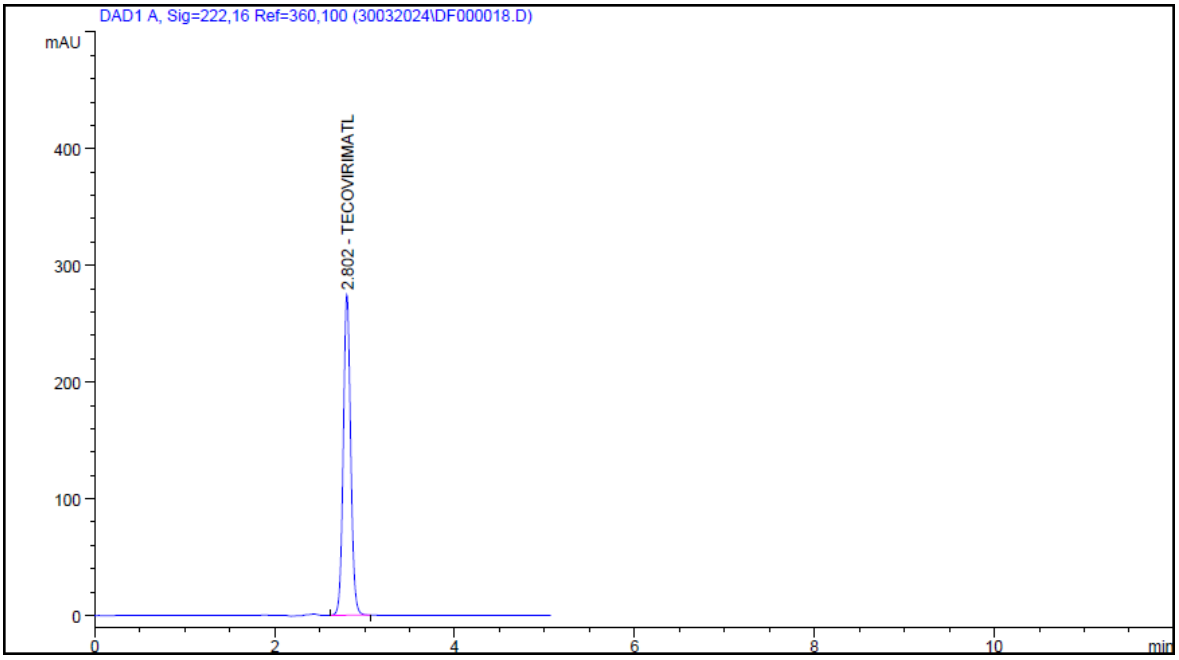

Fig No 10: Chromatogram of final Trial 8 Table.No.18. Final

Trial-8 of chromatogram of Tecovirimat

No.	RT[min]	Area[mV*s]	TP	TF
1	2.802	1573.43738	5942	0.90

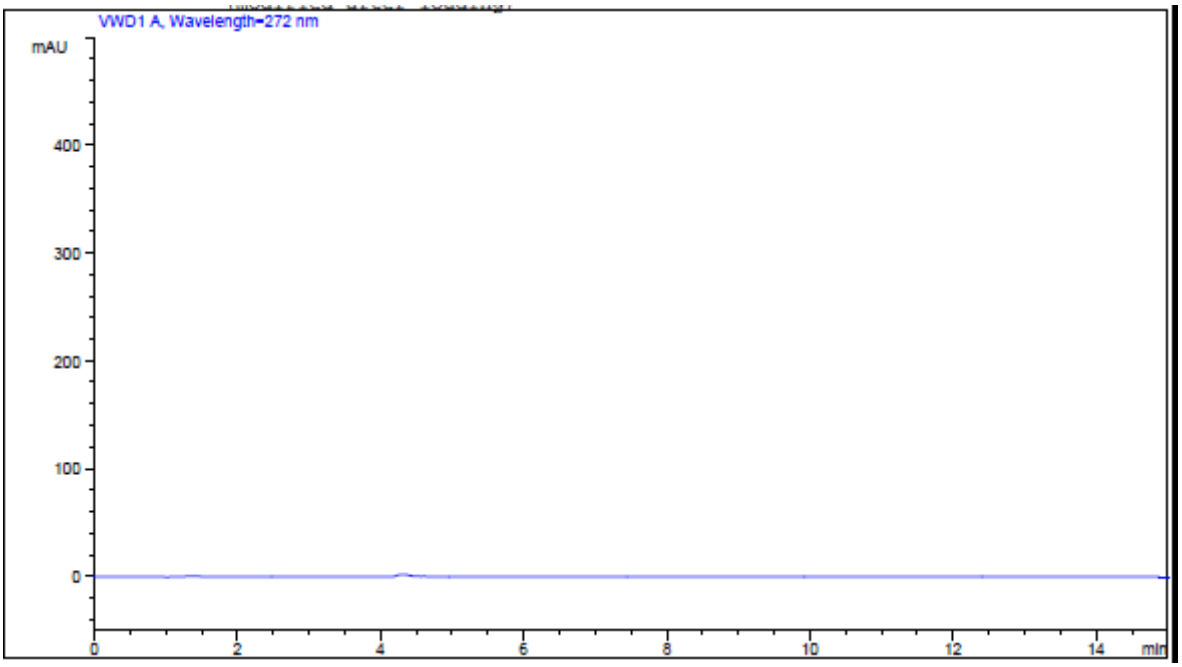

Fig No 11: Chromatogram of blank

➢ **The final chromatographic conditions selected were as follow:**

- Analytical column : waters C18 Column (150mm x 4.6mm, 5µm partical size).
- Injection volume : 20µl.
- Flow rate : 1 ml/min.
- Mobile phase : Methanol : 0.1% Acetic acid (72: 38% V/V)
- Detection : 222 nm.
- Run Time : 15 min.

➢ **Preparation of Standard chromatogram of Tecovirimat**

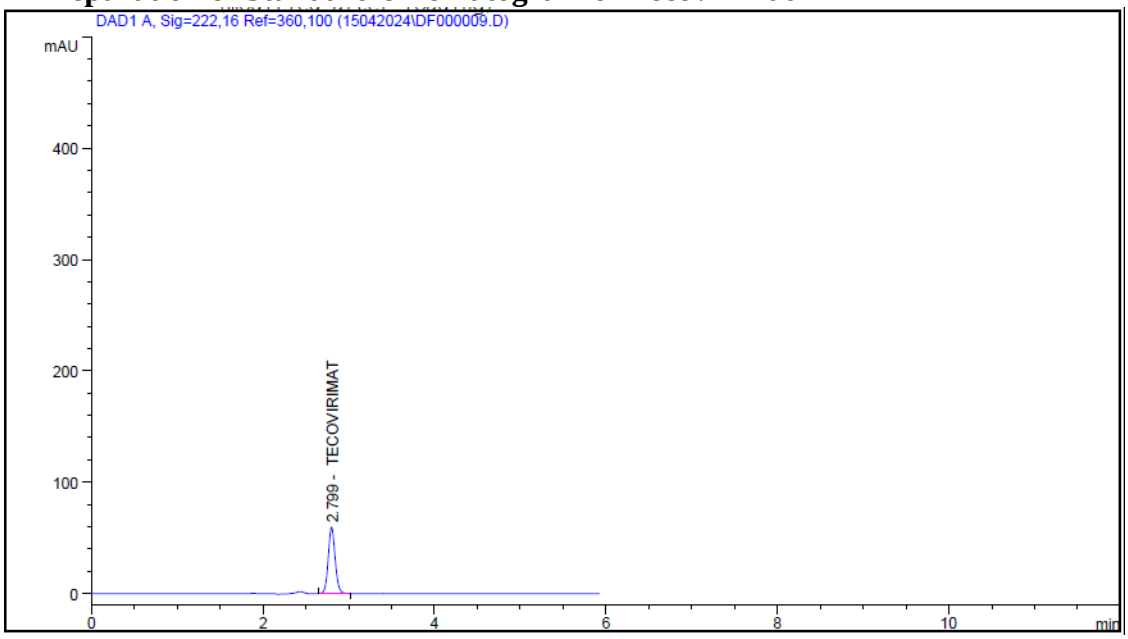

Fig No.12: Chromatogram of standard Tecovirimat Table.No.19. Details of chromatogram of standard Tecovirimat

No.	Name	RT[min]	Area[mV*s]	TP	TF
1	TVR	2.798	628.7930	5926	0.89

In the standard of Tecovirimat theoretical plates were found above 2000 i.e. for Tecovirimat 5926 at minimum RT 2.798.

Calibration experiment

➢ **RP-HPLC Method :**

The data obtained in the calibration experiments when subjected to linear regression analysis showed a linear relationship between peak areas and concentrations in the range 5-25µg/mL for Tecovirimat (**Table No:20)**depict the calibration data of Tecovirimat The respective linear equation for Tecovirimat was Y= 62.77x+11.78 where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Tecovirimat is depicted in (**FigNo.23).**

Table No 20: Linearity data for Tecovirimat

Method	Conc µg/ml	Peak area(µV.sec)		Average peak area (µV.sec)	S.D. of Peak Area	% RSD of Peak Area
		1	2			
RP-HPLC Method	5	334.4977	333.844	334.1709	0.46	0.14
	10	628.7930	627.131	627.9622	1.17	0.19
	15	944.7210	943.752	944.2367	0.68	0.07
	20	1287.4742	1285.84	1286.6579	1.15	0.09
	25	1575.1557	1573.43	1574.2965	1.22	0.08
	Equation		Y= 62.77x+11.78			
	R^2		0.999			

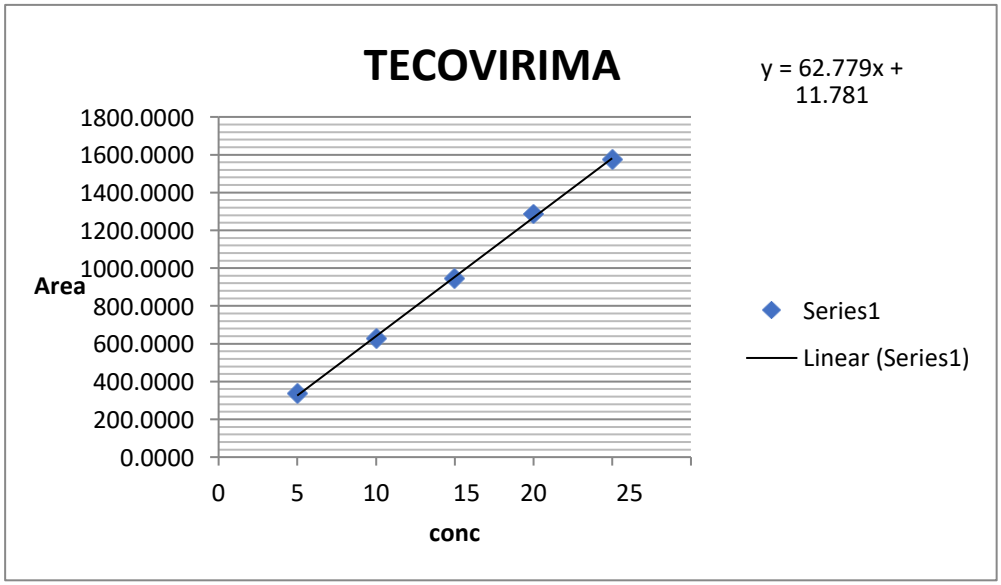

Fig.No.12: Calibration curve of Tecovirimat (HPLC)

The RP-HPLC Method for respective linear equation for Tecovirimat was y = 62.77x +11.78where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Tecovirimat is depicted in **Fig 23.**

Analytical of Method Validation:

1. Linearity:

From Tecovirimat standard stock solution, different working standard solution (5- 25µg/ml) were prepared in mobile phase 20 µl of sample solution was injected into the chromatographic system using mixed volume loop injector Chromatograms were recorded. The area for each conce ntration was recorded (**Table No. 21)** The Calibration curves are shown in **[Fig. No.36]**

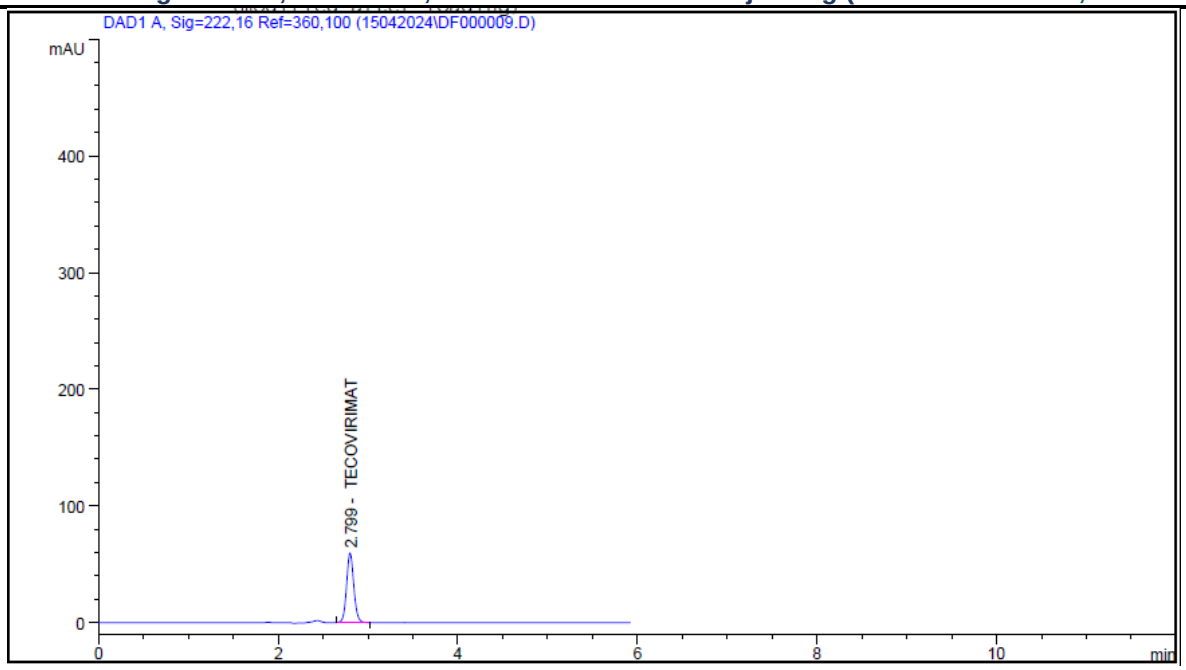

Fig.No.13.Chromatogram of linearity

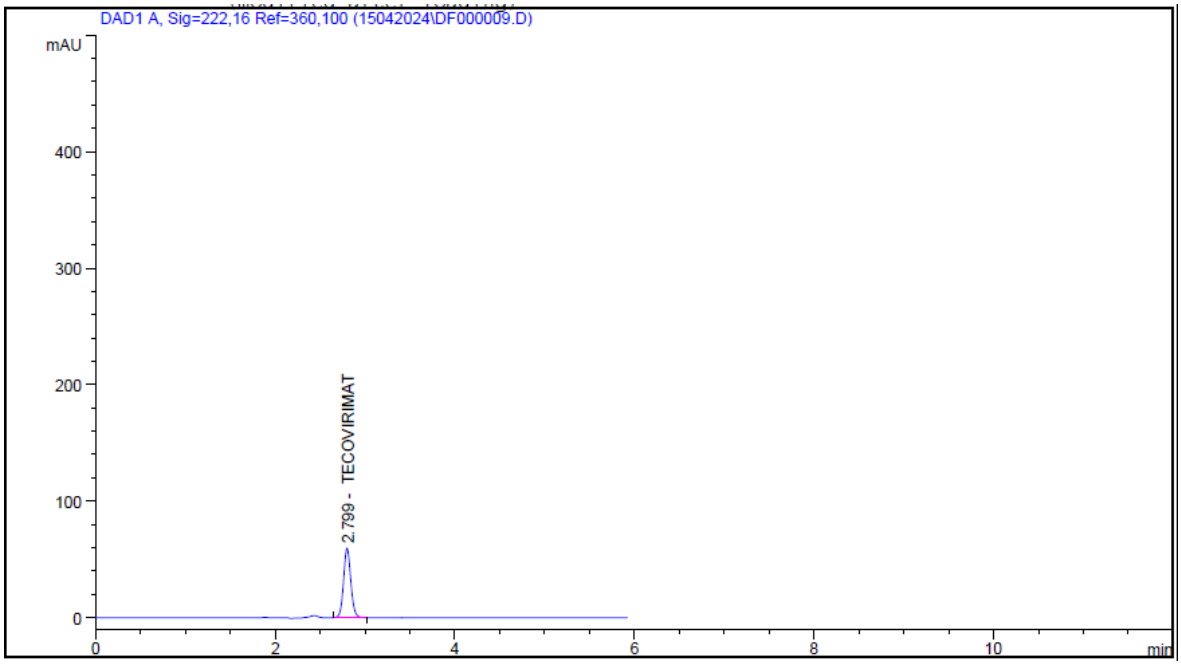

Fig.No.14.Chromatogram of linearity (5 mcg)-01

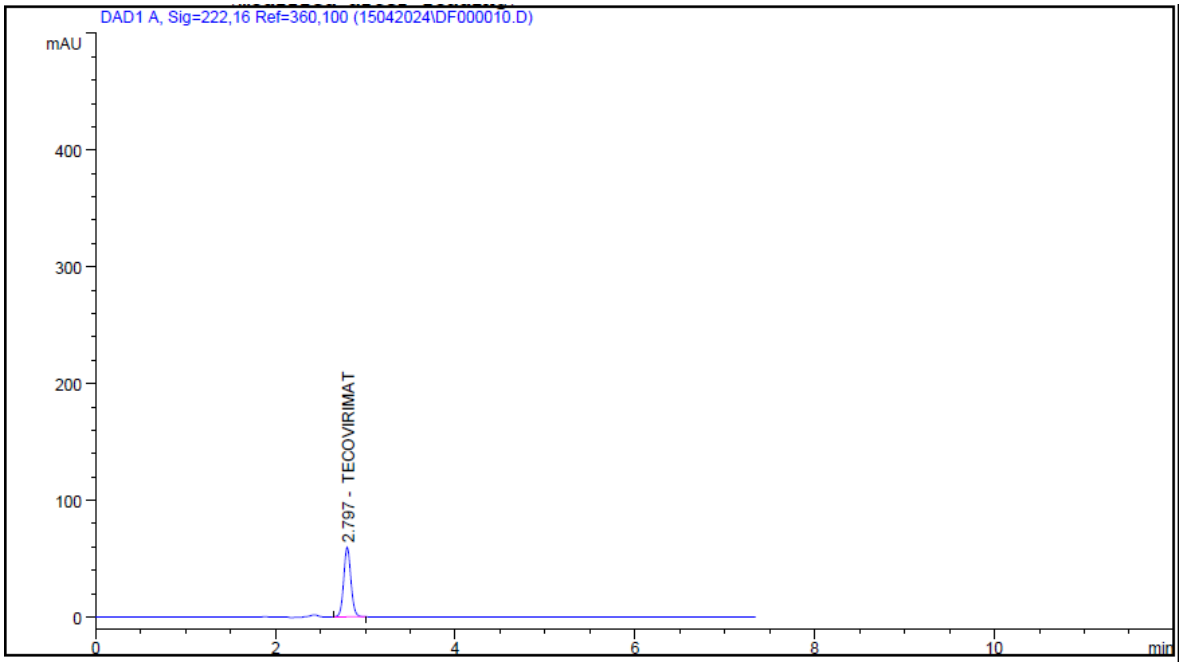

Fig.No.15.Chromatogram of linearity (5 mcg)-02

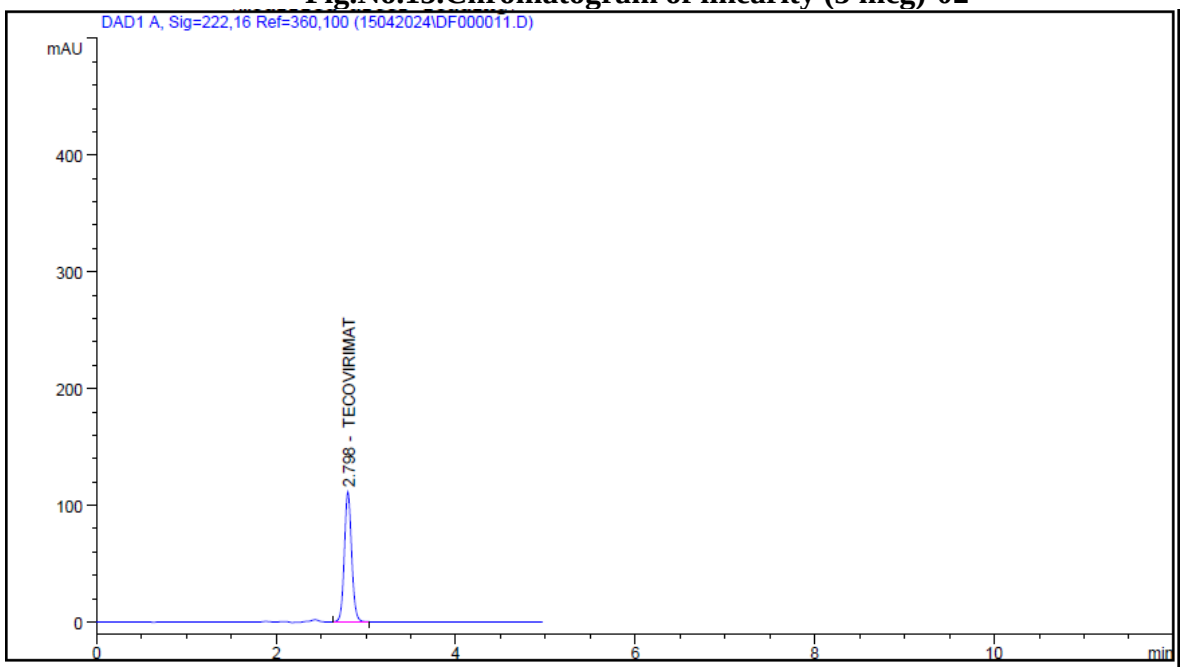

Fig.No.16.Chromatogram of linearity (10 mcg)-01

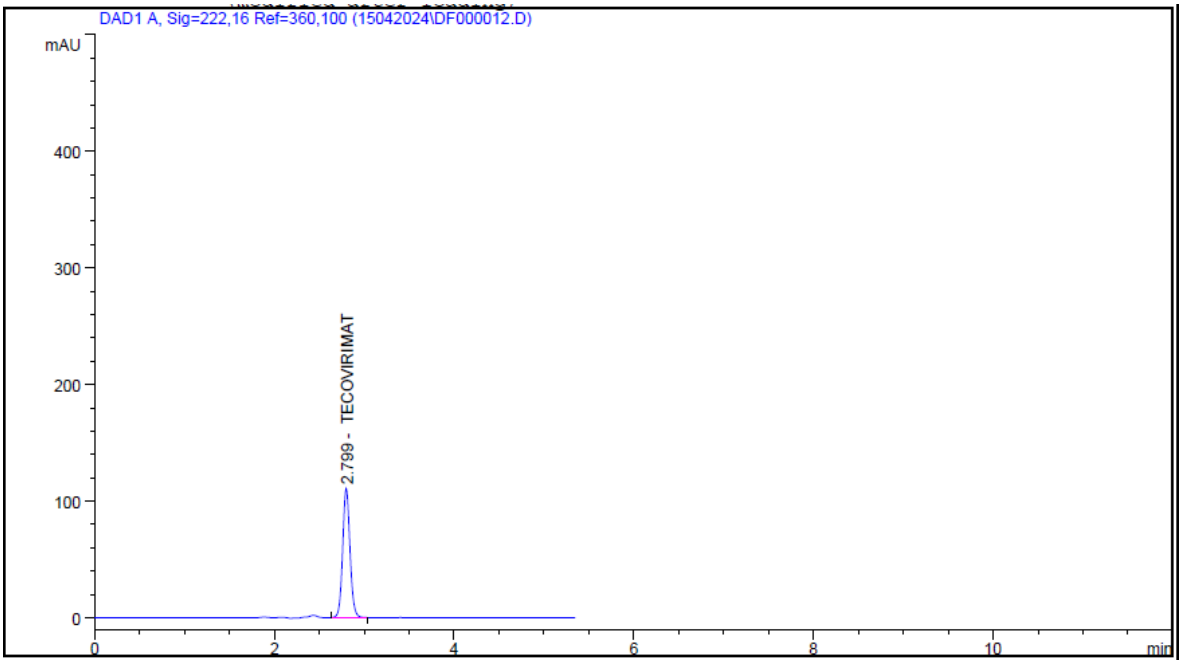

Fig.No.17.Chromatogram of linearity (10 mcg)-02

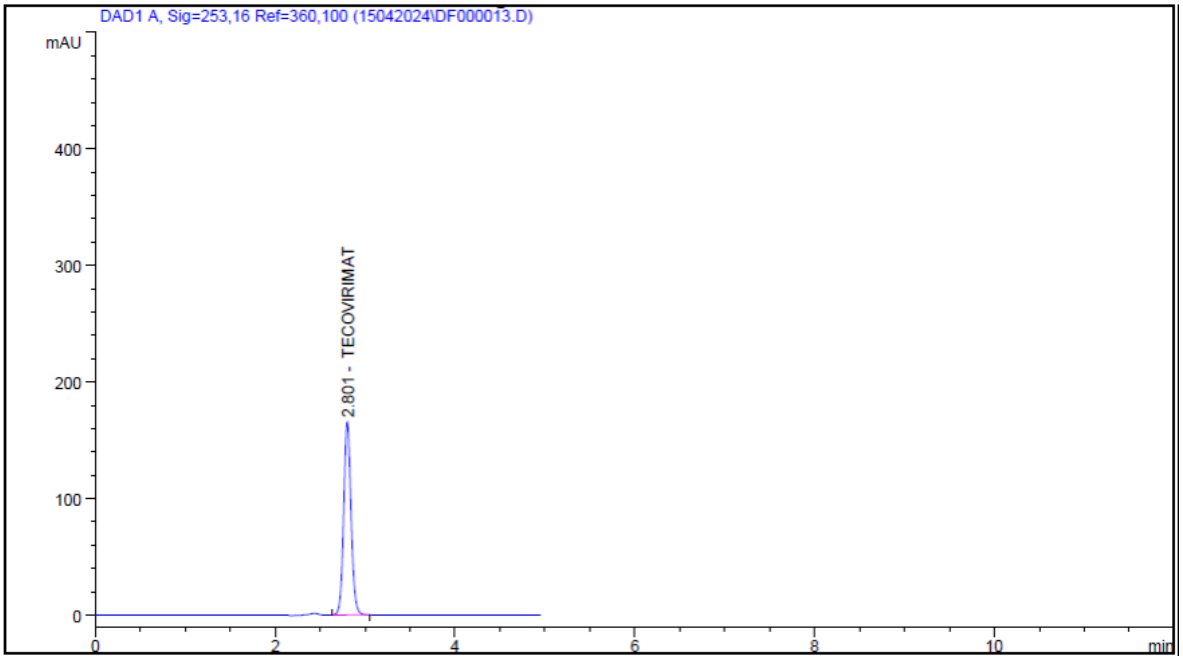

Fig.No.18.Chromatogram of linearity (15 mcg)-01

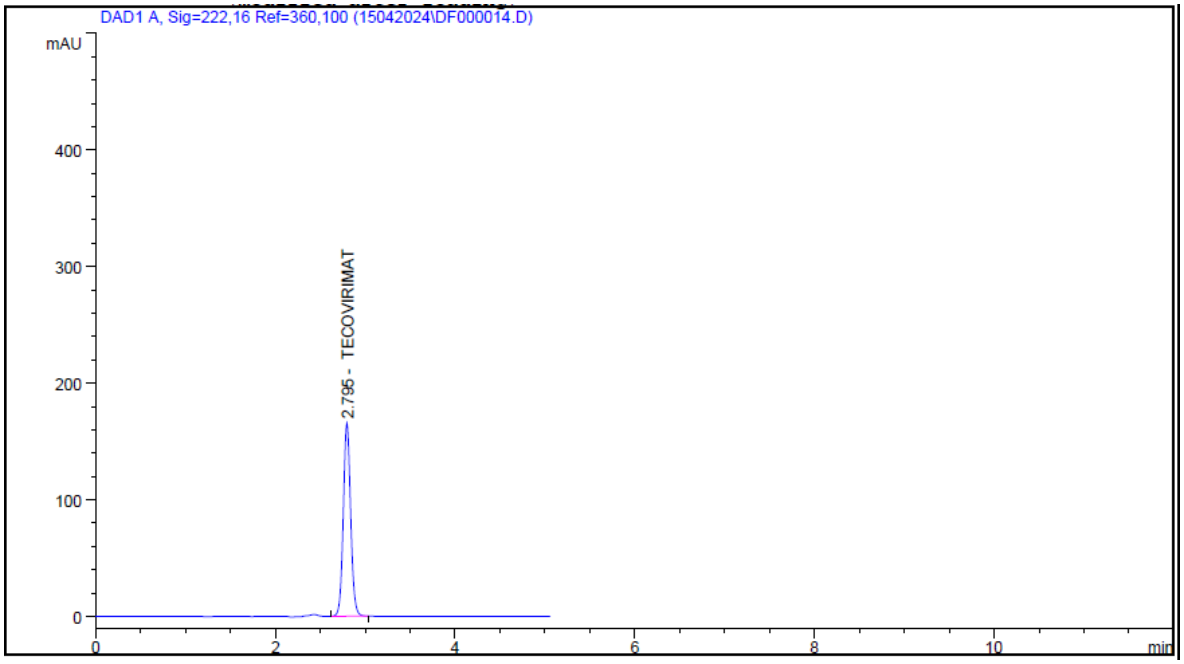

Fig.No19.Chromatogram of linearity (15 mcg)-02

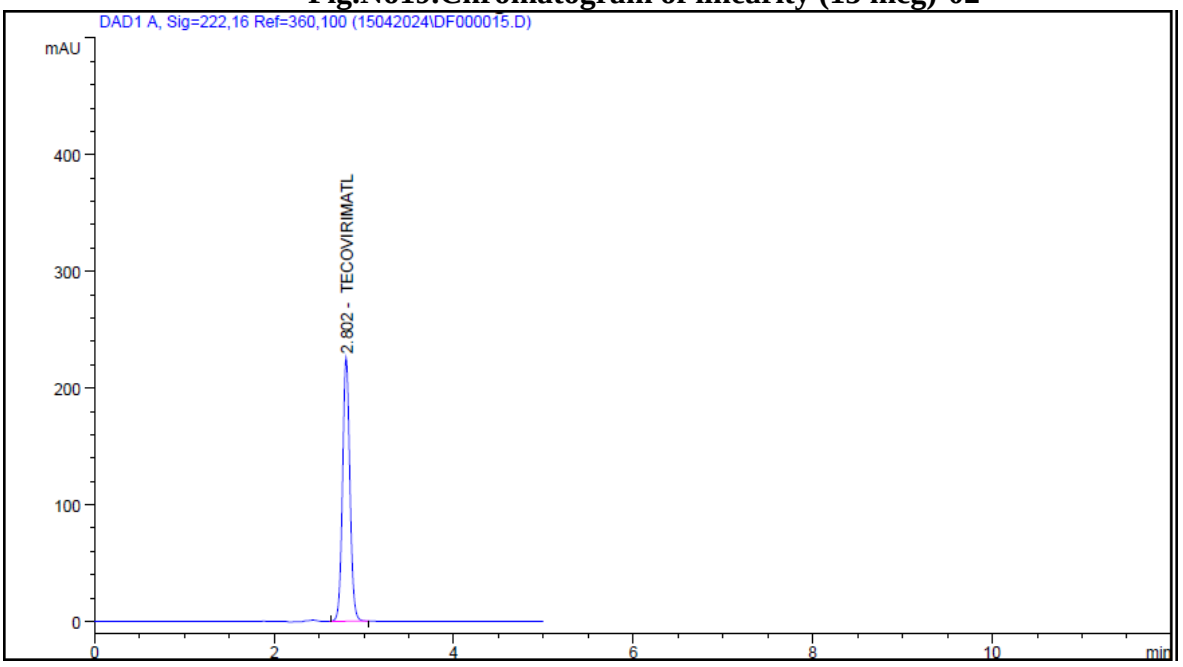

Fig.No.20.Chromatogram of linearity (20 mcg)-01

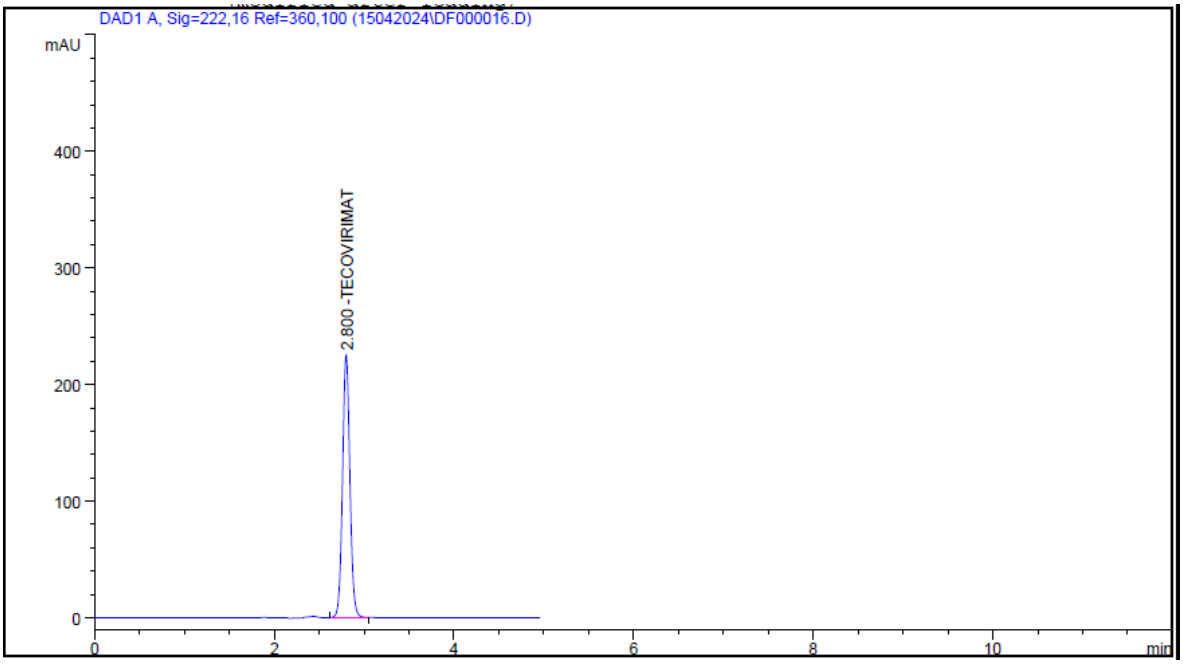

Fig.No.21.Chromatogram of linearity (20 mcg)-02

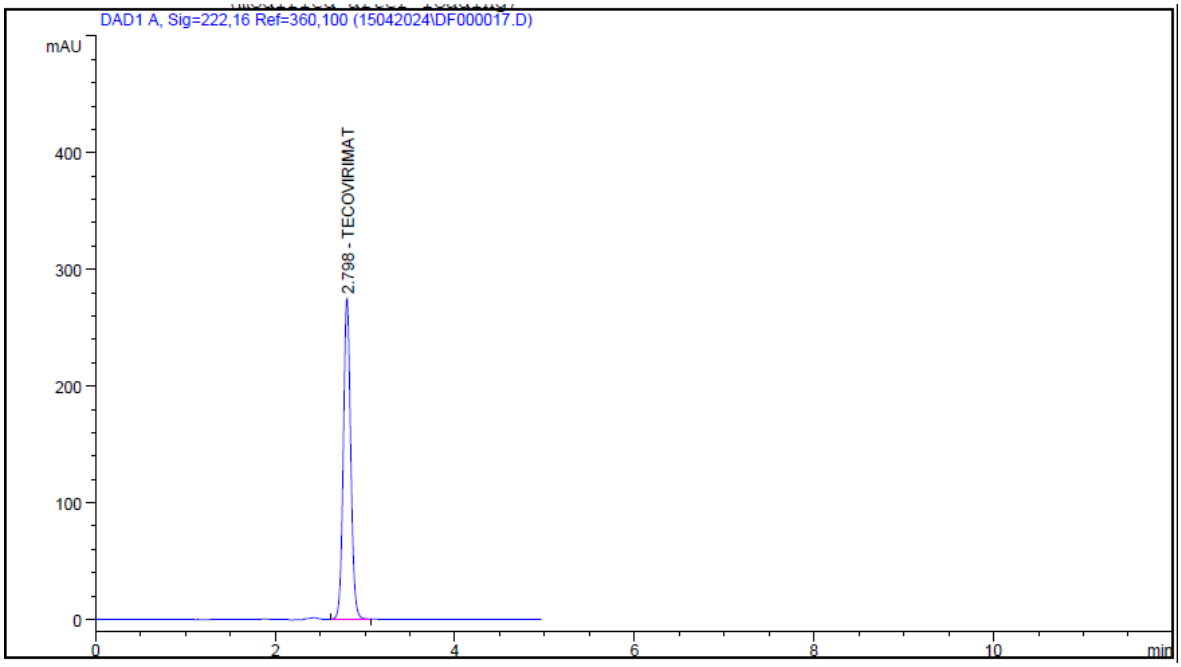

Fig.No.22.Chromatogram of linearity (25 mcg)-01

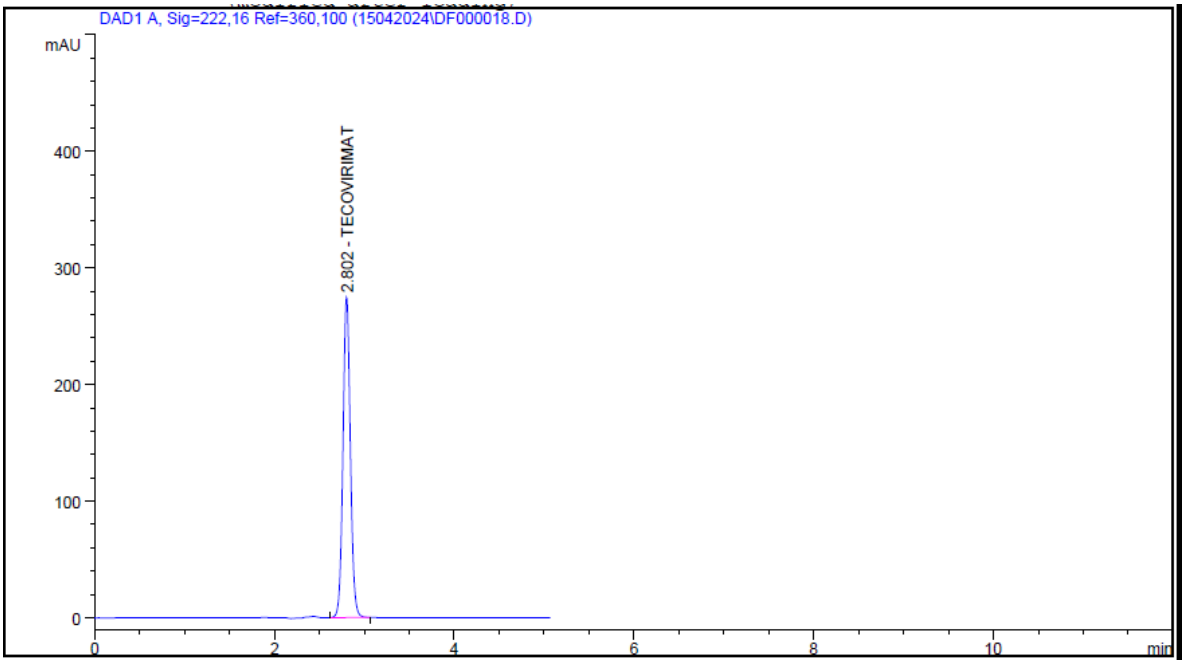

Fig.No.23.Chromatogram of linearity (25 mcg)-02

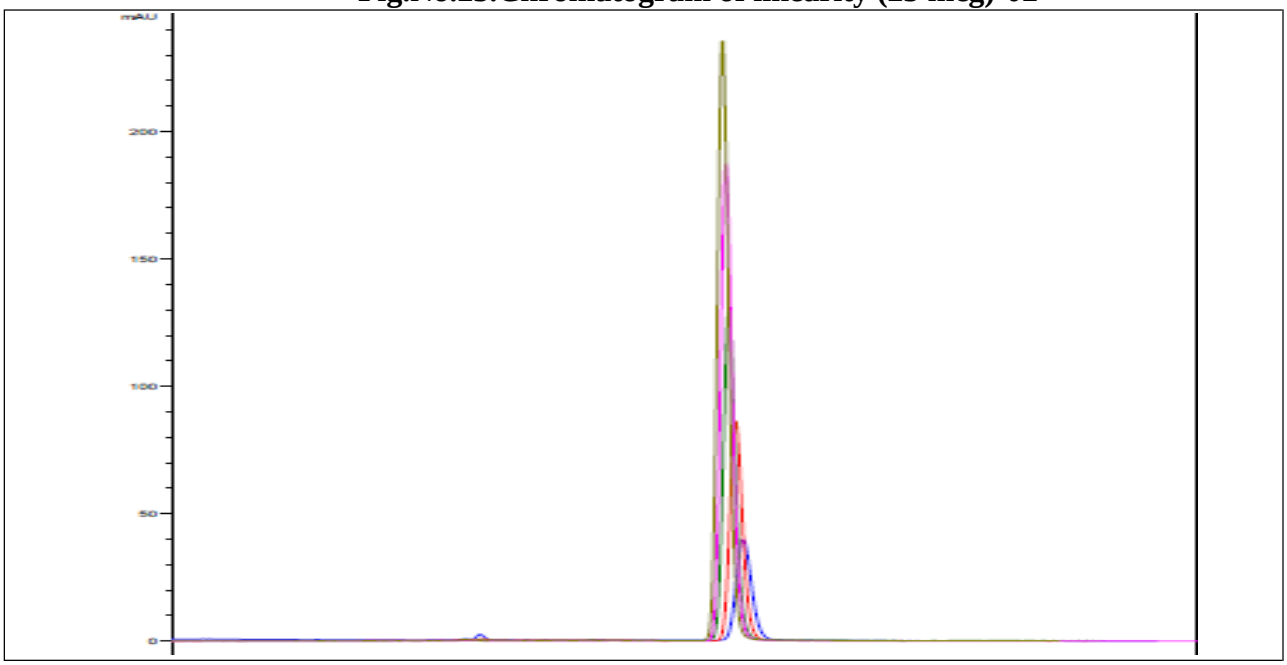

Fig.No.24.Overlay Chromatogram of linearity Table No 21. Linearity of Tecovirimat

Sr. No.	Concentration µg/ml Tecovirimat	Area Tecovirimat
1	5	334.1709
2	10	627.9622
3	15	944.2367
4	20	1286.6579
5	25	1574.2965

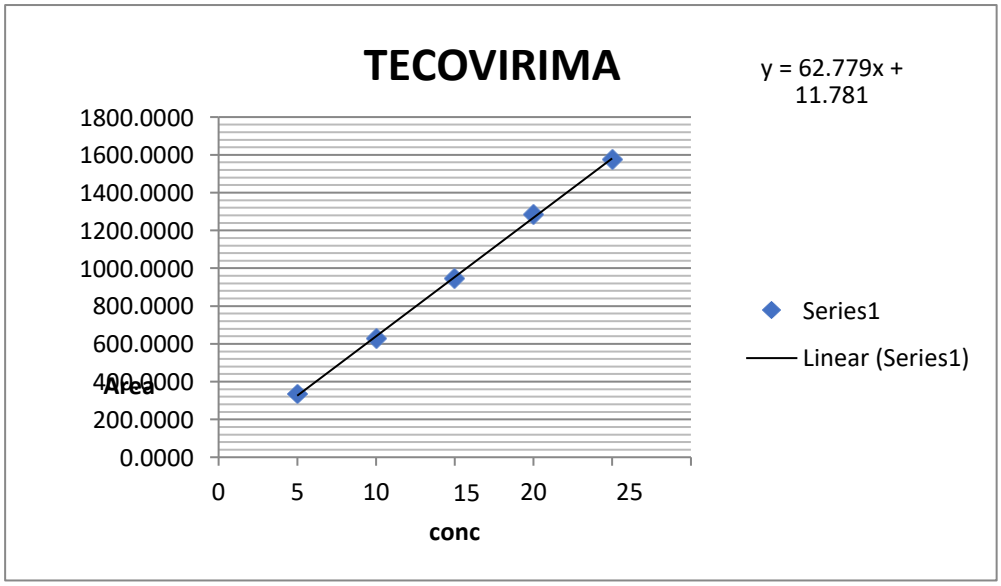

Fig.No.25. Calibration curve of Tecovirimat for HPLC method Table No 22.

Regression equation data for Tecovirimat

Regression Equation Data Y=mx+c	
Slope(m)	62.77
Intercept(c)	11.78
Correlation Coefficient	0.999

Linearity of of Tecovirimat was observed in the range of 5-25µg/ml Detection wavelength used was 222 nm.

The plot should be linear passing through the origin; Correlation Coefficient should not be less than 0.999.that concluded. **(Table. No. 22)**

2. Accuracy:-

Recovery studies were performed to validate the accuracy of developed method. To pre analyzed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed **(Table No.37).** Statistical validation of recovery studies shown in **(Table No. 39).**

Accuracy 80%

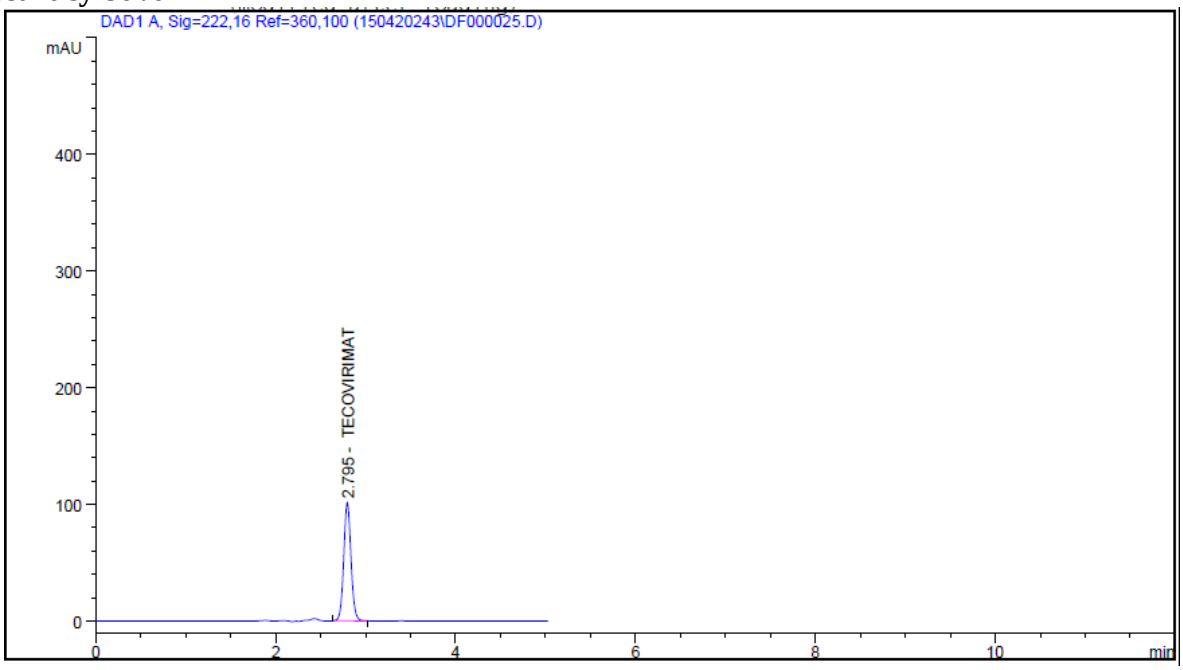

Fig.26. Chromatogram of Accuracy 80%

Accuracy 100%

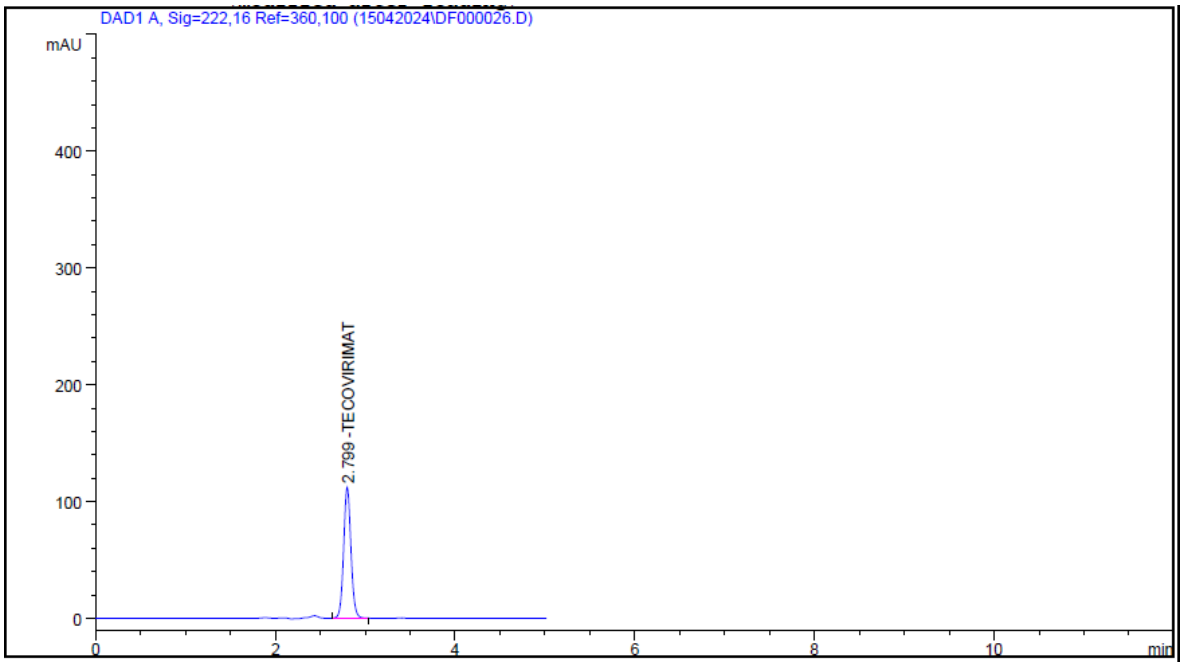

Fig.27. Chromatogram of Accuracy 100%

Accuracy 120%

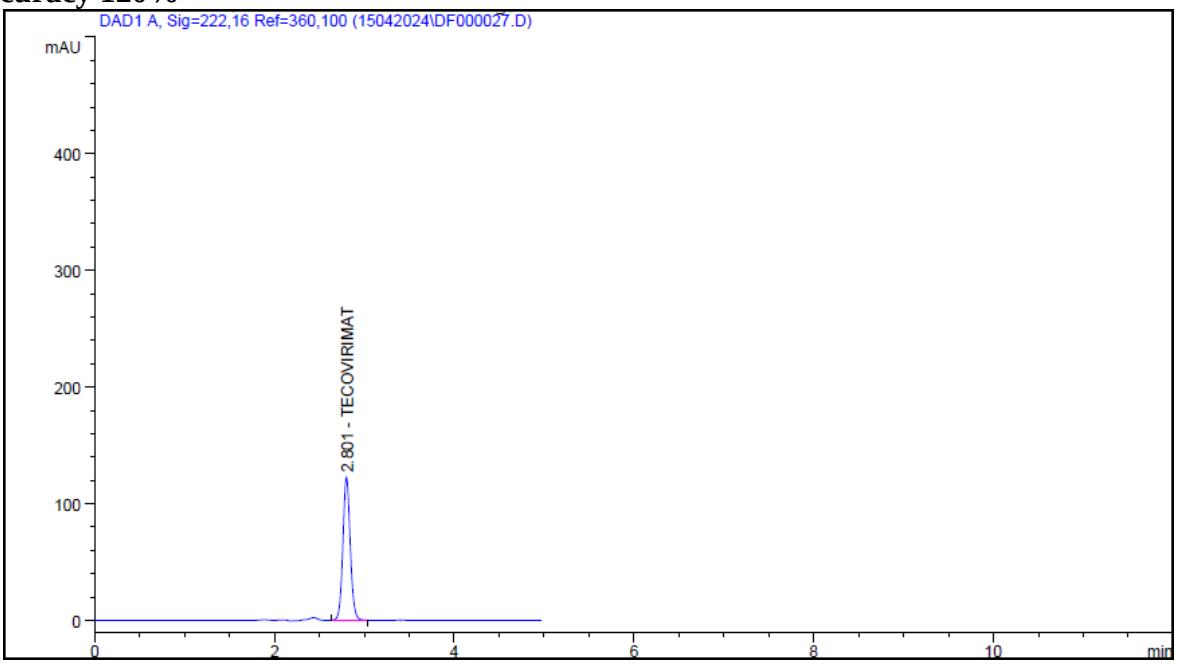

Fig.28. Chromatogram of Accuracy 120% Table .23. Result of Recovery data for Tecovirimat

METHOD	Drug	Level (%)	Amt. taken (µg/ml	Amt. Added (µg/ml	Absorbance Mean* ± S.D.	Amt. recovered Mean *±S.D.	%Recovery Mean *± S.D.
RP-HPLC Method	TVR	80%	5	4	9.03±0.0.01	4.04±0.011	100.99±0.27
		100 %	5	5	9.92±0.019	4.92±0.019	99.59 ±1.99
		120 %	5	6	10.93±0.013	5.93±0.013	98.75±0.21

***mean of each 3 reading for RP-HPLC method**

Table.24. Statistical Validation of Recovery Studies Tecovirimat

METHOD	Drug	Level (%)	Mean % Recovery	Standard Deviation*	% RSD
RP-HPLC Method	TVR	80%	99.09	0.27	0.27
		100%	97.27	0.019	0.38
		120%	98.36	0.21	0.22

***Denotes average of three determinations for RP-HPLC method**

Accuracy of RP-HPLC method is ascertained by recovery studies performed at different levels of concentrations (80%, 100% and 120%). The % recovery was found to be within 97-102% **(Table No. 26, 27)**.

3. System suitability parameters :(Repeatability)

To ascertain the resolution and reproducibility of the proposed chromatographic system for estimation of Tecovirimat system suitability parameters were studied. The result shown in below **(Table No.25).**

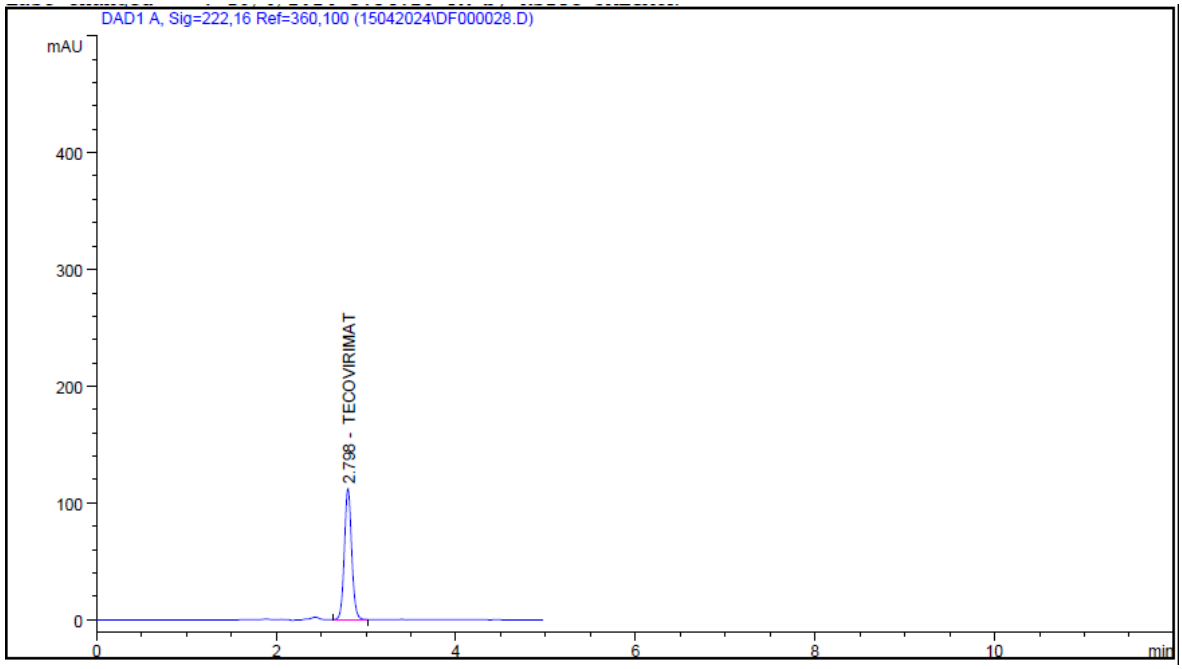

Fig No.29: Chromatogram of System suitability -1 (10 mcg)

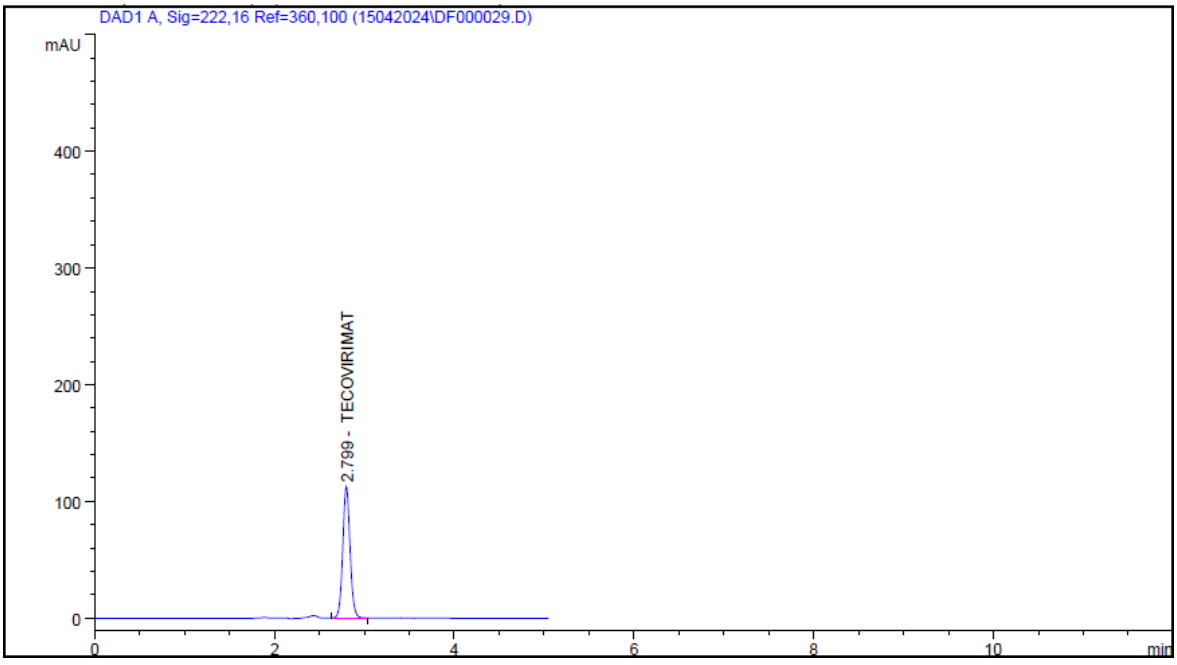

Fig No 30: Chromatogram of System suitability No-2

Table No.25: Repeatability studies on RP-HPLC for Tecovirimat

Sr.No.	Concentrationof Tecovirimat (mg/ml)	Peak area	Amount found (mg)	% Amount found
1	10	634.469	21.02	99.22
2	10	630.770		
		Mean	632.62	
		SD	2.62	
		%RSD	0.14	

Repeatability studies on RP-HPLC method for Tecovirimat was found to be ,The %RSD was less than 2%, which shows high percentage amount found in between 98% to 102% indicates the analytical method that concluded .**(Table No.25)**

4. Precision:-

The method was established by analyzing various replicates standards of Tecovirimat. All the solution was analyzed thrice in order to record any intra-day & inter-day variation in the result that concluded. The result obtained for intraday is shown in**(Table No. 26)** respectively.

Chromatogram of Precision:

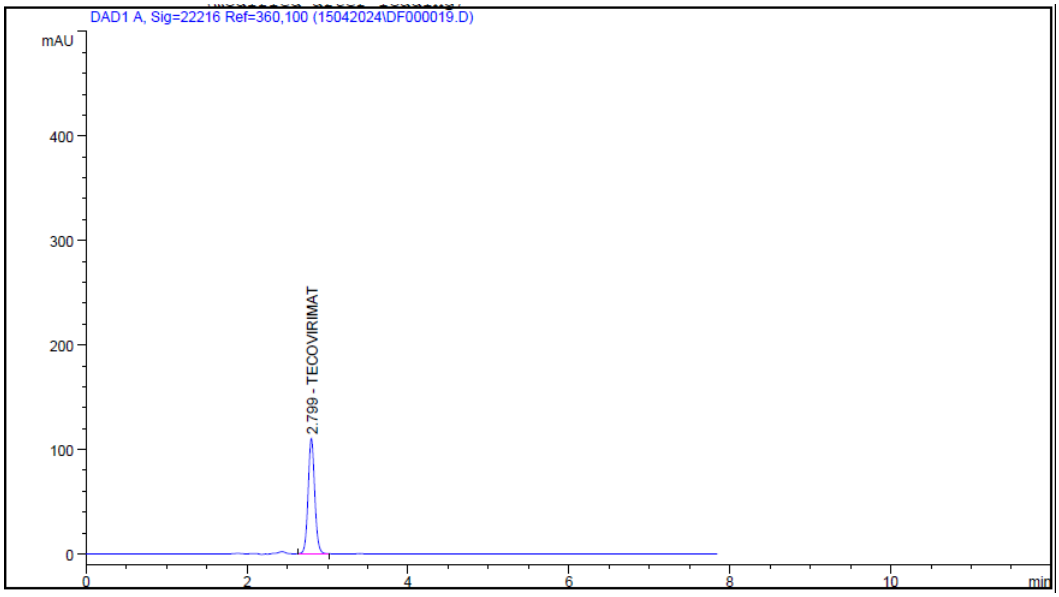

Fig No .31: Chromatogram of Precision

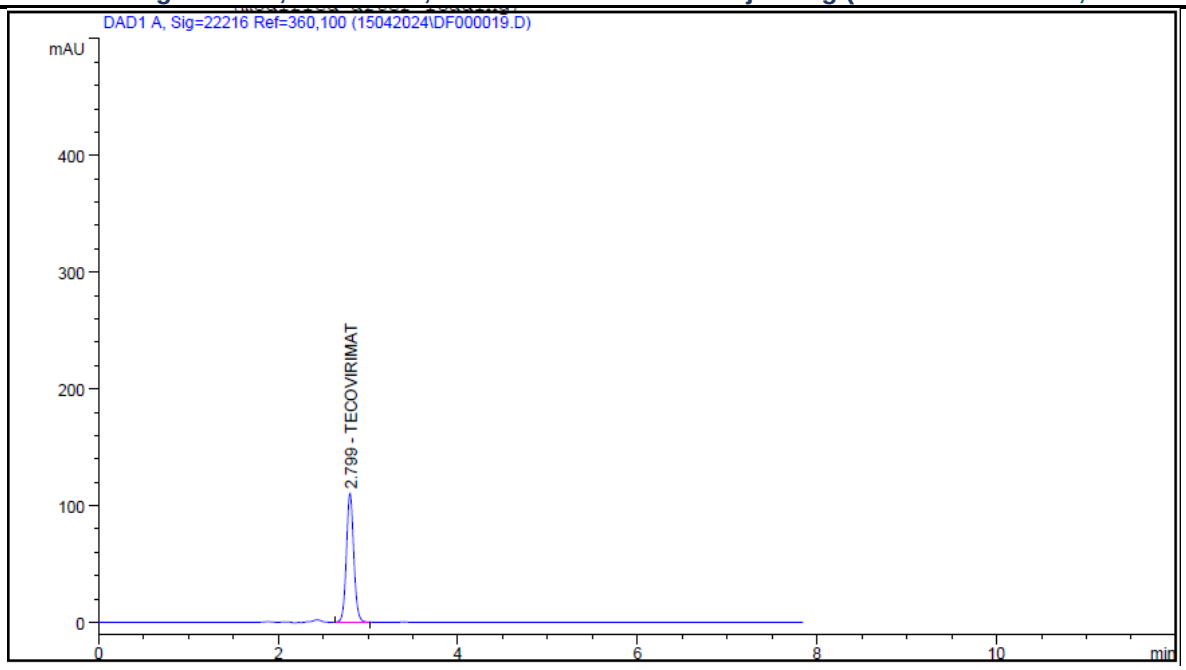

Fig No.32: Chromatogram Intra-day precision 10 mcg-01

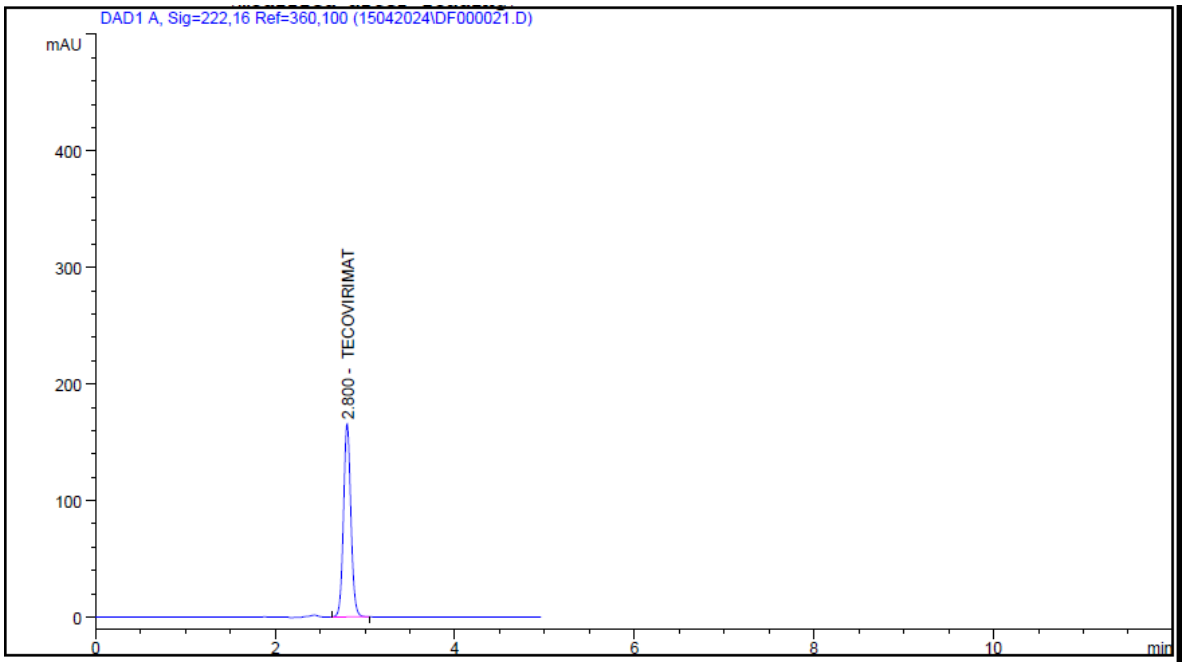

Fig No.33: Chromatogram Intra-day precision 15 mcg-01

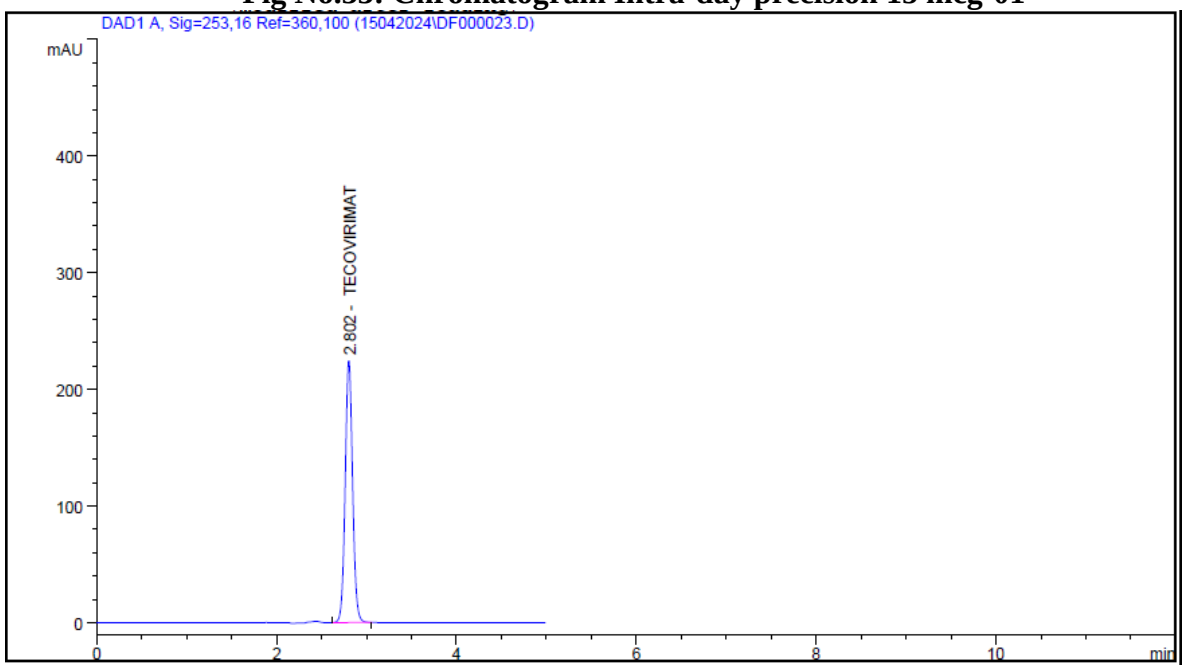

Fig No.34: Chromatogram Intra-day precision 20 mcg-01

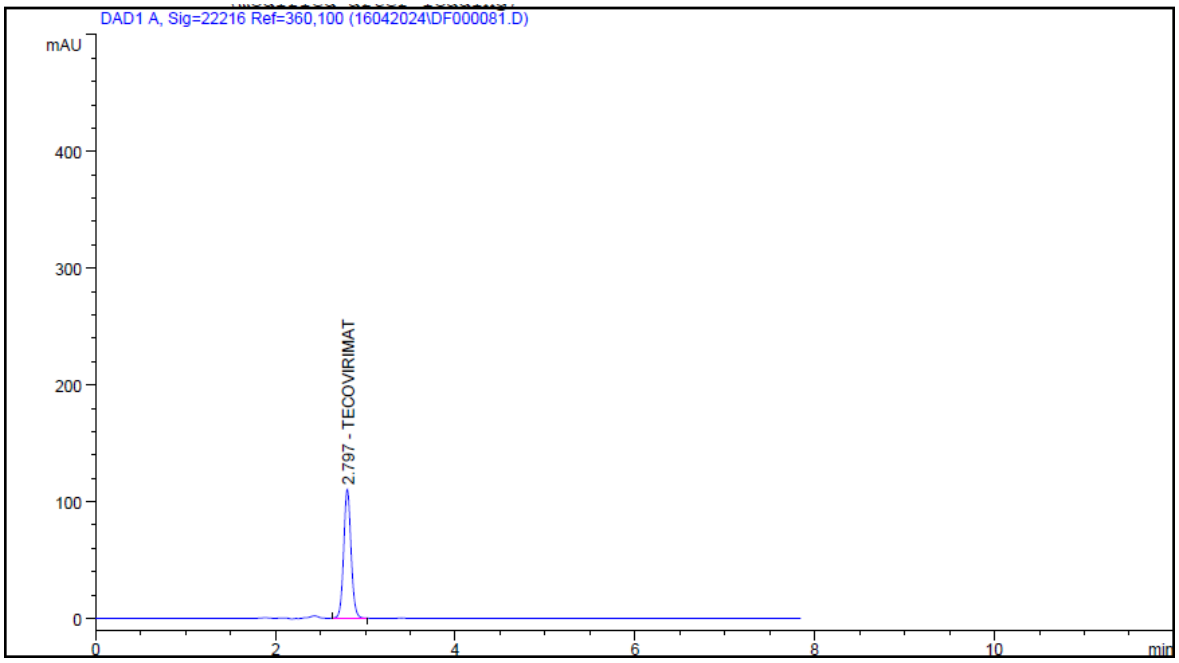

Fig No.35: Chromatogram Inter-day precision-10mcg-01

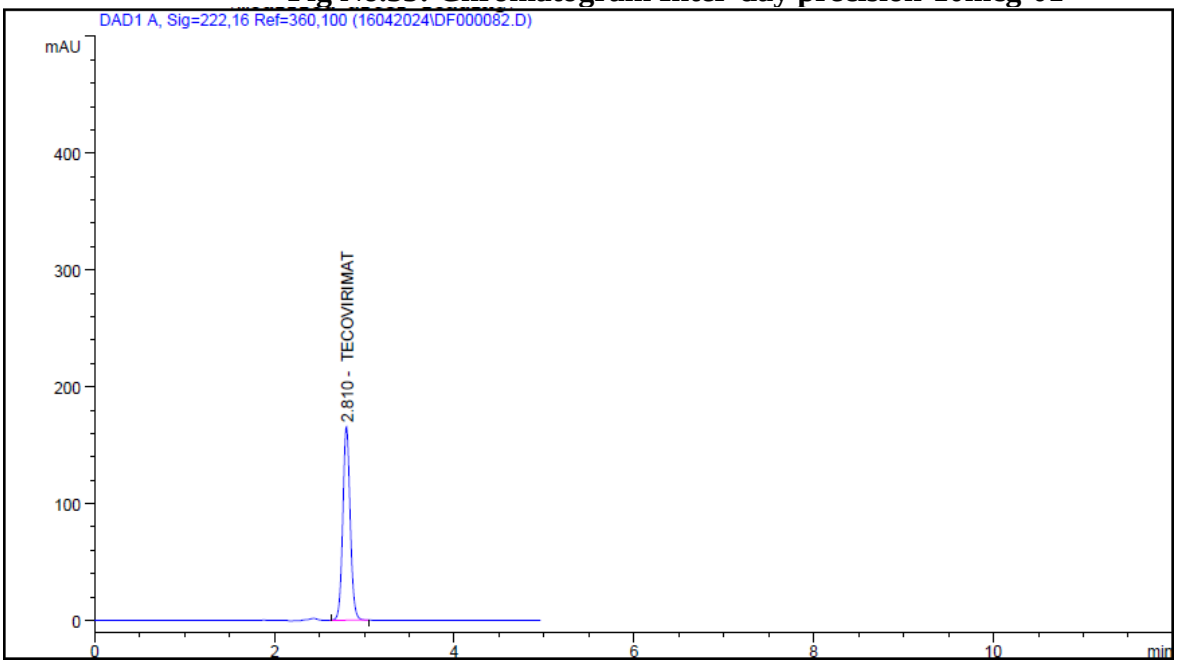

Fig No.36: Chromatogram Inter-day precision-15 mcg-01

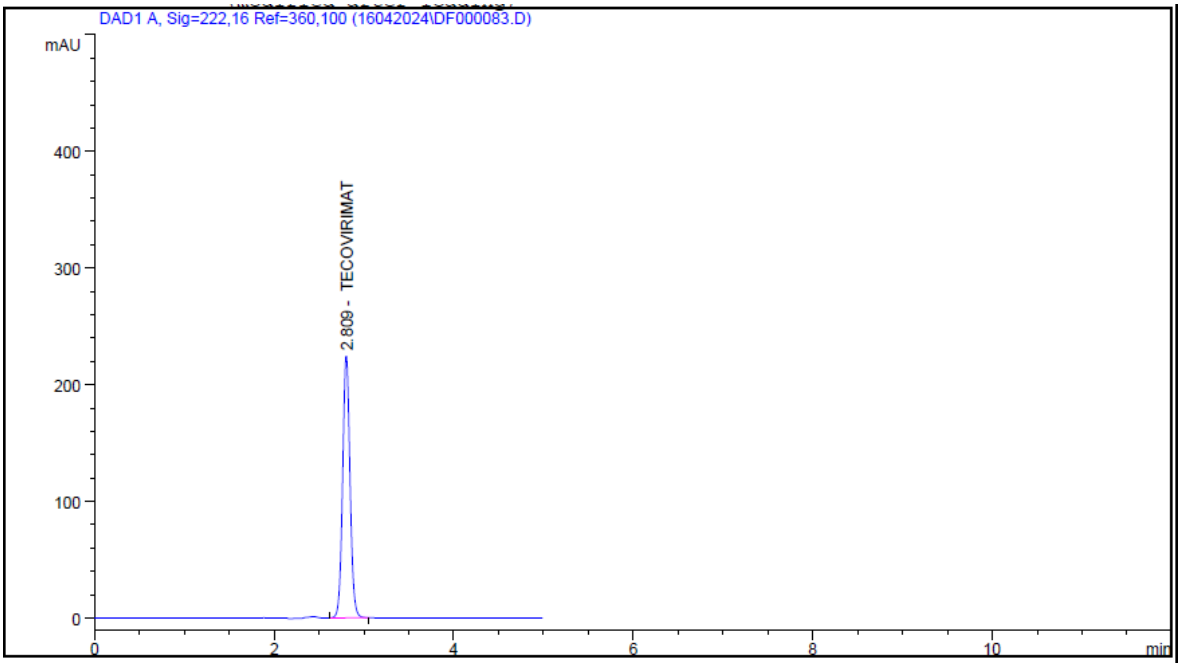

Fig No.37: Chromatogram Inter-day precision-20 mcg-01

Table No .26: Result of Intraday and Inter day Precision studies on RP-HPLC method for Tecovirimat

METHOD	Drug	Concⁿ (µg/ml)	Intraday Precision		Interday Precision	
			Mean± SD	%Amt Found	Mean± SD	%Amt Found
HPLC METHOD Rp-	TVR	10	630.65±1.22	98.60	632.70±7.93	98.47
		15	949.02±1.13	99.54	955.01±0.91	100.18
		20	1288.13±0.08	101.67	1284.81±6.45	101.40

SUMMARY AND CONCLUSIONS:

The present work deals with the Development and validation of RP-HPLC method for determination of Tecovirimat by pure and Capsule dosage form

Summary of RP-HPLC method:

Attempts were made to develop RP-HPLC method for estimation of Tecovirimat from Capsule. For the RP - HPLC method,Agilent (Autosampler) GradientSystem DAD Detect or and C18 (waters) with 150mm x4.6 mm i.d and 5µm particle size Methanol : 0.1% Acetic Acid (72:38v/v) pH 3.1 was used as the mobile phase for the method. The detection wavelength was 222 nm and flow rate was 1 ml/min. In the developed method, the retention time of Tecovirimat were found to be 2.799 min.

The ICH recommendations were followed in the validation of the devised approach. The ICH recommendations' limitations were met by the linearity, accuracy, range, and robustness. The approach was therefore determined to be straightforward, accurate, precise, affordable, and repeatable.

So, it is worthwhile that, the proposed methods can be successfully utilized for the routine quality control analysis Tecovirimat in bulk drug as well as in formulations.

Conclusion:

Simple, rapid, accurate and precise RP-HPLC method have been developed and validated for the routine analysis of Tecovirimat in API and Capsule dosage forms. Both methods are suitable for the determination of Tecovirimat in Single-component formulations without interference of each other. The developed methods are recommended for routine and quality control analysis of the investigated drugs component pharmaceutical preparations. The amount found from the proposed methods was in good agreement with the label claim of the formulation. Also the value of standard deviation and coefficient of variation calculated were satisfactorily low, indicating the suitability of the proposed methods for the routine estimation of Capsule dosage forms.

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