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METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF IVACAFTOR AND TEZACAFTOR BY RP-HPLC

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ABSTRACT

A novel RP-HPLC technique was developed for simultaneous quantification of Tezacaftor and Ivacaftor drugs which is used to cure cystic fibrosis. The method was developed using Inertial -ODS C18 (250 x 4.6 mm, 5) column, flow rate was 1.0 ml/min, mobile phase ratio was Methanol: Buffer (45:55), and detection wavelength was 254 nm. The chromatographic conditions for the separation of Tezacaftor and Ivacaftor were effectively created.

Main Words: Tezacaftor, Ivacaftor, RP-HPLC, Method Development, Validation.

LIST OF ABBREVATIONS

BP	=	British Pharmacopoeia
cm	=	Centimetre
Conc.	=	Concentration
Cps	=	Centipoises
CRDDS	=	Controlled Release Drug Delivery System
°C	=	Degree Centigrade
DSC	=	Differential Scanning Calorimetry
F	=	Formulation
FTIR	=	Fourier Transform Infrared Spectroscopy
g	=	Gram
GIT	=	Gastrointestinal tract
h	=	Hour

HPMC = Hydroxypropyl methylcellulose
IP = Indian Pharmacopoeia
Kg = Kilogram
LD = Lethal Dose
MCC = Microcrystalline cellulose
mcg = Microgram
MDT = Mean dissolution time
MEC = Minimum Effective Concentration
Mg = Milligram
min = Minute
mL = Milliliter
mPa s = Milli Pascal Second
MS = Magnesium Stearate
MSC = Maximum Safe Concentration
n = Diffusion coefficient
N = Normality
nm = Nanometer
No. = Number
PEO = Polyethylene Oxide
rpm = Revolutions per minute
SD = Standard Deviation
S. No. = Serial Number
SR = Extended-Release
USP = United States Pharmacopoeia
UV = Ultraviolet
w/w = Weight by weight
% = Percentage
β = B

INTRODUCTION

Pharmaceutical analysis is used to determine the purity, safety and quality of drugs and chemicals.

It involves a series of process for identification, determination, quantification & purification of a substances, separation of the components of a solution of mixture or determination of structure of chemical compounds.

There are two types of chemical analysis.

- ❑ Qualitative (Identification).
- ❑ Quantitative (Estimation or Determination).

Pharmaceutical research is critical to the safety and quality control of bulk medicines and their formulations. Pharmaceutical analysis is a specialized field of analytical chemistry that isolates, classifies, and calculates the relative quantities of components in a sample of matter. This is concerned with the chemical characterization of matter in both quantitative and qualitative terms.

In recent years, many analytical methodologies have evolved.

SPECTROPHOTOMETRIC METHODS

In general, small-scale industry is favoured for spectrophotometry as equipment costs are reduced and maintenance difficulties are minor. The analytical approach is based on the measurement by colourless substances of the absorption of monochromatic light into the near ultraviolet spectrum (200-380nm). The photometric analysis techniques are based on the Bouger-Lambert-Beer equation, which determines the solution's absorption is directly proportional to the analysis concentration. In the light of the defined interval of wavelength pass through a cell with a solvent and into the photoelectric cell that changes radiant energy into electro energy recorded by the galvanometer, the fundamental principle of operations of UV region-decking spectrophotometers.

HPLC METHOD DEVELOPMENT

The word 'chromatographing' includes those techniques aiming at dividing different mixture species into one fixed and mobile phase based on their distribution characteristics.

MODES OF CHROMATOGRAPHY

Chromatographic modes are fundamentally established based on the nature of the interactions between the solution and the stationary phase that may result from hydrogen binding, wall

forces of Vander, electrostatic forces, or hydrophobic forces or from particle size (e.g. Size exclusion chromatography).

Different modes of chromatography are as follow.

- Adsorption Chromatography or Normal Phase Chromatography
- Reversed Phase Chromatography
- Reversed Phase – ion pair Chromatography
- Ion-Exchange Chromatography
- Size Exclusion Chromatography

Adsorption Chromatography or Normal Phase Chromatography

The stationary phase is a polar adsorbent and, in general, the mobile phase is a mix of non-aquatic solvents in normal phase chromatography.

At the end of the silica structure are soaked by groups of silanol. These OH groups are throughout the entire surface statistically disturbed. In the stationary stage, the silanol groups reflect the active locations (highly polar).

This occurs when one or more atoms with a single pair of electron or dual bond are present in the molecular. In the following order, the strength of adsorption and hence k' (elution series) values rise. Saturated hydrocarbons < olephines < aromatic < sulphide < ethers < aldehydes < ketones < sulphones < amides < Carboxylic acids. The intensity of interactions depends not only on the functional groups, but also on steric variables in the sample molecule. The most polar molecule influences the reaction characteristics, if it has a number of functional groups.

Aminopropyl and cyanopropyl phases give potential for particular interactions between analytes and stationary phases, offering new alternatives to optimize separations.

Water resolution can be most readily performed in the weak mobile phase by drying the solvents and then adding to the mobile phase a constant concentration of water or a highly polar modifier such as acetic acid or trityl amine (TEA). In addition to these polar modificators, the polar form and the reproducibility of retention times are deactivated.

High Performance Liquid Chromatography (HPLC) is a column chromatography special branch in which the high-speed mobile phase is pushed across the column. This reduces the test time by 1-2 order of magnitude relative to conventional column chromatography and

makes it feasible to considerably increase the efficiency of the column by use of far smaller adsorbent or supporting particles.

A sample, a stationary phase, the detector and the recorder are the main devices, which comprises of the eluent, the reservoir, a high-pressure pump and an injector for injecting the sample. The creation of very effective micro particle bonded phases has enhanced the technical flexibility and the analysis of multi-component blends considerably.

Reversed Phase Chromatography

The aim was to produce polar or non-polar to separate water soluble polar chemicals with polar solvents. The ionic nature is now reversed, i.e., since it is neither polar nor reversed in the phase. The chromatography is called the reversed phase chromatography using this silica.

Many chemically bound stationary silica-based phases are commercially accessible. In reverse-phase chromatography, silica-based fixed phases are still most common, while alternative polymer-based adsorbents (styrene-divinyl benzene copolymer) slowly grow.

The fewer water-soluble chemicals (i.e., the more non-polar) are better retained by the reversed phase surface. In the following order, retention decreases: Aliphatic > dipole induced (i.e., CCl4) > persistent dipoles (e.g., CHCl3) > low bases of Lewis (ethers, aldehydes, ketones) > strong foundations of Lewis (amines) > weak Lewis's acid (alcohols, phenols) (carboxylic acids). The number of carbon atoms also rises with retention.

As a rule, retention is increased by increasing contact area between the sample molecules and the stationary stage, i.e., by increasing the volume of water molecules produced during compound adsorption. Branched chain compounds are eluted faster than typical isomers.

The most popular stationary stage in the pharmaceutical business is chemically binding octadecyl silane (ODS) to alkaline with 18 atoms of carbon. Because most pharmaceutical chemicals are polar and water soluble, ODS HPLC columns are utilized to provide quality assurance in most HPLC techniques used for decomposition study, and quantitative analysis. In reverse phase chromatography the solvent strength is reversed from the chromatographic adsorption (silica gel) as mentioned previously. Water interacts significantly with the groups of silanol, thereby limiting the adsorption of sample molecules and so quickly eluting them.

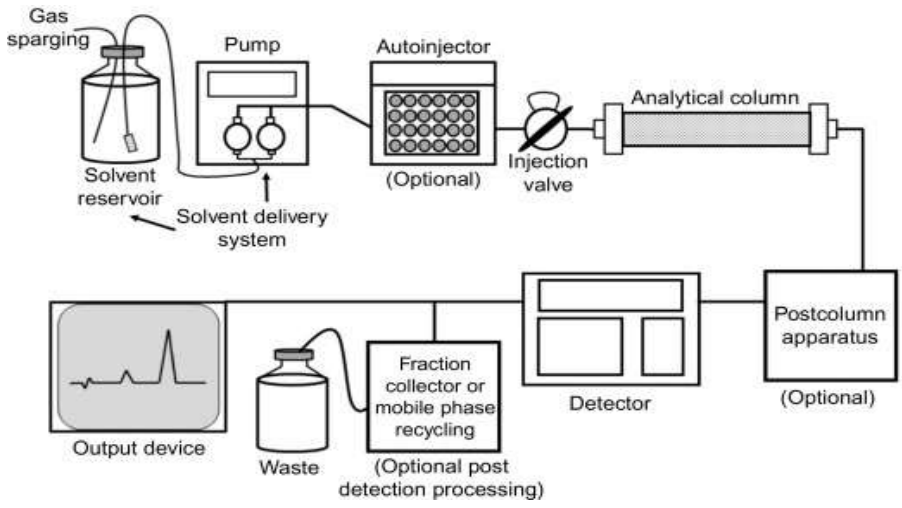
In reversed phase water does not wet non-polar (hydrophobic) alkylic groups like C18 in the ODS phase and hence does not interact with bound moiety. The opposite is true. Water is therefore the weakest solvent of all, with the slowest rate of elution. With increasing amounts of water in the mobile phase, elution time (retention time) rises in reverse phases.

Principle of HPLC

- The principle of HPLC is separation in normal phase mode and reverse phase mode is adsorption.
- When a mixture of components are introduced into a HPLC column, they travel according to their relative affinities towards the stationary phase.
- The component which has more affinity towards the adsorbent, travels slower.
- The component which has less affinity towards the stationary phase travels faster.
- Since no two components have the same affinity towards the stationary phase, the components are separated.

Having discussed the different components of an HPLC system.

Solvent container — Pump — Damping unit — Injection port --Column
|
Recorder — Detector --- Effluent



LITERATURE REVIEW

The objective is to create a new verified stability indicating the method of ivacaftor and tezacaftor simultaneous estimation by RP-HPLC. In WATERS, software: Empower, 2695 separation module, UV detector fitted with YMC (4.6 x 150mm, 5 peanutm), 50% buffer of orthophozyme and 50% mobile isocratic phase with a flow rate of 1mL/min with a 20µl volume of injection, chromatographical analyse was done. The retention times were 2.52min

and 2.03min respectively of ivacaftor and tezacaftor. The linearity of the ivacaftor and tezacaftor was demonstrated for concentrations of 100-500 µg/mL and 40-200 µg/mL. The average recoveries for ivacaftor and tezacaftor were 100.21 percent and 100.15 percent. The technique presented has been verified according to the ICH standards and effectively utilized in its combination tablet dose form for the estimation of ivacaftor and tezacaftor.

METHOD VALIDATION PARAMETERS

ACCURACY

The closeness of test results obtained by that method to the true value.

LINEARITY

The ability of an analytical procedure to produce test results that are directly proportional to the concentration of analyte in the sample.

PRECISION

The closeness of agreement between the obtained values by analyzing the same sample for multiple times under prescribed conditions.

SPECIFICICTY

The ability to measure the analyte specifically in the presence of components that may be expected to be present.

RANGE

The interval between upper and lower concentration (amounts) of analyte.

RUGGEDNESS

Degree of reproducibility of test results obtained by the analysis of same samples under a variety of conditions.

ROBUSTNESS

The capacity of a method to remain unaffected by small, deliberate variations in method parameters.

LIMIT OF QUANTIFICATION (LOQ)

Lowest amount of analyte which can be quantitatively determined.

LOQ = 10 σ /S

LIMIT OF DETECTION (LOD)

Lowest amount of analyte in a sample which can be detected but not necessarily quantitated.

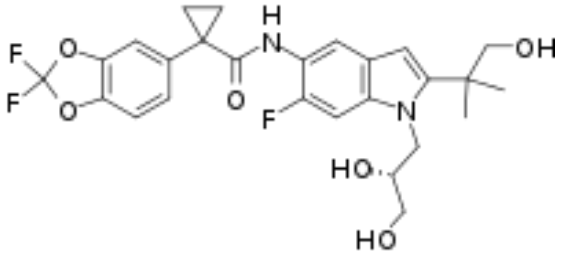
LOD = 3.3 σ /S

DRUG PROFILE

TEZACAFTOR

GENERAL DATA

Chemical Structure.



IUPAC: 1-(2,2-Difluoro-1,3-benzodioxol-5-yl)-N-[1-[(2R)-2,3-dihydroxypropyl]-6-fluoro-2-(2-hydroxy-1,1-dimethylethyl)-1H-indol-5-yl]-cyclopropanecarboxamide

Molecular formula: $C_{26}H_{27}F_3N_2O_6$

Molecular weight: 520.505 g/mol

Pharmacodynamics

After Tezacaftor/Ivacaftor Therapy, clinical trials have demonstrated a substantial drop in sweat chloride and an increase in forced expiratory volume (FEV). Phase 3 clinical tests demonstrate that the forced expiratory volume increased significantly when this medication was started at 4-8 weeks. The actions mentioned lead to alleviation of cystic fibrosis' respiratory symptoms. Tezacaftor does not cause QT prolongation to be clinically meaningful. 2 Tezacaftor may cause liver transaminase increases when taken with ivacaftor. Transaminases (ALT and AST) should be tested each 3 months before and every year thereafter, before commencing this combination. Patients with a transaminase history should be more regularly checked.

Mechanism of Action: The transportation of loaded ions across cell membranes is usually done by the activities of the protein of the CFTR. This protein serves as a conduit for chloride and sodium transmission. This process regulates water flow in and out of the tissues and affects mucus formation that lubricates and protects various organ and body tissue, including

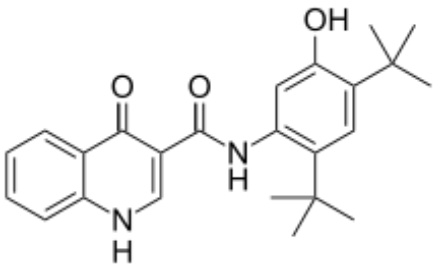
the pulmonary tissue. One amino acid is removed from position 508 in the F508del mutation of the CFTR gene, which impairs the operation of the CFTR channel, which results in spreading the mucus discharges. CFTR-correctors like tezacaftor are intended to fix cellular error processing F508del.

Half Life: The apparent half-life of tezacaftor is approximately 57.2 hours.

IVACAFTOR

GENERAL DATA

Chemical Structure.



IUPAC: N-(2,4-Di-tert-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide

Molecular formula: $C_{24}H_{28}N_2O_3$

Molecular weight: 392.499 g/mol

Pharmacodynamics

The use of Ivacaftor has been demonstrated to alleviate both CF symptoms and the pathophysiology of the illness. The possibility of CFTR protein opening (or gating) in patients with deteriorated gating mechanisms is promoted. This is done by This. This is in contrast to Lumacaftor, another CF drug which works by preventing misfolding of the CFTR protein and therefore leading to enhanced protein treatment and trade on the cell surface.

Mechanism of Action

The Cystic Fibrosis phenotype is linked with a broad array of CFTR mutations, and the levels of seriousness of the disease vary. The most common mutation in approximately 70% of patients with CF worldwide is F508del- CFTR or delta-F508 (along the lines of F 508), which results in a decreased production of the CFTR protein by a deletion of phenylalanine in amino acid at position 508 causing considerable reductions in the number of cell membranes ion transporters. In monotherapy Ivacaftor did not reveal any advantage for patients with

mutations of delta-F508, probably because the cell membrane lacked enough protein to interact and potentiate by the drug.

Half-life: In a clinical study, the apparent terminal half-life was approximately 12 hours following a single dose of ivacaftor.18 One source mentions the half-life ranges from 12 to 14 hours.

PURPOSE AND SCOPE OF WORK

(Aim and Plan of Work)

1. To anticipate Tezacaftor and Ivacaftor in tablet dosage forms using the RP-HPLC method at the same time.
2. The procedure must be put through its paces in accordance with ICH recommendations.

WORK PROJECT PLAN

The following was the proposed experimental work:

The creation of the novel analytical RP-HPLC system Tezacaftor and Ivacaftor combination is based on an analysis of the Tezacaftor and Ivacaftor literature regarding their physical and chemical properties, as well as numerous analytical methods conducted for Tezacaftor and Ivacaftor.

RP-HPLC METHOD DEVELOPMENT

1. Diluent solvent selection and mobile phase selection.

Selecting a solvent in which the product is soluble and stable. They must be simple to use, inexpensive, and HPLC quality.

2. Steps to take on your mobile phase.

The use of an organic or aqueous eluent is the first feature of the mobile process to be determined. It is necessary to provide either an aqueous eluent or a highly polar organic solvent such as methanol or acetonitrile for the RP-HPLC procedure. If the K 'values for an aqueous solvent are too high, we can consider an organic solvent. If the K 'value with an organic solvent is too low, a mixture of two solvents with differing characteristics should be used to separate the particles. The K'-capacity factor is a computation that determines the degree to which the value peak in respect to the void volume, i.e., Period of Elution of the unretained materials, is situated. The value of K is usually more than two. A buffer's pH and ionic strength may be determined using a buffer.

3. To identify the wavelength for the study, a thorough examination of the drug's Ultraviolet absorbance spectra will be carried out.

4. A thorough examination of the drug's structure and physicochemical characteristics; selection of chromatographic parameters.

5. Method selection for chromatography quantitative research. Determining the level of concentration at work.

6. The system was validated by following the ICH standards.

METHODOLOGY VALIDATION

(Materials and Methods**)**

Instruments-Instruments

- HPLC –Waters Model NO.2690/5 series Compact System Consisting of Inertsil-C18 ODS column.
- Electronic balance (SARTORIOUS)
- Sonicator (FAST CLEAN)

Substances containing chemicals

- Methanol HPLC Grade.
- Buffer (KH_2PO_4) Hplc Grade.

Raw Equipment (Unprocessed Materials)

Tezacaftor and Ivacaftor are working standards.

Instrument Used: HPLC Waters

Model: 2690/95

Detector: Photo Diode Array (PDA)

Software: Empower

Standard Stock Preparation

Take 10mg of each drug in 10ml Volumetric flask and add 7ml ethanol to it and sonicate for half an hour. After half an hour add remaining 3ml up to the mark and sonicate it to 5mins (i.e., 1000ppm).

Working Standard Preparation

Take 1ml of Ivacaftor and 1ml Tezacaftor standard solution in 10ml volumetric flask. Make up the volume up to mark with methanol and sonicate it to 5mins (100ppm).

DEVELOPMENT OF AN RP-HPLC METHOD

The goal of this study was to improve the assay technique for simultaneous quantification of Tezacaftor and Ivacaftor on literature surveys. As a result, the trials detailed below show how the optimization was accomplished.

Trail: 1

Mobile Phase: Degassed Acetonitrile: Methanol 50:50.

Chromatographic Conditions

Flow rate	: 1.0ml/min
Column	: Inertsil - C18, ODS column
Detector wavelength	: 210nm
Column temp	: Ambient
Injection volume	: 20µl
Run time	: 10min
Retention time	: 2.6min for Tezacaftor and 2.7 for Ivacaftor.

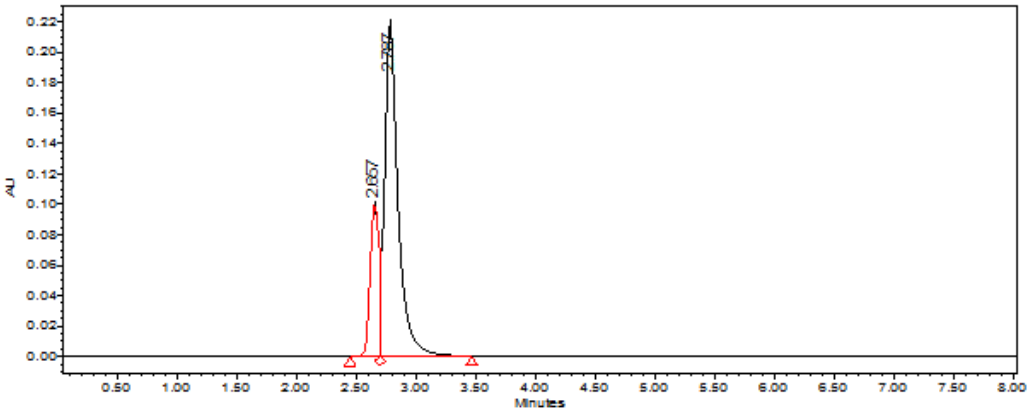


Fig 1: Trial 1 Chromatogram.

Inference: The two summits are entirely blended and cannot be distinguished.

S.NO	Name of the peak	Retention time(min)
1	**Tezacaftor**	2.6
2	**Ivacaftor**	2.7

Trail: 2

Mobile Phase: Degassed Acetonitrile and methanol in the ratio of 90:10 V/V.

Chromatographic Conditions

Flow rate : 1ml/min

Column : Inertsil -C18, BDS column

Detector wavelength : 210nm

Column temp : Ambient

Injection volume : 20µl

Run time : 10min

Retention time : 2.7 min for Tezacaftor and 4.0 min for Ivacaftor.

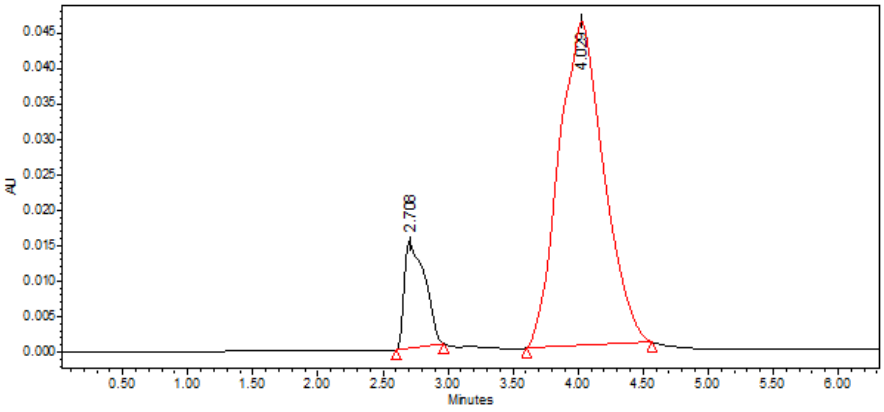


Fig 2: Trial 2 chromatogram.

Inference: The forms of the peaks are not appealing.

S.NO	Name of the peak	Retention time(min)
1	**Tezacaftor**	2.7
2	**Ivacaftor**	4.0

Trail: 3

Mobile Phase: Degassed Acetonitrile and Methanol in the ratio of 80:20 V/V.

Chromatographic Conditions

Flow rate : 1.0ml/min

Column : Inertsil - C18, BDS column

Detector wavelength : 210 nm

Column temp : Ambient

Injection volume : 20µl

Run time : 10min

Retention time : 2.7 min for Tezacaftor and 3.1 min for Ivacaftor.

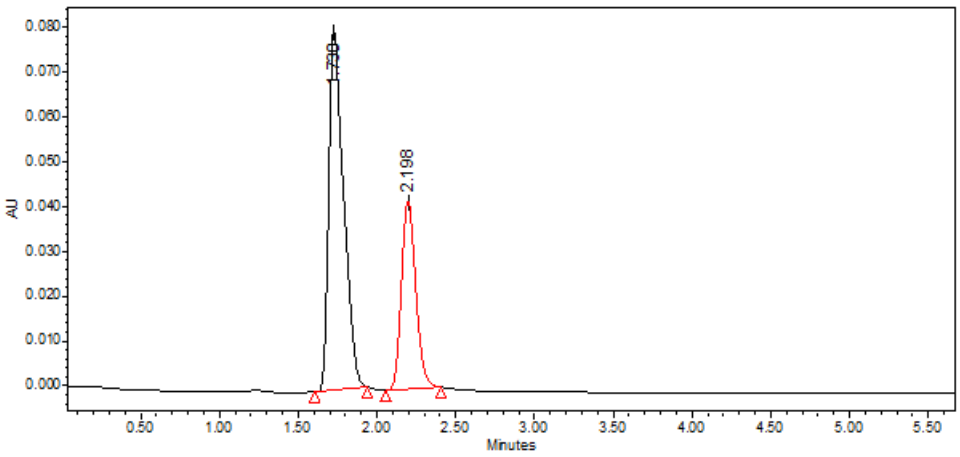


Fig 3: Trial 3 chromatogram.

Inference: The summits are not fully separated.

S.NO	Name of the peak	Retention time(min)
1	**Tezacaftor**	1.7
2	**Ivacaftor**	2.1

RESULTS AND DISCUSSIONS

ADVANCED METHOD (OPTIMIZED METHOD**)**

Mobile Phase: Degassed Methanol and Buffer in the ratio of 45:55 V/V.

Preparation of pH 3.4 Phosphate buffer: 2.7218g of KH2PO4 was weighed and transferred into a 1000ml beaker, later it was dissolved and diluted to 1000ml with HPLC water, and the pH was adjusted to 3.4 with orthophoshoric acid.

Chromatographic conditions that have been optimized.

Parameters	Method
Stationary phase (column)	Inertsil -ODS C_{18} (250 x 4.6 mm, 5 µ)
Mobile Phase	Methanol: Buffer (45:55)
Flow rate (ml/min)	1.0 ml/min
Run time (minutes)	12 min
Column temperature (°C)	Ambient
Volume of injection loop (ml)	20
Detection wavelength (nm)	210 nm
Drug RT (min)	4.977min for Tezacaftor and 7.077 for Ivacaftor.

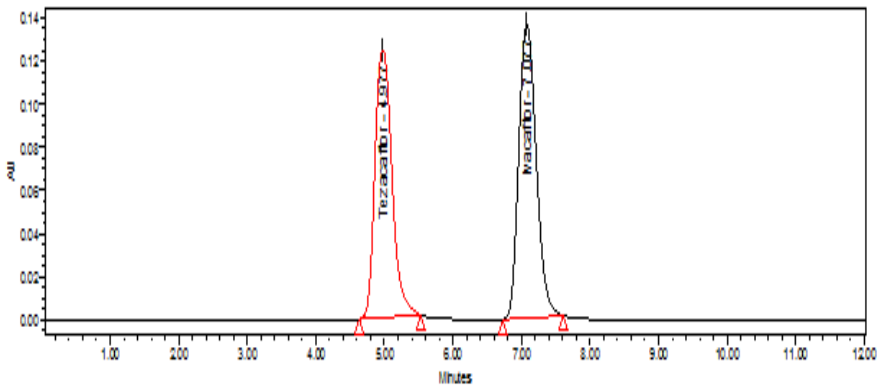


Fig 4: Standard chromatogram,

Inference: At RTs of 4.977 minutes for Tezacaftor and 7.077 minutes for Ivacaftor, a chromatogram was obtained.

S.NO	Name of the peak	Retention time(min)
1	**Tezacaftor**	4.977
2	**Ivacaftor**	7.077

VALIDATION DATA

1. System Suitability

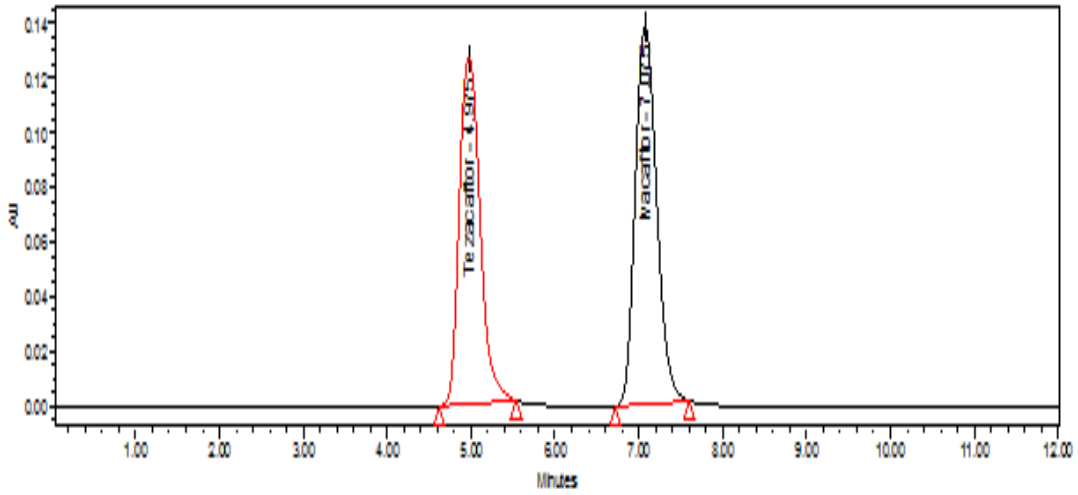
Table 1(a): Data on Tezacaftor System Suitability.

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	4.975	674753	10953.6097	1.153539
2	4.976	674261	10951.0146	1.155271
3	4.974	675298	10003.2730	1.157740
4	4.975	679221	10986.9427	1.159499
5	4.979	688636	10946.8723	1.152820
Mean	4.975205	678433.8	10768.3467	1.155774
SD	0.001483	6031.135	-------	-------
% RSD	0.031506	0.888979	-------	-------

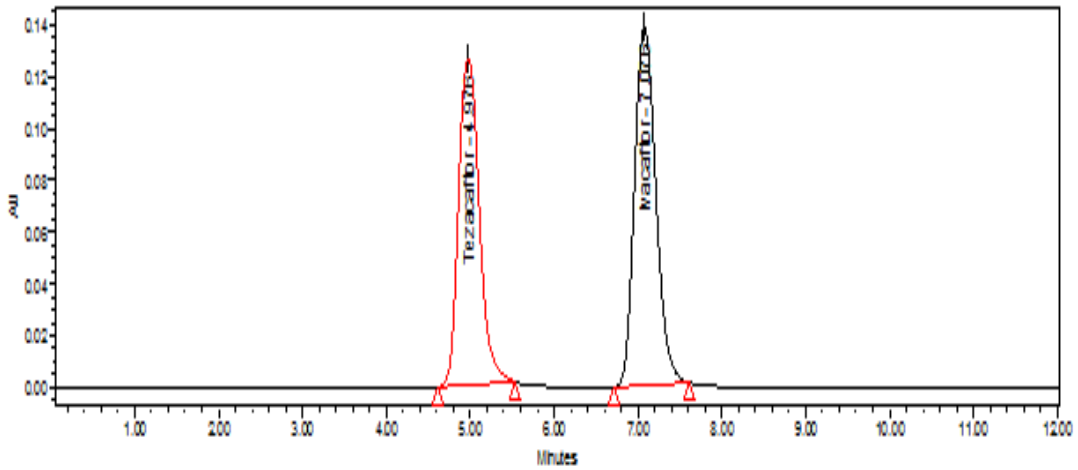
Table 1(b): Data on Ivacaftor System Suitability.

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	7.075	1218805	9478.3171	0.899633
2	7.076	1214014	9452.1967	0.893423
3	7.074	1215474	9569.9285	0.894443
4	7.070	1227655	9619.6337	0.882222
5	7.075	1267019	9749.9072	0.892316
Mean	7.0763	1228593	9573.9971	0.892407
SD	0.002408	122124.07	-------	-------
% RSD	0.036039	1.800764	-------	-------

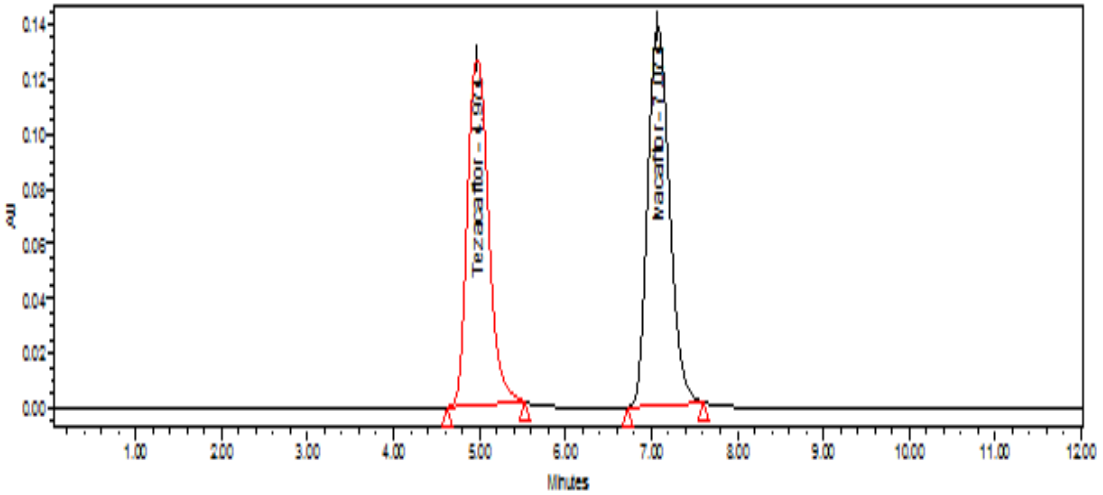
Fig: 6-10 System suitability chromatograms (standards 1-5)



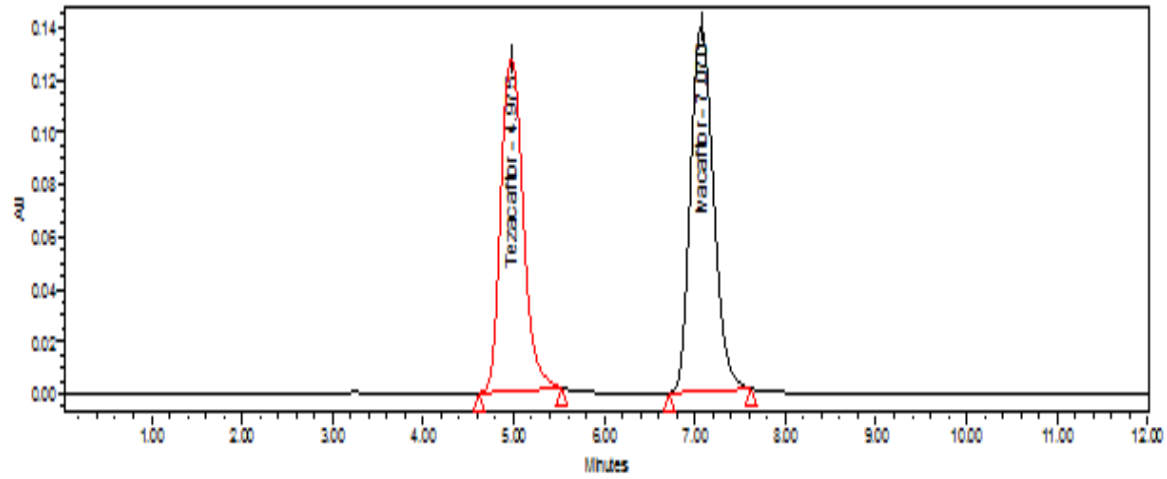
Inference: Standard Chromatogram-1 System Suitability.



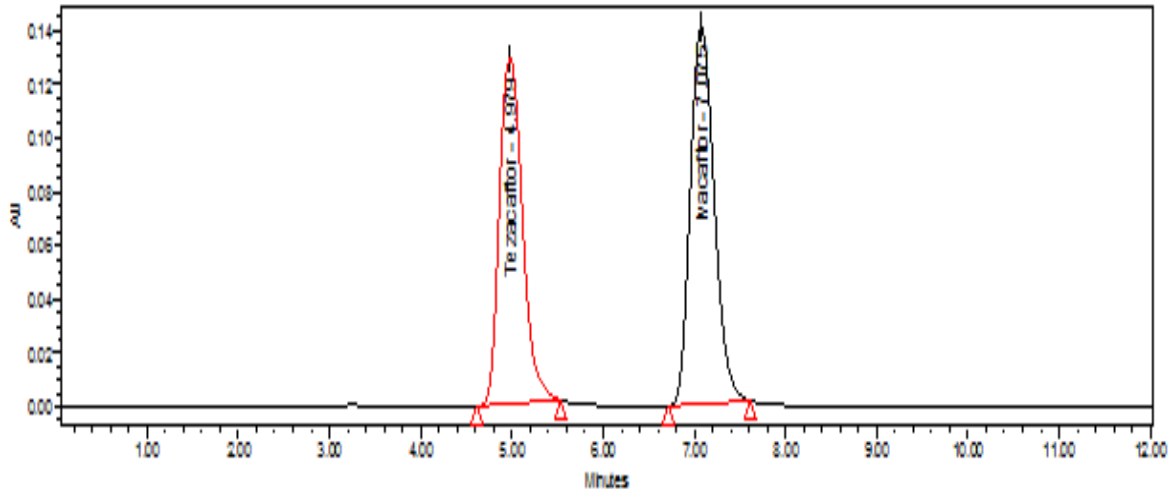
Inference: Norm Chromatogram-2 device appropriateness.



Inference: For standard Chromatogram-3, a suitable system is required.



Inference: Applicability of the device for routine Chromatogram-4.



Inference: Norm Chromatogram-5 device appropriateness.

2. SPECIFICITY

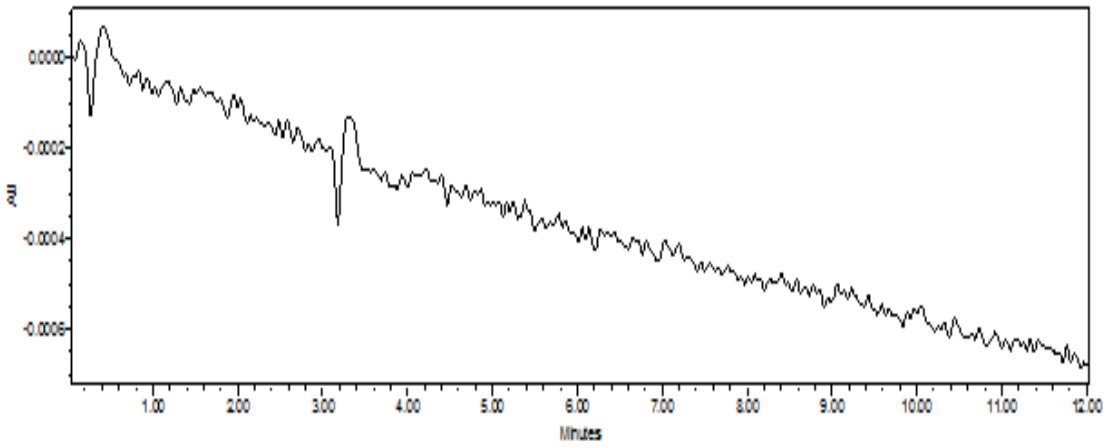


Fig 11: Blank Chromatograph.

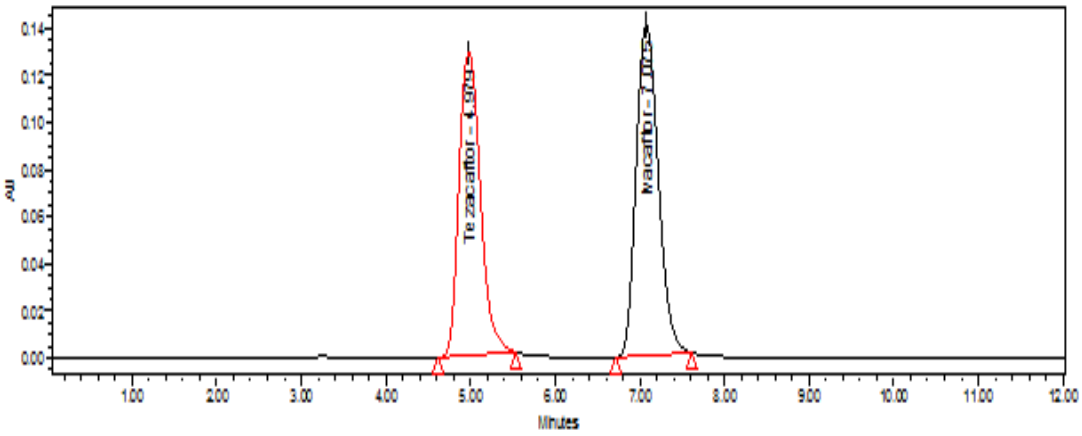


Fig 12: Chromatogram Standard.

Inference: For Tezacaftor, a Rt of 4.979min was obtained, while for Ivacaftor, a Rt of 7.075min was obtained.

3. PRECISION

(a) Precise system (System precision).

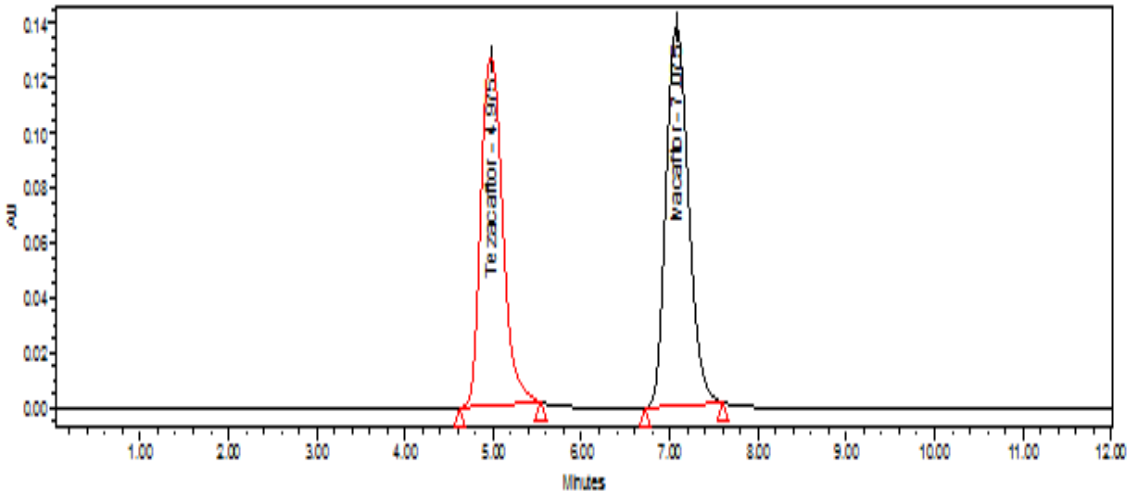
Table-3(i): Data of Repeatability (System precision) for Tezacaftor.

	Injection	Peak Areas of Tezacaftor	%Assay
Concentration 100ppm	1	674753	98.66
	2	674261	99.30
	3	675298	101.53
	4	679221	100.53
	5	688636	99.98
Statistical Analysis	**Mean**	678433.8	100.00
	SD	6031.135	1.107678
	% RSD	0.888979	1.10

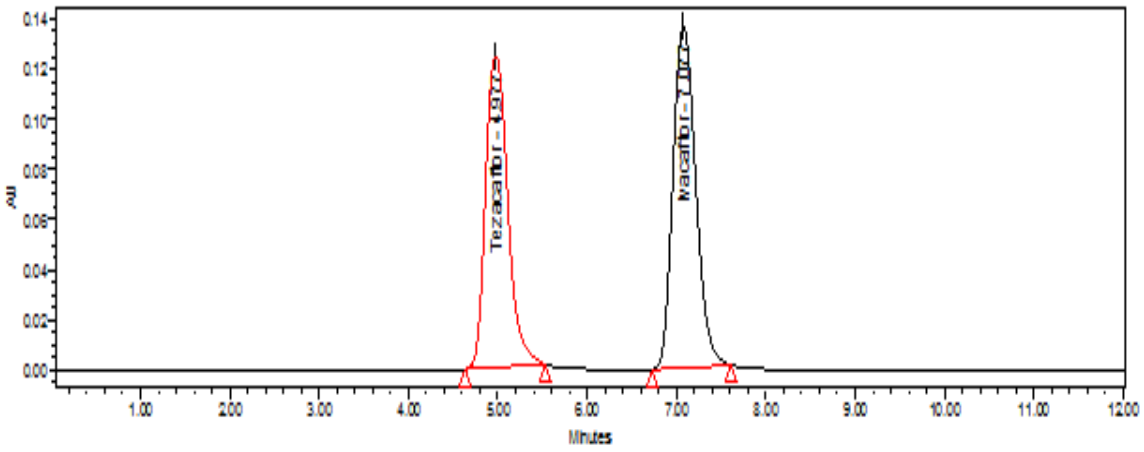
Table-3(ii): Information on Ivacaftor Reliability (System Precise).

	Injection	Peak Areas of Ivacaftor	%Assay
Concentration 100ppm	1	1218805	99.95
	2	1214014	100.24
	3	1215474	100.06
	4	1227655	99.30
	Injection	**Peak Areas of Ivacaftor**	**%Assay**
	5	1267019	100.00
Statistical Analysis	**Mean**	1228593	99.91
	SD	22124.07	0.35819
	% RSD	1.800764	0.35

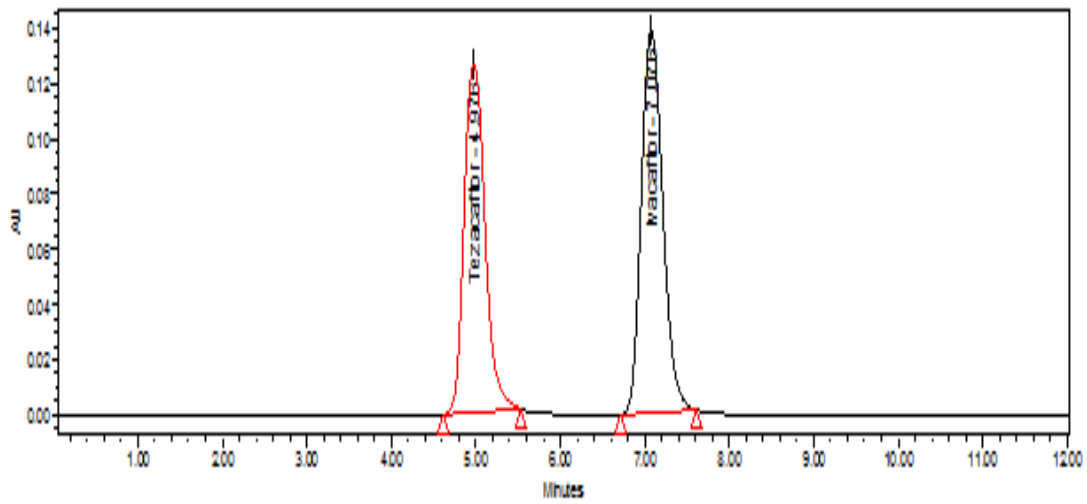
Fig 13-17 Detailed chromatograms of systems.



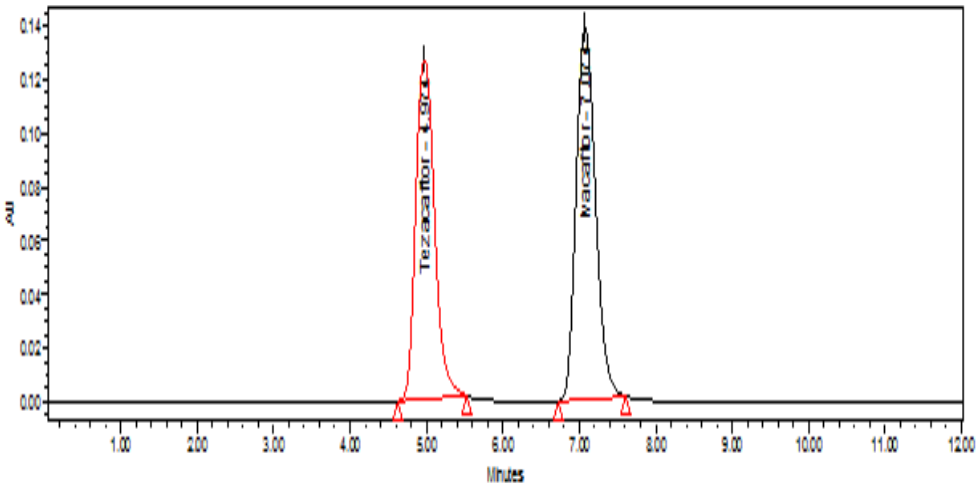
Inference: Precision chromatograph devices (standard-1).



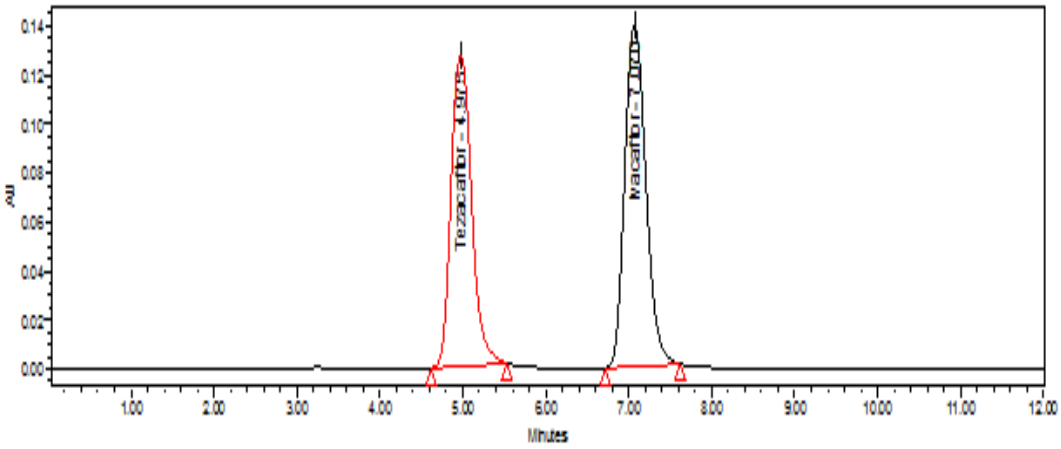
Inference: Precision chromatograph devices (standard-2).



Inference: Precision chromatograph devices (standard 3).



Inference: Precision chromatograph devices (standard 4).



Inference: Precision chromatograph devices (standard 4).

(b) Method Precision

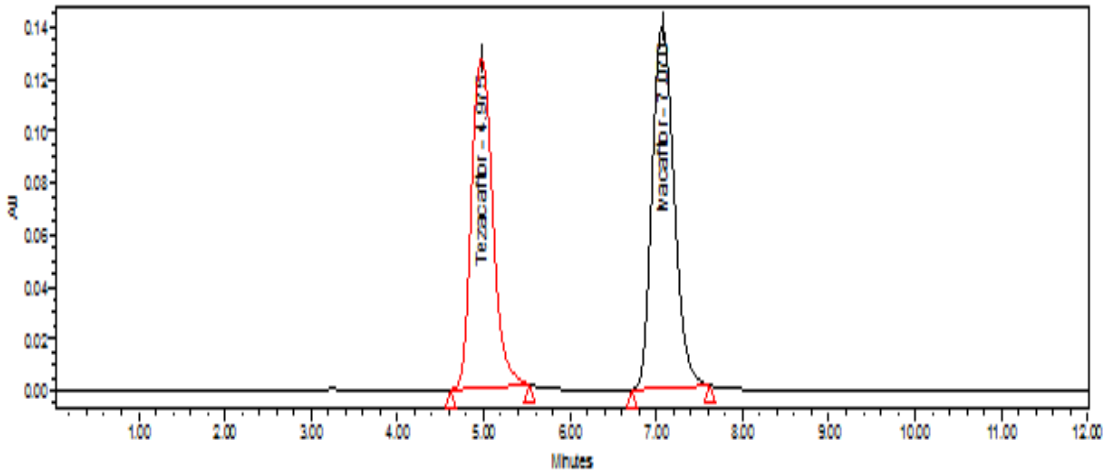
Table 3(i): Data of Repeatability (Method precision) for Tezacaftor.

	Injection	Peak Areas of Tezacaftor	%Assay
Concentration 100ppm	1	633495	98.55
	2	635992	98.88
	3	639828	99.40
	4	639098	99.30
	5	648289	100.53
	6	631322	98.28
Statistical Analysis	**Mean**	637312	99.278
	SD	5988.879	0.827236
	% RSD	0.0891	0.83

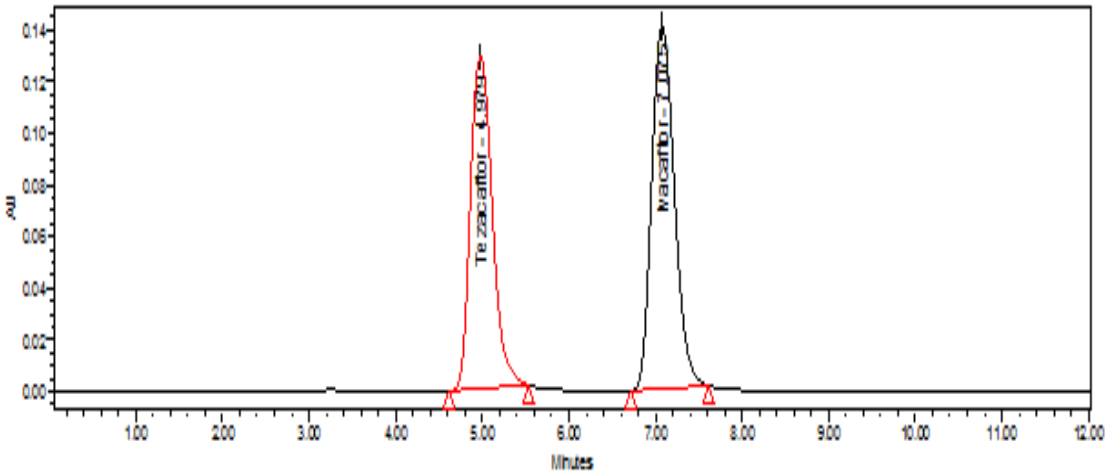
Table 3(ii): Data of Repeatability (Method precision) for Ivacaftor.

	Injection	Peak Areas of Ivacaftor	%Assay
Concentration 100ppm	1	1202110	98.6
	2	1203700	99.02
	3	1201851	98.12
	4	1202255	98.31
	5	1203283	98.81
	6	1202349	98.36
Statistical Analysis	**Mean**	1202687.6	98.48
	SD	771.5483	0.352647
	% RSD	0.1358	0.35

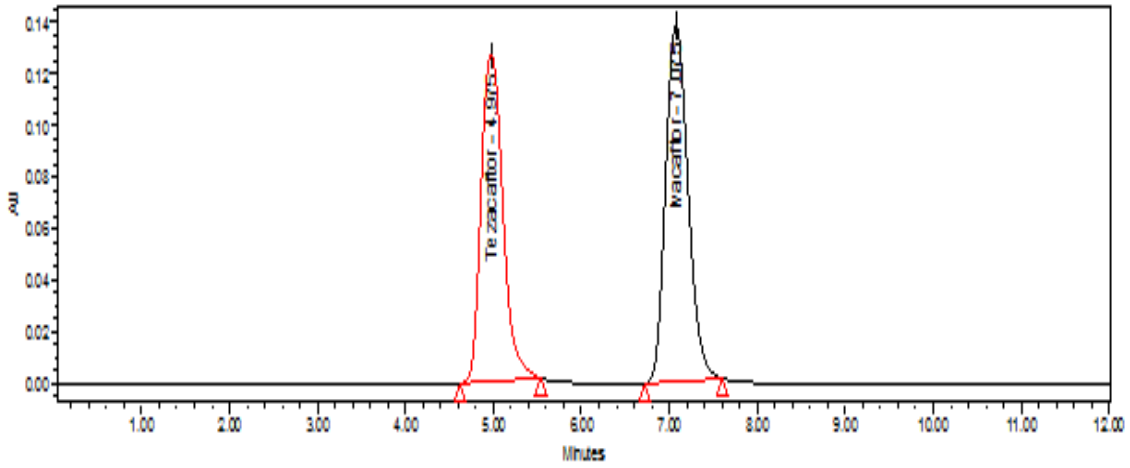
Fig 18-23: Repeatability chromosomes (Repeatable Chromatograms)



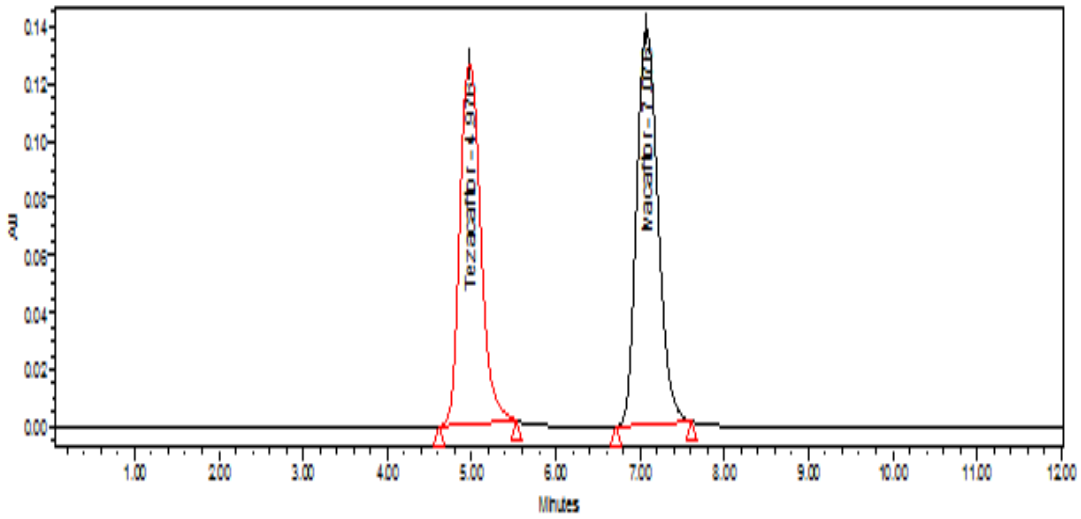
Inference: Chromatograph with high repeatability (Standard-1).



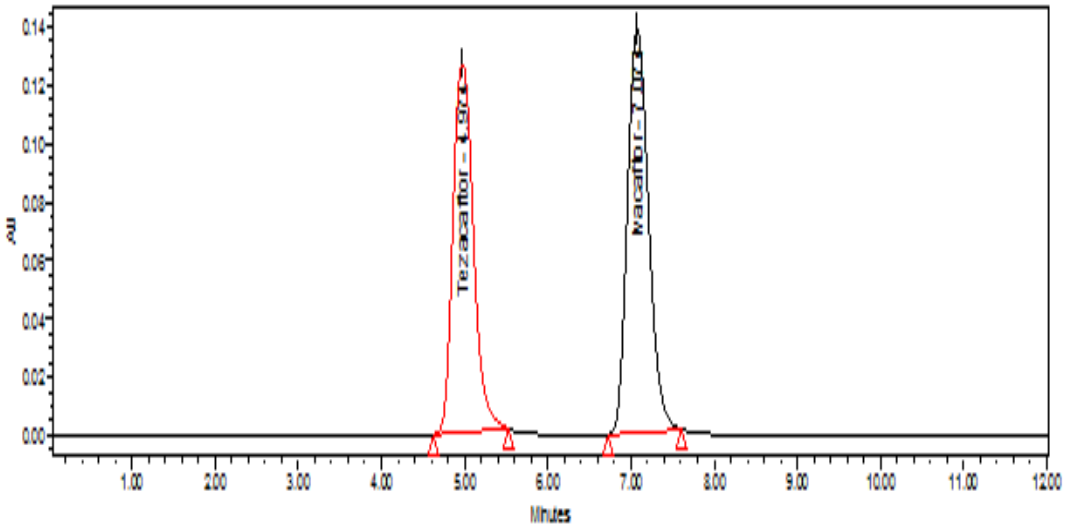
Inference: Chromatograph with high repeatability (Standard-2).



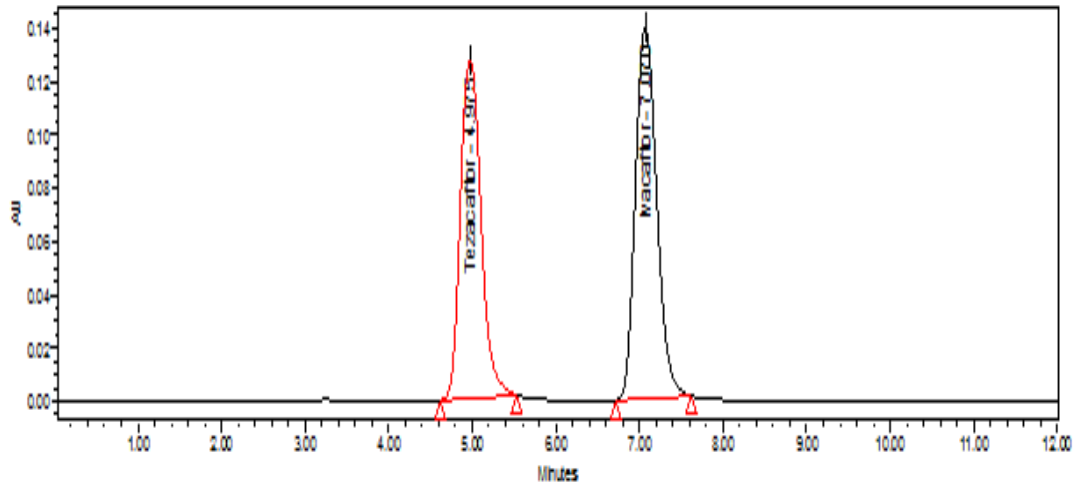
Inference: Chromatograph with high repeatability (Standard-3).



Inference: Chromatograph with high repeatability (Standard-4).



Inference: Chromatograph with high repeatability (Standard-5).



Inference: Chromatograph with high repeatability (Standard-6).

c) Intermediate precision

For Analyst 1 ref: Table3.

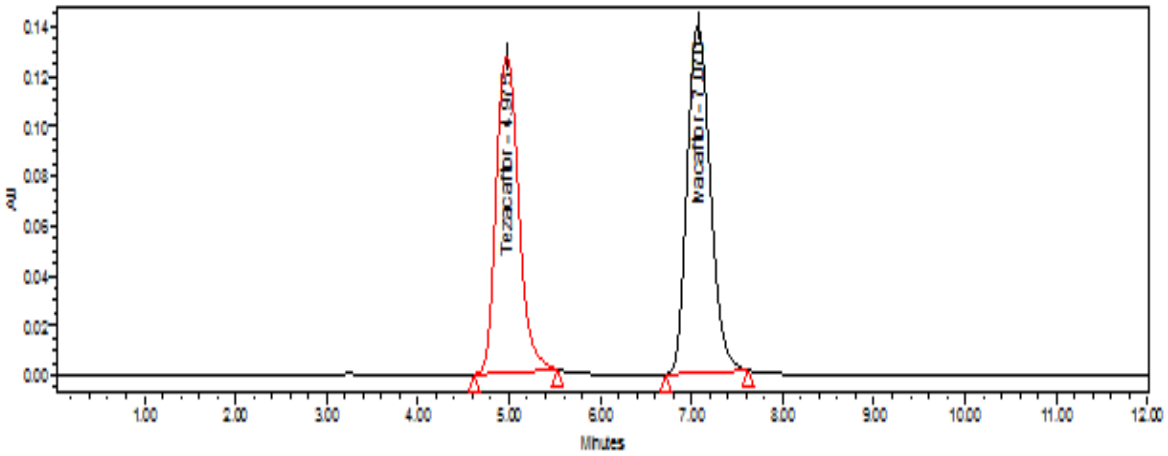
(i) Data of Intermediate precision (Analyst 2) for Tezacaftor.

	Injection	Peak Areas of Tezacaftor	%Assay
Concentration 100ppm	1	636792	99.99
	2	634360	99.66
	3	655696	101.53
	4	644147	99.98
	5	644127	99.97
	6	652525	101.10
Statistical Analysis	**Mean**	644607.8	100.37
	SD	6392.59	0.753536
	% RSD	1.183	0.75

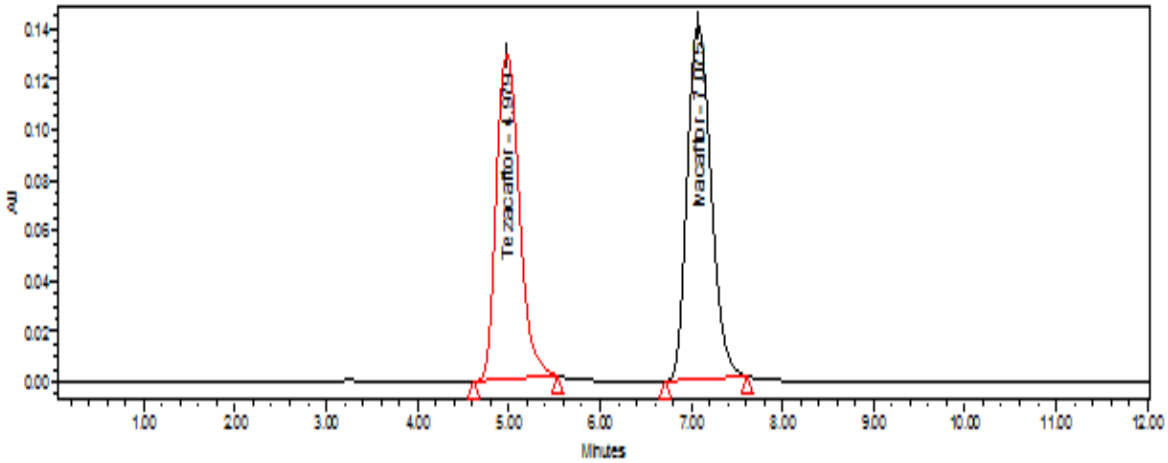
(ii) Specifications for Ivacaftor Intermediate (Analyst 2)

	Injection	Peak Areas of Ivacaftor	%Assay
Concentration 100ppm	1	1205267	99.78
	2	1205625	99.95
	3	1205840	100.00
	4	1202735	98.55
	5	1208991	101.50
	6	1208543	101.37
Statistical Analysis	**Mean**	1206333.5	100.19
	SD	12572.599	1.100898
	% RSD	1.24	1.09

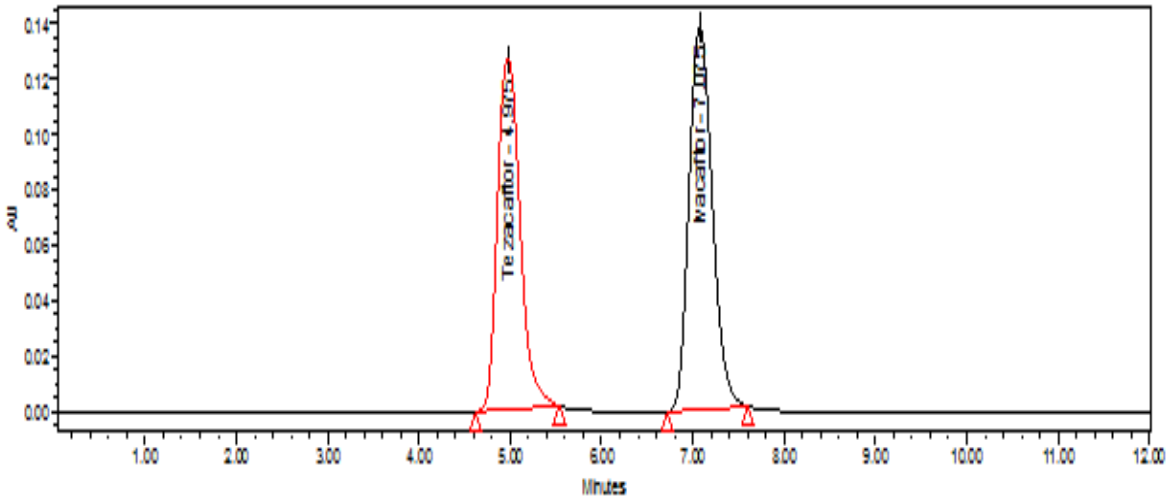
Fig 24-29: Chromatograms of Intermediate Precision



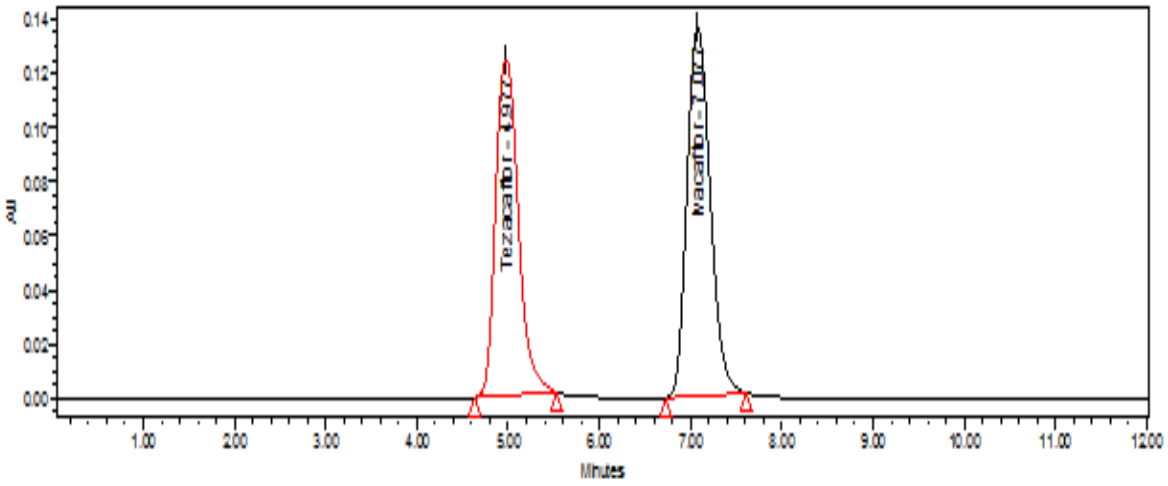
Inference: Chromatograph with a medium precision.1.



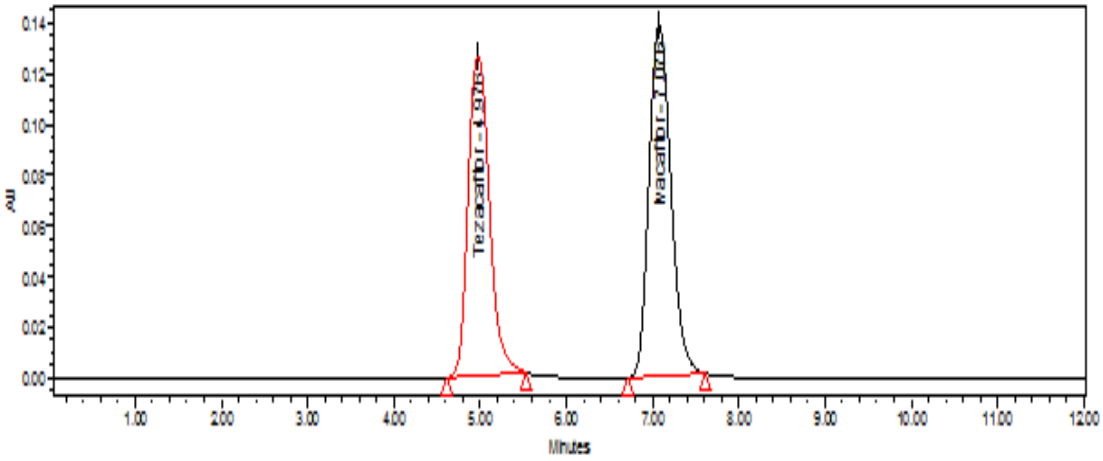
Inference: Chromatograph with a medium precision.2.



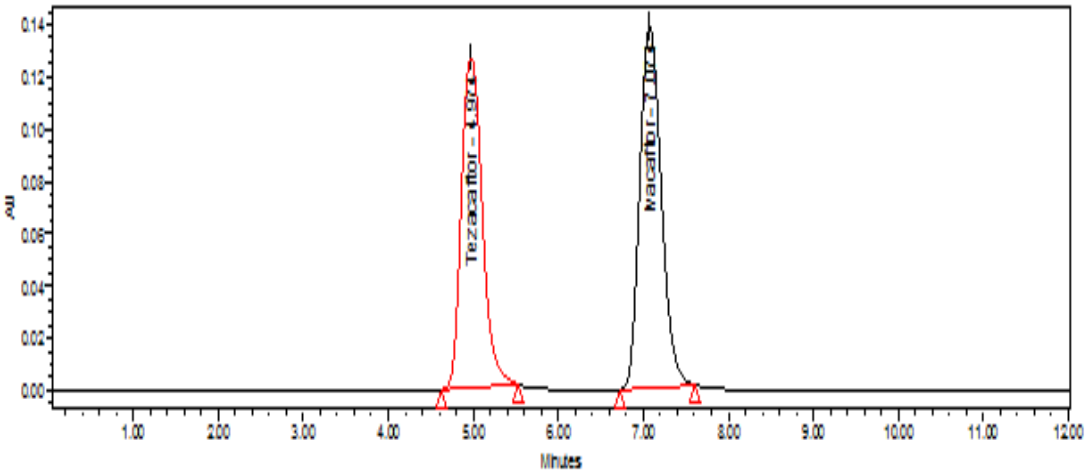
Chromatograph with a medium precision.3.



Chromatograph with a medium precision.4.



Chromatograph with a medium precision.5.



Chromatograph with a medium precision.6.

4. ACCURACY

Tabal. 4.

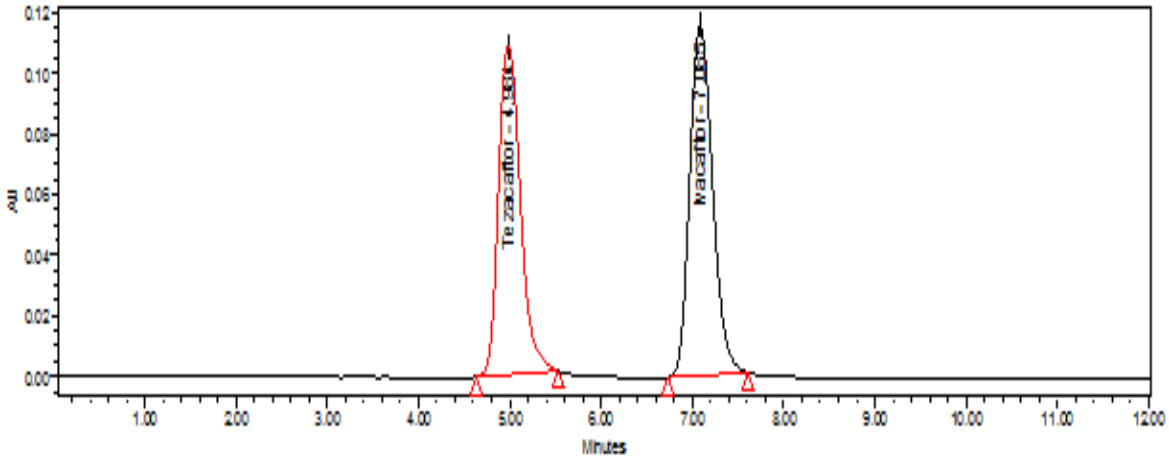
(i) Tezacaftor data with accuracy

Concentration % of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50% Injection 1	20	20.15	100.75	**MEAN**	99.69333
50% Injection 2	20	19.86	99.31	-	-
50% Injection 3	20	19.80	99.02	**%RSD**	0.92
100 % Injection 1	40	39.88	99.70	**MEAN**	99.83333
100 % Injection 2	40	40.12	100.30	--	
100% Injection 3	40	39.80	99.50	**%RSD**	0.41
150% Injection 1	60	60.12	100.21	**MEAN**	99.97333
150% Injection 2	60	59.76	99.61	-	-
150% Injection 3	60	60.06	100.10	**%RSD**	0.31

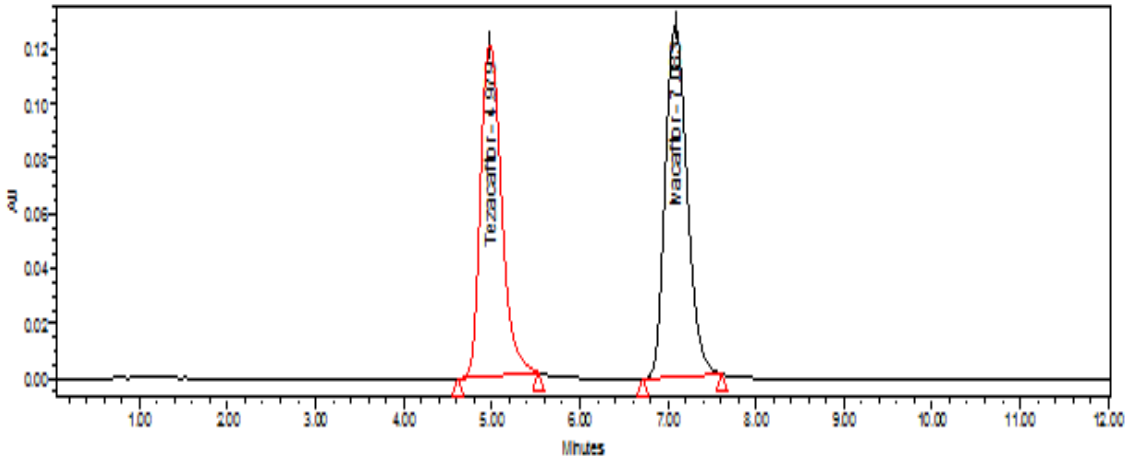
(ii) Ivacaftor data with accuracy

Concentration % of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50% Injection 1	20	20.04	100.22	**MEAN**	100.06
50% Injection 2	20	19.97	99.85	-	-
50% Injection 3	20	20.02	100.11	**%RSD**	0.18
100 % Injection 1	40	40.01	100.02	**MEAN**	100.04
100 % Injection 2	40	40.05	100.14	-	-
100% Injection 3	40	39.98	99.96	**%RSD**	0.091
150% Injection 1	60	60.08	100.14	**MEAN**	100.02
150% Injection 2	60	59.97	99.96	-	-
150% Injection 3	60	59.98	99.98	**%RSD**	0.09

Fig 30 -31: Chromatographic precision (50 percent)

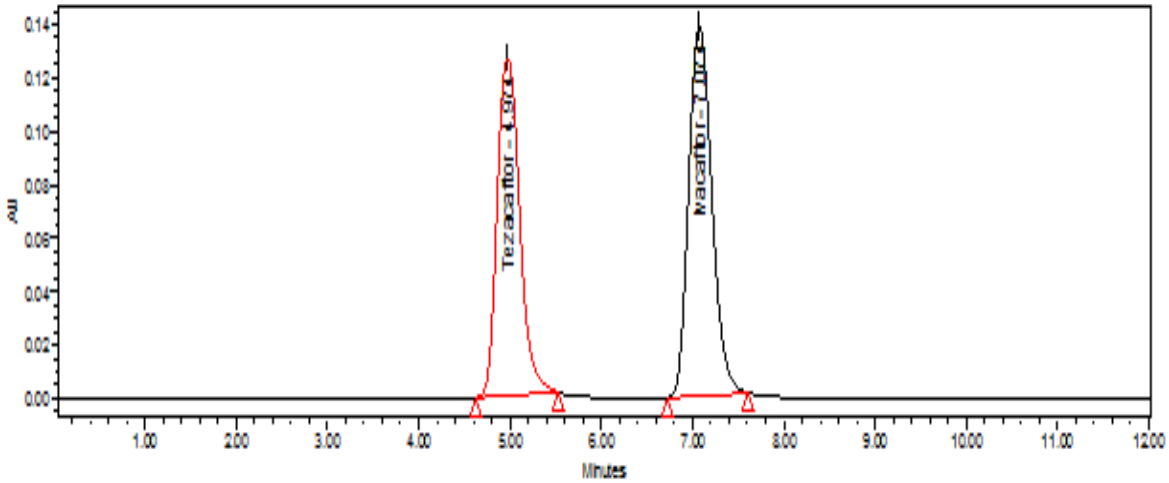


Inference: Standard 1 chromatogram.

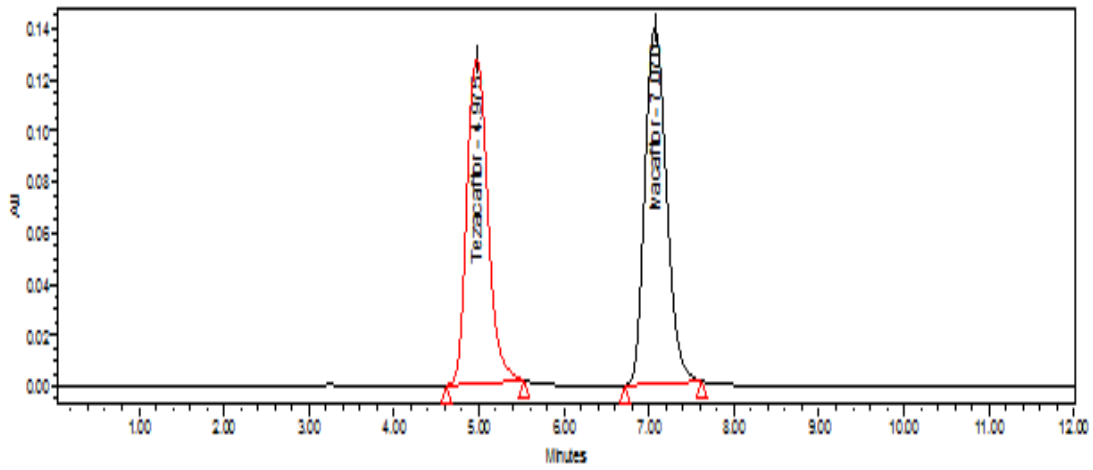


Inference: Standard 2 chromatogram.

Fig 32-33: Chromatograms with extreme accuracy (100 per cent)

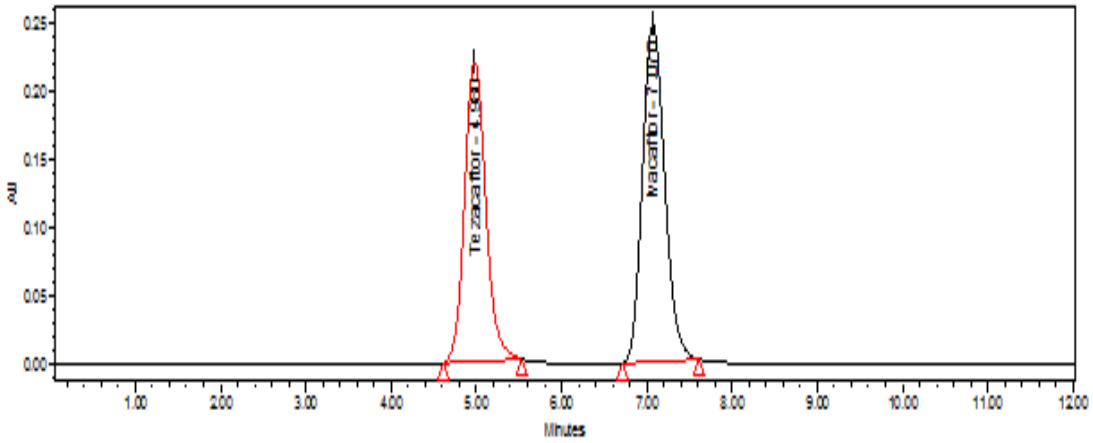


Inference: Standard 1 chromatogram.

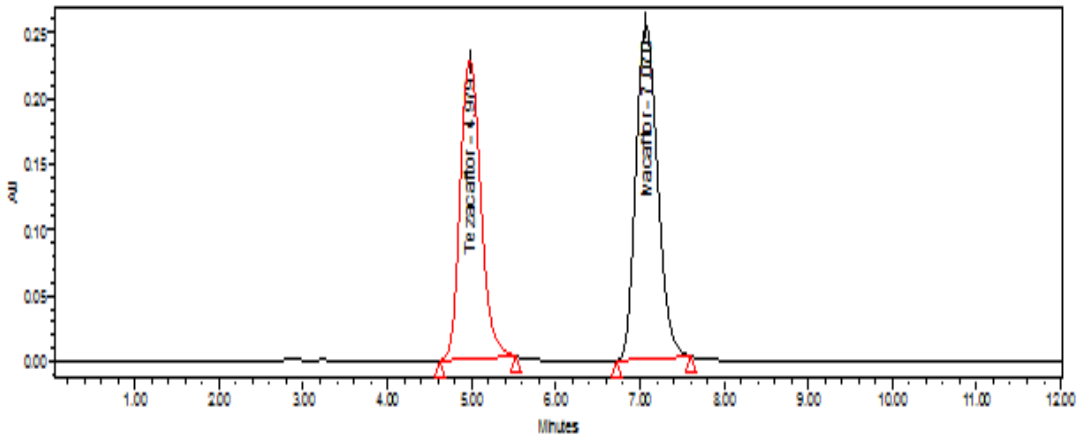


Inference: Standard 2 chromatogram.

Fig 34-35: Chromatograms are used to ensure precision (150 per cent)



Inference: Standard 1 chromatogram.



Inference: Standard 2 chromatogram.

5. LINEARITY

Tabal 5.

(i) Data of Linearity (Tezacaftor)

Concentration (ppm)	Average Area	Statistical Analysis	
0	0	Slope	18600
20	632546	y-Intercept	276.2
30	658296	Correlation Coefficient	1
40	694400		
50	730308		
60	916282		
70	9402046		

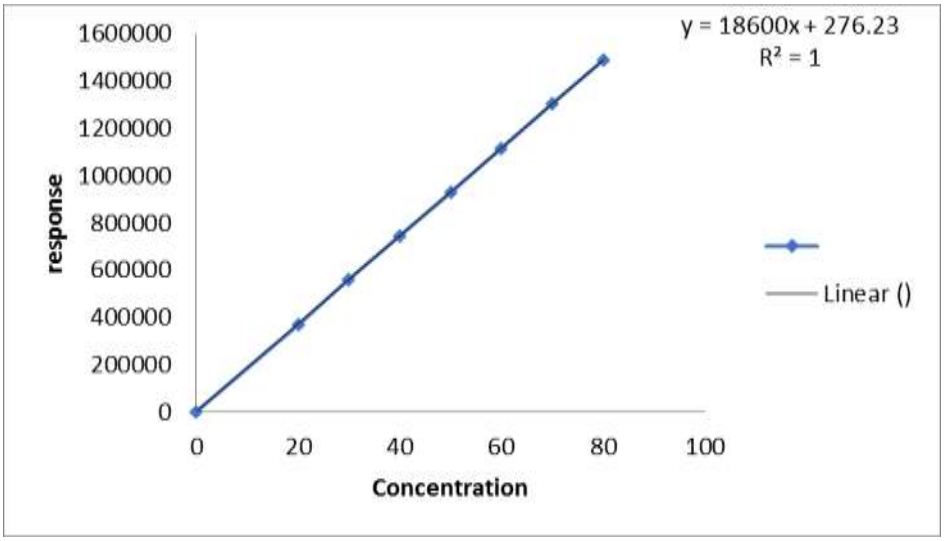


Fig 35 (a) Tezacaftor' s Linearity Plot (concentration vs response).

(ii) Details on linearity (Ivacaftor)

Concentration (ppm)	Average Area	Statistical Analysis	
0	0	Slope	5140
20	1202965	y-Intercept	114.7
30	1254371	Correlation Coefficient	1
40	1295856		
50	1297167		
60	1308577		
70	1359903		

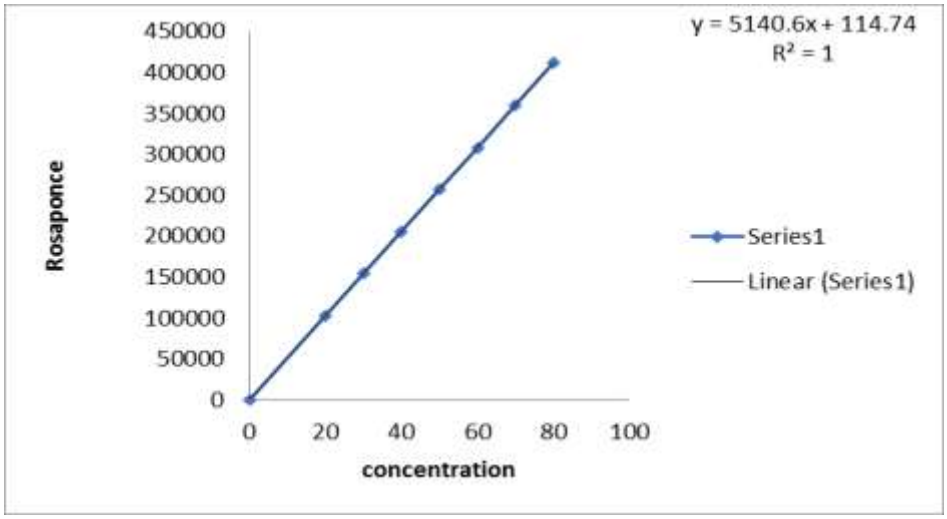
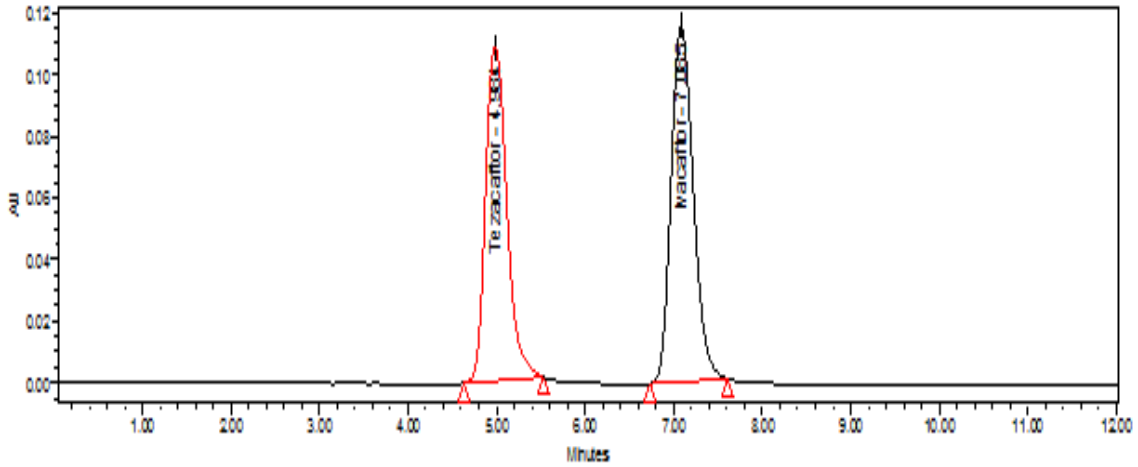


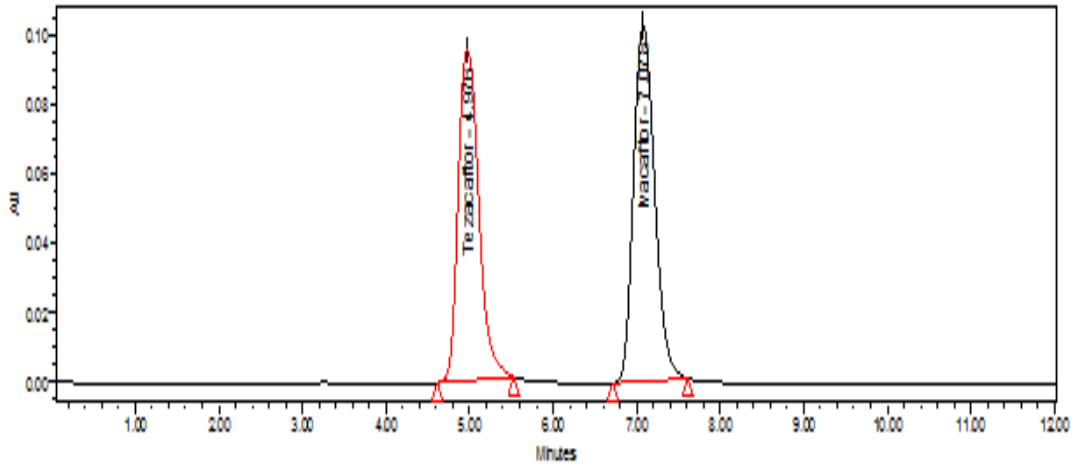
Fig 35 (b) Plot of Ivacaftor Linearity (Concentration Vs Answer).

Fig: 36 The chromatograms at 20 ppm are as follows.

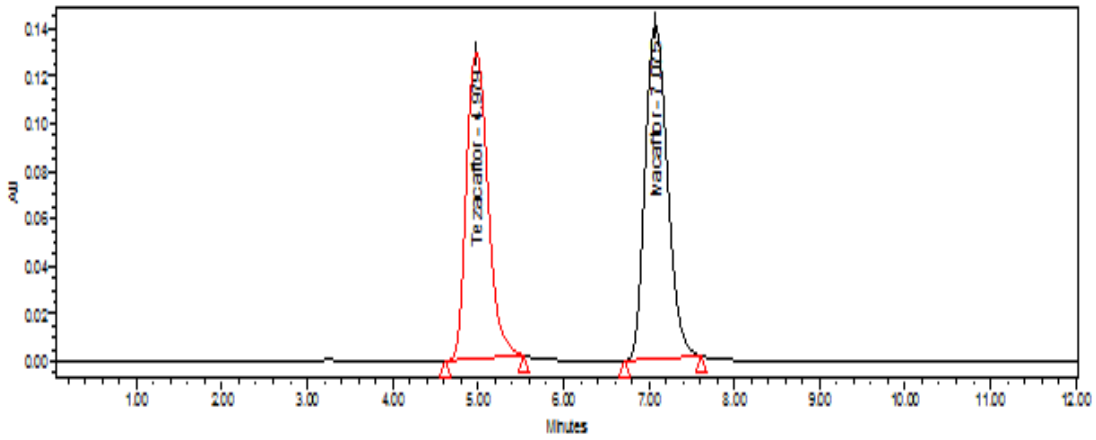


Inference: The standard chromatogram of 20 ppm.

Fig: 37-38 For chromatograms at 30ppm and 40ppm

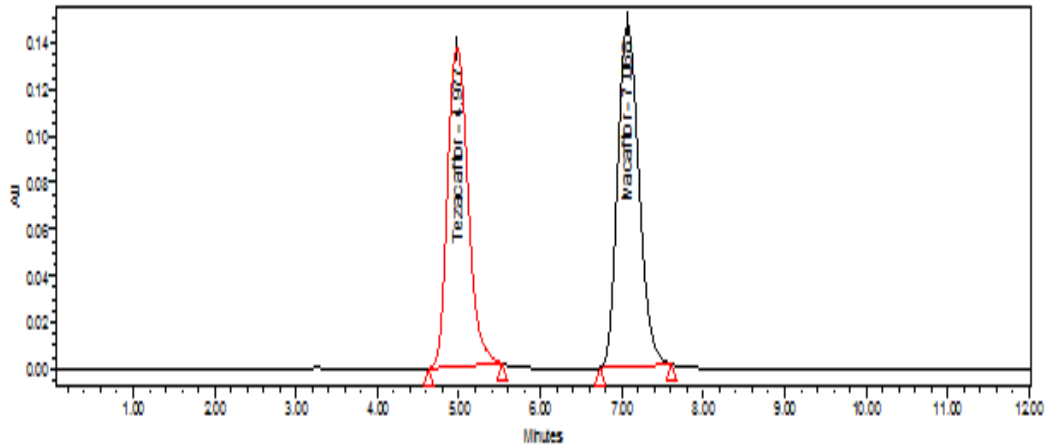


Inference: The standard chromatogram of 30 ppm.

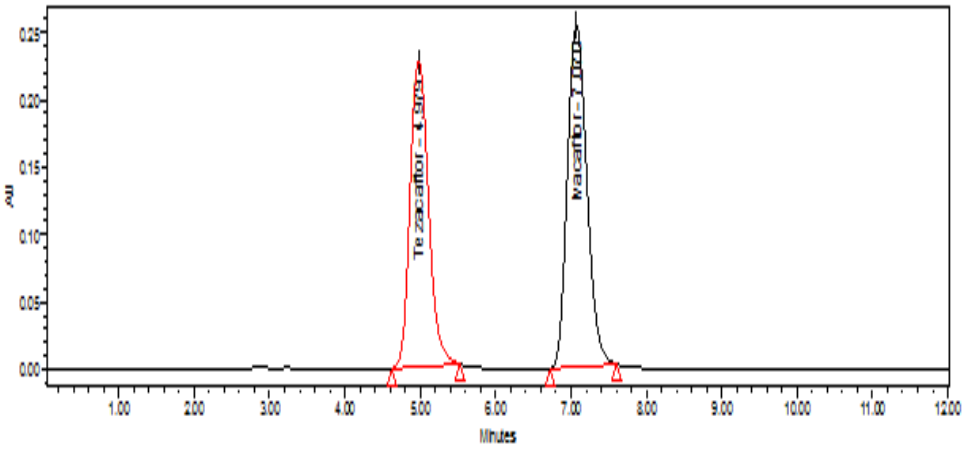


Inference: The standard chromatogram of 40 ppm.

Fig 39:40: -50 ppm chromatograms, 60 ppm chromatograms.

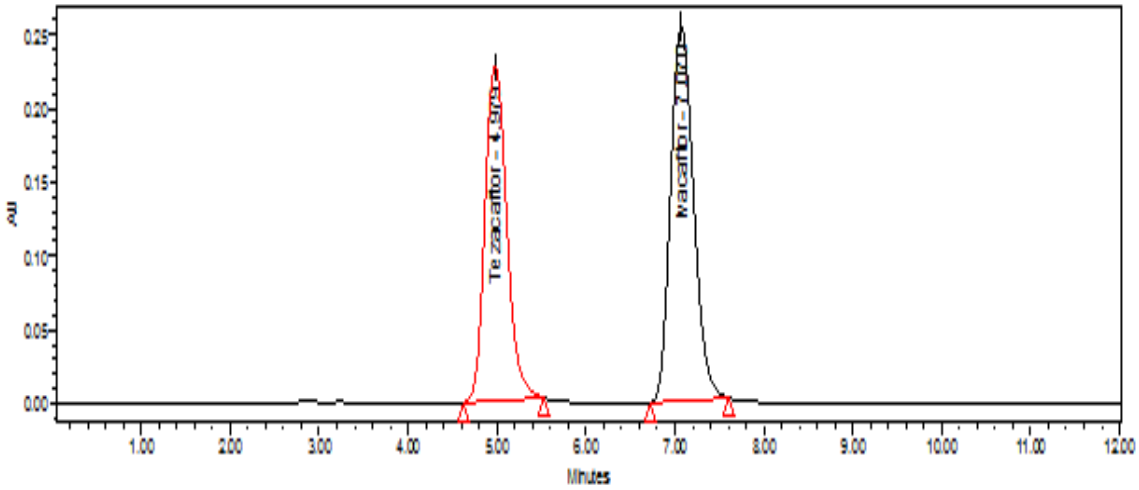


Inference: The standard chromatogram of 50 ppm.



Inference: The standard chromatogram of 60 ppm.

Fig 41: There are chromatograms available. 70 parts per million.



Inference: The standard chromatogram of 70 ppm.

6. Ruggedness

Variability from system to system (System to System variability)

Refer to Table3 for System 1.

Table: 6.

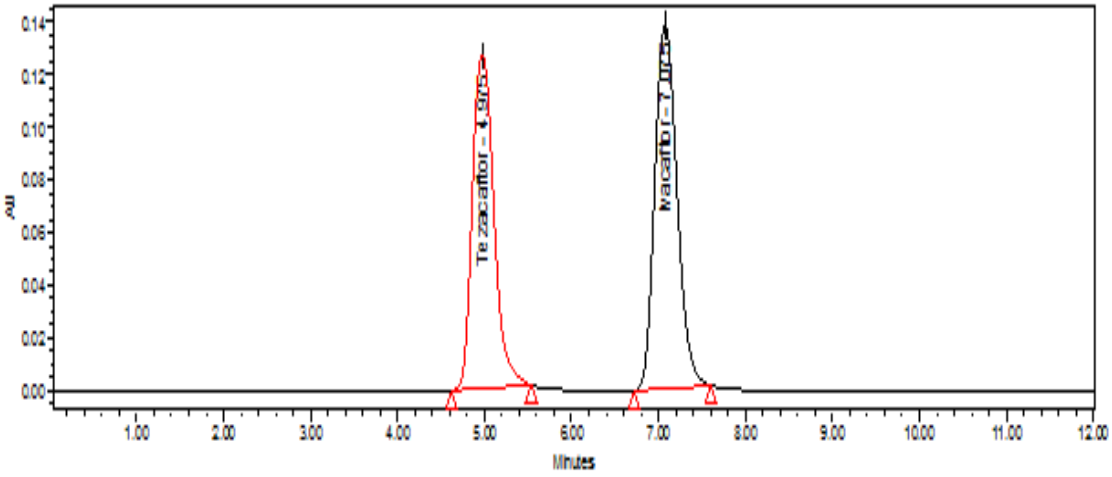
(i) Data on System Variability (Tezacaftor) System-2

S.NO:	Peak area	Assay % of Tezacaftor
1	634360	98.65
2	634098	98.63
3	635696	98.86
4	633289	98.52
5	634147	98.63
6	633495	98.55
Mean	634180.8	98.64
%RSD	0.019	0.12

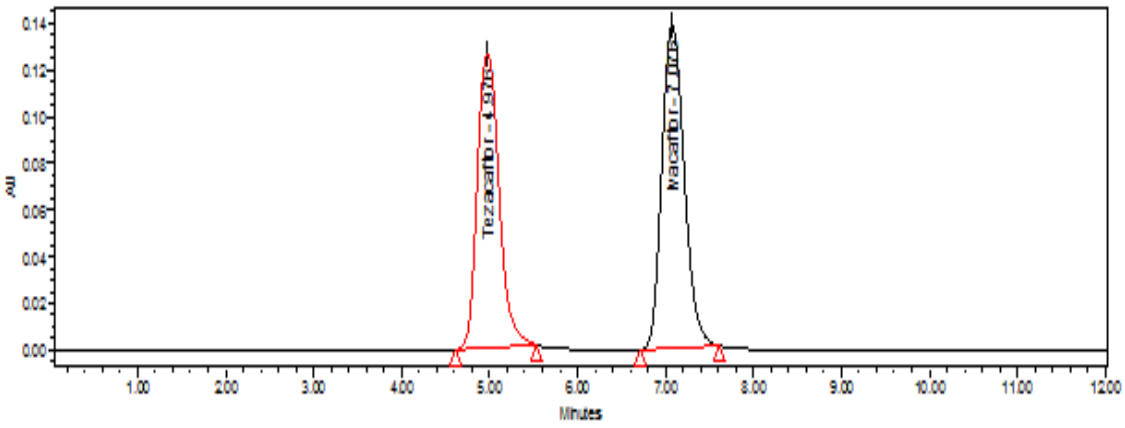
(i) Data on device variations (Ivacaftor) System-2

S.NO:	Peak area	Assay % of Ivacaftor
1	1203625	99.98
2	1202225	99.30
3	1202840	98.60
4	1204283	99.30
5	1202735	98.55
6	1203110	98.73
Mean	1203136.3	99.07667
%RSD	1.35	0.56

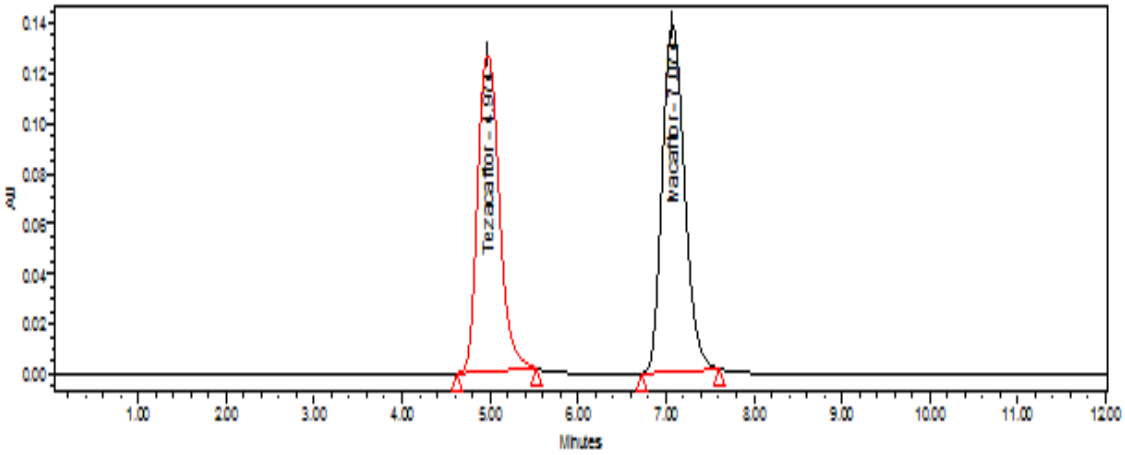
Fig42-47, System to system variability chromatograms.



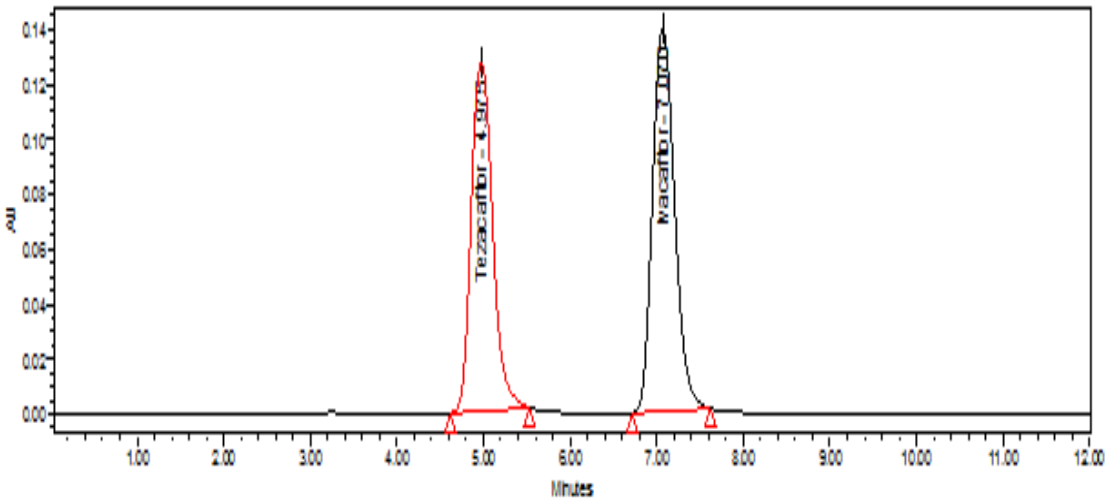
Inference: std- 1 chromatogram showing system-to-system variability.



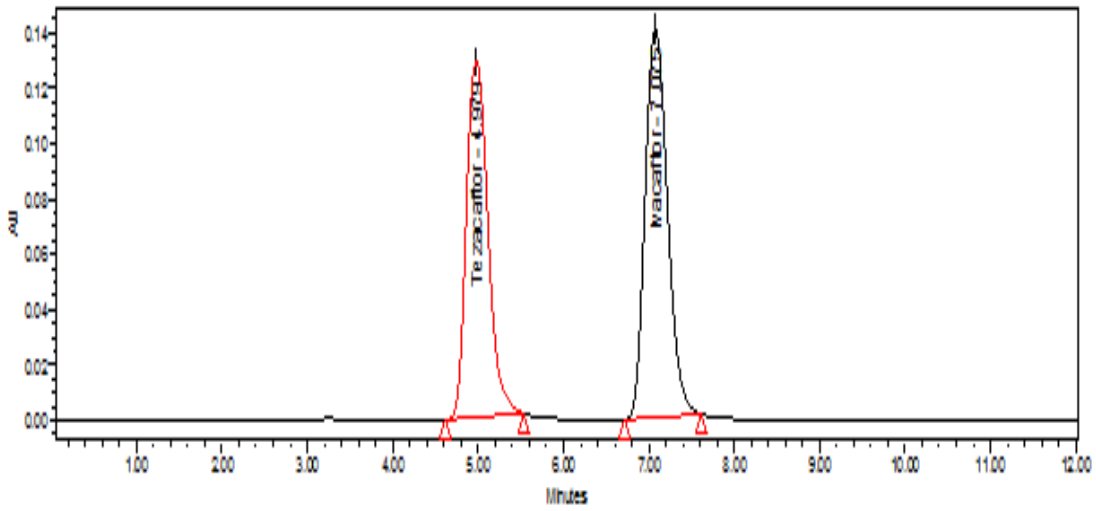
Inference: std- 2 chromatogram showing system-to-system variability.



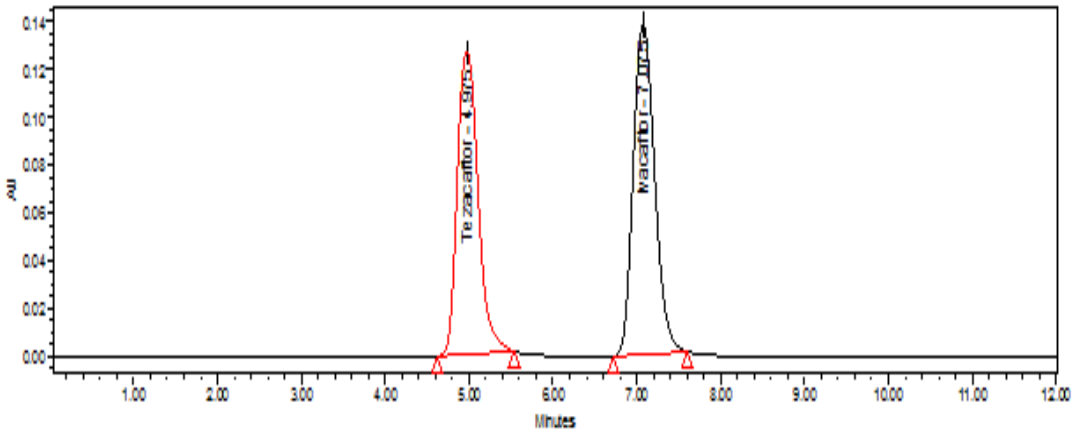
Inference: std- 3 chromatogram showing system-to-system variability



Inference: std- 4 chromatogram showing system-to-system variability.



Inference: std- 5 chromatogram showing system-to-system variability.



Inference: std- 6 chromatogram showing system-to-system variability.

7. Robustness

Table: 7.

(i) There's proof that flux rate variability has an impact (Tezacaftor).

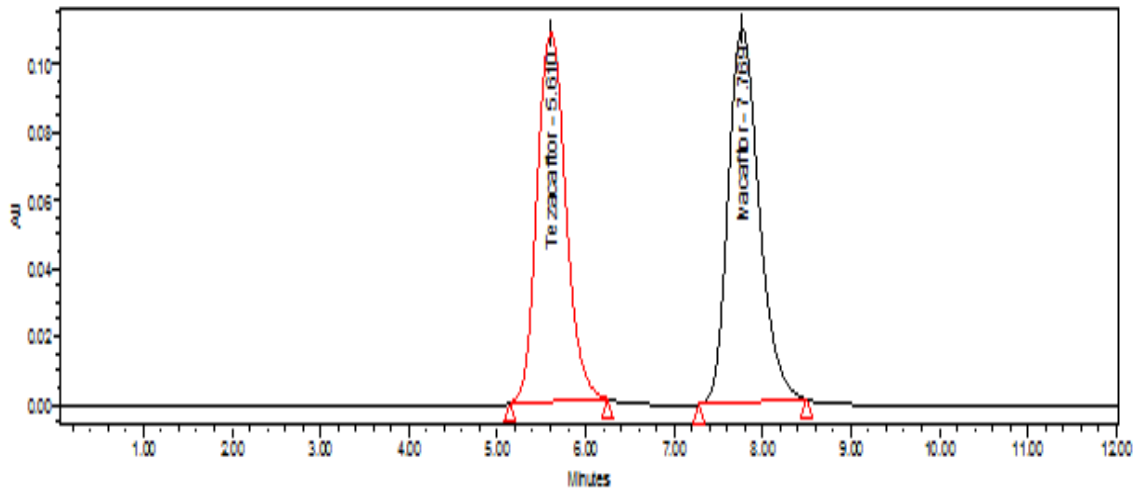
Flow 0.8 ml	Std Area	Tailing factor	Flow 1.0 ml	Std Area	Tailing factor	Flow 1.2 ml	Std Area	Tailing factor
	620286	1.322089		634322	1.604878		602077	1.285372
	619282	1.331920		635792	1.584354		601854	1.319385
	621337	1.296438		634360	1.543805		602403	1.292055
	620456	1.315454		635696	1.568590		603421	1.304561
	620765	1.326551		633147	1.559986		602465	1.294621
Avg	620425	1.31849	**Avg**	634663.4	1.572323	**Avg**	602444	1.299199
SD	754.0018	0.013728	**SD**	1100.917	0.023367	**SD**	599.8833	0.013223
%RSD	0.086	1.04	**%RSD**	0.184	1.48	**%RSD**	0.09	1.01

(ii) Flow rate shift impact data (Ivacaftor).

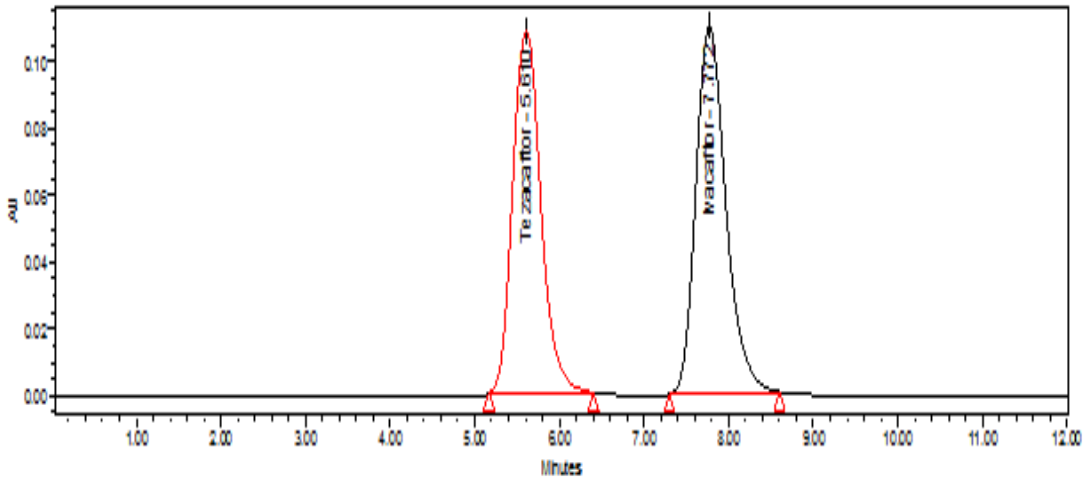
Flow 0.8 ml	Std Area	Tailing factor	Flow 1.0 ml	Std Area	Tailing factor	Flow 1.2 ml	Std Area	Tailing factor
	1273707	1.362089		1206349	1.280574		1266195	1.285372
	1273211	1.352617		1205267	1.279932		1265885	1.299385
	1273948	1.376926		1205625	1.261721		1266303	1.308063
	1273465	1.345752		1205840	1.276089		1267243	1.274662
	1273862	1.374925		1205735	1.250640		1265762	1.267630
Avg	1273638.6	1.362462	**Avg**	1205763.2	1.269791	**Avg**	166277.6	1.287022
SD	3301.369	0.013609	**SD**	392.1635	0.01314	**SD**	582.9758	0.016786
%RSD	1.041	0.99	**%RSD**	0.19	1.03	**%RSD**	0.35	1.3

Fig 48-49, Robustness chromatograms.

a) Variation in flow rate (for 0.8 ml/min flow) has an effect.

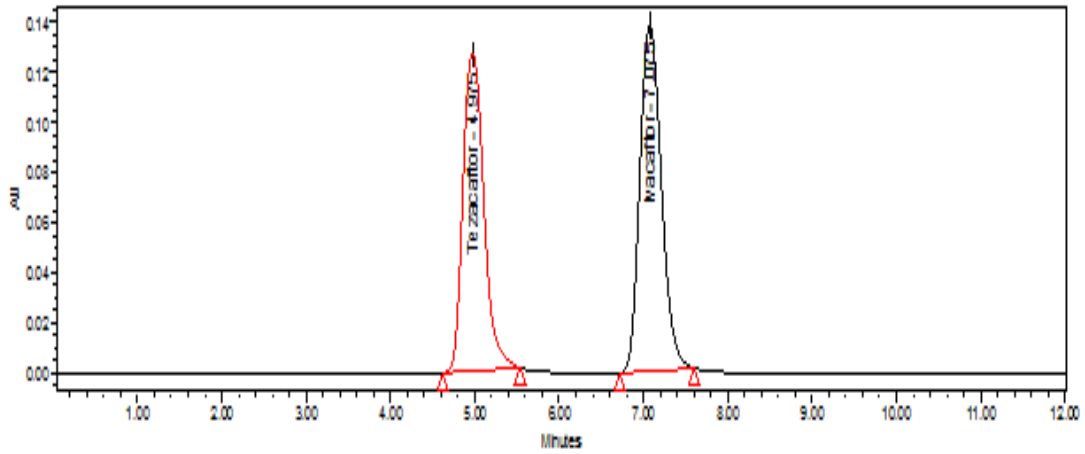


Inference: Standard for robustness chromatogram – 1.

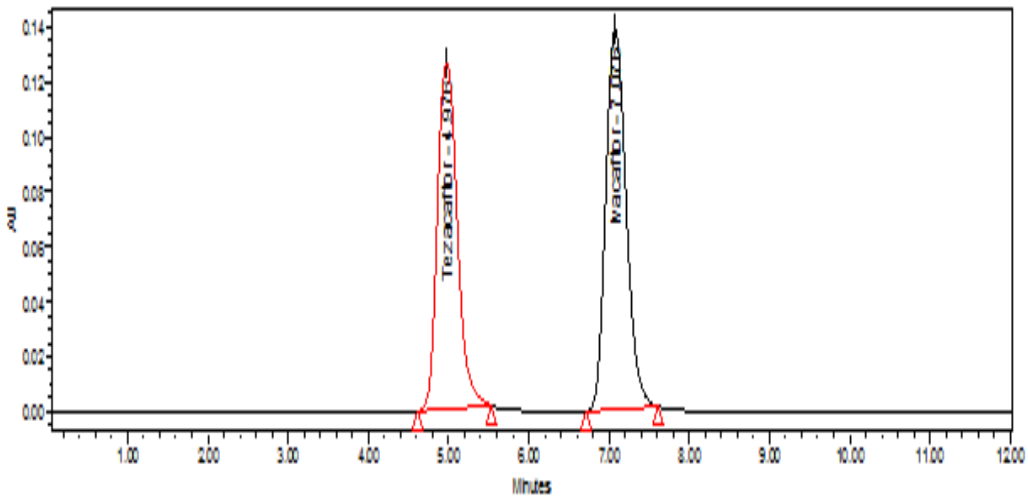


Inference: Standard for robustness chromatogram – 2.

Fig 50-51: chromatograms for a 1ml/min flow rate.

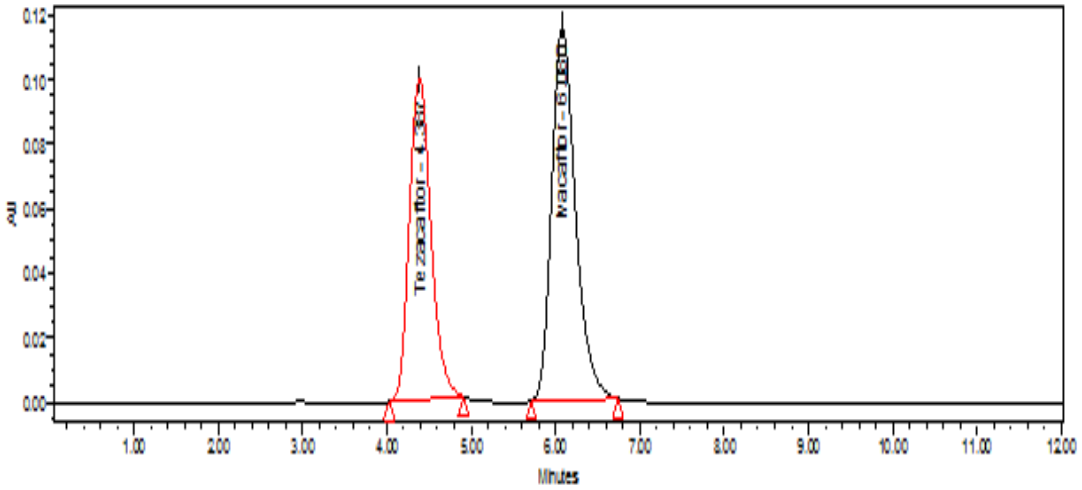


Inference: Standard for robustness chromatogram – 1.

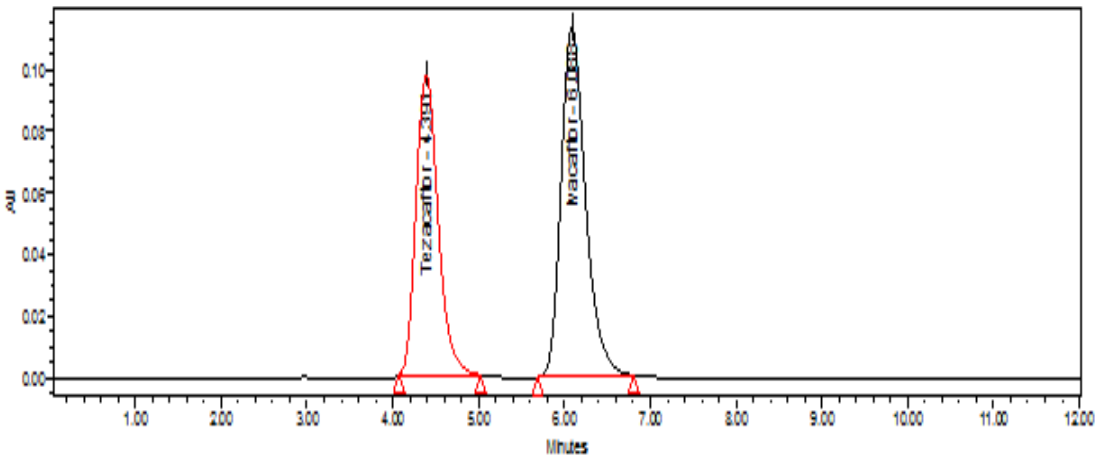


Inference: Standard for robustness chromatogram – 2.

Fig 52-53: 1.2ml/min chromatograms



Inference: Standard for robustness chromatogram – 1.



Inference: Standard for robustness chromatogram – 2.

8. LIMIT OF DETECTION & LIMIT OF QUANTITATION (LOD & LOQ)

Tezacaftor

From the linearity plot the LOD and LOQ are calculated.

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

$$= \frac{3.3 \times 867.0705}{5140} = 0.56$$

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

$$= \frac{10 \times 867.0705}{5140} = 1.69$$

Where,

σ = 867.0705

S = 5140

Ivacaftor

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

$$= \frac{3.3 \times 3244.904}{18600} = 0.57$$

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

$$= \frac{10 \times 3244.904}{18600} = 1.74$$

Where,

σ = 3244.904

S = 18600

CONCLUSION AND SUMMARY

The analytical method was developed by studying different parameters. First, maximum absorbance was found to be at 241nm for Tezacaftor and 254nm for Ivacaftor. Common wavelength will be 254nm and the peaks purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was Inertsil C18, ODS chosen good peak shape. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area, satisfactory retention time and good resolution. Different ratios of mobile phase were studied, mobile phase with ratio of Methanol: Buffer (45:55) was fixed due to good symmetrical peaks and for good resolution. So, this mobile phase was used for the proposed study.

Both system and method precision were found to be accurate and well within range. Linearity study was correlation coefficient and curve fitting were found to be. The analytical method was found linearity over the range of 20-70ppm of the target concentration for both the drugs. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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