ANALYTICAL METHOD DEVELOPMENT FOR SELECTED CARDIOVASCULAR DRUGS AND ITS APPLICATION FOR IMPURITY PROFILING, DEGRADATION AND *IN VITRO* DRUG-DRUG INTERACTION STUDIES

Thesis submitted to
The Tamil Nadu Dr. M. G. R. Medical University, Chennai

For the award of Degree of
DOCTOR OF PHILOSOPHY

In
PHARMACY

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DECEMBER 2017

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1. INTRODUCTION

The rapid drug discovery in modern era is pragmatic due to the advancement in computer aided drug designing and modernevaluation methods.The drug discovery process became more efficient and introduces large number of therapeutic molecules for the treatment of various ailments. The role of modern analytical techniques that offer high throughput and sensitivity are inevitable in drug development. The selection of analytical method is based on the protocol specification from research perspective, method appropriateness and availability.

Drug testing and analysis will provide a meaningful exploration to the pharmacokinetic, clinical and metabolic studies in in-vitro and in-vivo models. Different kinds of instrumental methods employed for quantification of drugs in formulations, impurity profiling and in vitro interaction studies include spectrophotometric methods like UV-Visible, Spectrofluorimetry, chromatographic methods like HPLC, HPTLC and hyphenated techniques like LC-MS, LC-MS/MS etc. UV spectroscopic methods are one of the most widely employed simple and rapid analytical techniques, while spectrofluorimetric methods posses the merit of high specificity, sensitivity and selectivity. Chromatographic techniques have several advantages over the spectroscopic techniques. HPTLC is rapidly becoming a routine analytical technique due to its advantages of low operating costs, need for minimum sample preparation, and high sample throughput. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase. The results produced are of a high resolution and are easy to read, and the tests are easily reproduced via the automated process. The hyphenated technique like LC-MS is a versatile technique for the determination of related substances in the bulk drug /dosage forms.

Pharmaceutical impurities are unwanted fractions present in the active pharmaceutical ingredient which may come from the process of synthesis, formulation and storage. Identification of these impurities is of great concern for regulatory agencies because even traces of impurities in pharmaceutical products can reduce the efficiency

of drug products and cause adverse drug reactions. Traces of impurities in drug substance or drug product are inevitable. Therefore their level should be controlled and monitored[(1)].

Impurity profile is the description of identified and unidentified impurities present in new drug substances and products. Sometimes the effects produced by these impurities are teratogenic, mutagenic or carcinogenic. This can blemish the human health by effecting the QSE – quality, safety and efficacy of the product and cause serious health hazards. Hence identification and characterization of impurity is required. Their limits and threshold values should comply with the limit set and specified by official bodies and legislation (Pharmacopoeias and International conference on harmonization- ICH guidelines). The different Pharmacopoeias, such as the British Pharmacopoeia (BP), United States Pharmacopeia (USP), and Indian Pharmacopoeia (IP) are slowly incorporating limits to allowable levels of impurities present in the API's or formulations.According to ICH identification threshold for drug substance less than or equal to 2g/day is 0.10% or 1 mg/day intake (whichever is lower) and more than 2g/day is 0.05% [(2)].

Generally, a longer stay on the shelf life increases the possibility that impurities will occur. Such impurities can be caused by several conditions as mentioned below.

Hydrolysis

The presence of water leads to acid or base hydrolysis. Well known examples of functional groups in pharmaceutical compounds that undergo hydrolysis are esters, amides, lactones, lactams, imides, carbamates or derivatives of carboxylic acids. Hydrolysis is effected by pH, buffer salts, ionic strength, solvents, complexing agents, surfactants and excipients.

Oxidation

In pharmaceuticals, the most common form of oxidative decomposition is auto-oxidation through a free-radical chain process. Drugs that are prone to oxidation include conjugated dienes, nitroso and nitrite derivatives.

Photolysis

Photolytic cleavage on aging products occurs with APIs or drug products that are prone to degradation on exposure to light. The rate of degradation depends on the intensity of light and the quantity of light absorbed. It involves the generation of free radical intermediates. The oxidative photolytic reaction occurs through single oxidation or triple oxidation mechanism. The non-oxidative photolytic reaction includes isomerization, diamerization, cyclization, rearrangement, decarboxylation and haemolytic cleavage.

Thermal degradation

Impurities formed by exposure to temperatures include bond breakage i.e., pyrolysis. Any degradation mechanism that is enhanced at elevated temperatures are thermolytic pathways include hydrolysis, dehydration, isomerization, decarboxylation, rearrangement and polymerization reactions. In general rate of the reaction increases with temperature. Many APIs are sensitive to heat or topical temperature.

According to ICH guidelines - Q1A (R2), stress testing of the drug substance helps to identify the likely degradation products, which in turn help establish intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved[(4)].

Degradation studies

Forced degradation studies of new chemical entities or drug products are essential to help, develop or demonstrate the specificity of stability indicating methods. In addition to specificity, forced degradation studies can be used to study the degradation pathways and degradation products that would form during formulation development, manufacturing, packaging and storage. In general a 10% degradation of the drug is recommended[3,4].

ICH guidelines (Q_1A R_2)[5] on forced degradation study recommended conditions for hydrolysis, oxidation, thermal and photodegradation studies. A Stability Indicating Method (SIM) is a validated method that can accurately and precisely quantify the reductionin API content due to degradation, which is specific for the drug substance, without interference from excipients, impurities or degradation products.

***In vitro* drug-drug interaction studies**

A drug interaction is a situation in which a substance (usually another drug) affects the activity of a drug when both are administered together. This action can be synergistic or antagonistic or a new effect that neither of the molecule produces on its own. These chemical reactions are also known as pharmacological incompatibilities. The objective of drug-drug interaction studies is to determine whether potential interactions existbetween the selected drugs. It is also possible for interactions to occur outside an organism before administration of the drugs. This can occur when two drugs are mixed, for example, in a saline solution prior to intravenous injection.

Cardiovascular drugs encompass a large number of prescription medications that are used to control heart disease. They are complicated group of drugs with many being used for multiple heart conditions. Antiarrhythmic agents are used to suppress abnormal rhythms of the heart, such as atrial fibrillation and atrial flutter. Anticoagulants are a class of drugs that act directly on the factor Xa without the intervention of the antithrombin used in the treatment of stroke, pulmonary embolism and deep vein thrombosis. [6]

Apixaban, Rivaroxaban, Ibutilide and Dofetilide are very potent drugs in treating cardiac disorders. It is very essential to carryout degradation studies, impurity

profiling and in vitro drug-drug interaction studies in such molecules and this demand the development of analytical techniques that are validated in terms of linearity and range, accuracy (closeness of the assay value to true value), precision (reproducibility and repeatability of the value), robustness (effect of variations in the experimental conditions on the response), limit of detection, limit of quantitation, specificity and sensitivity according to the ICH guidelines Q2(R1) [7]. Statistical analysis ofexperimental resultsassures the reliability of the research study. Most commonly employed statistical parameters are standard deviation, Relativestandard deviation, Mean, Student t test, ANOVA etc.

2. AIM AND OBJECTIVE OF THE RESEARCH WORK

The current study aims on development of analytical techniques for the selected cardiovascular drugs and their formulations. The selection of drugs for research work is based on the approval, potency, physiochemical parameters and reported analytical procedures.

Apixaban, Rivaroxaban, Ibutilide fumarate and Dofetilide are the drugs selected for the study which are approved by the USFDA for specific conditions of Cardiovascular disorders in potent doses. The specific aim of the research work is

- To develop and validate various analytical methods for the selected cardiovascular drugs as per ICH Harmonised tripartite guidelines Q2(R1).
- To establish the inherent stability characteristics of the selected drugs by forced degradation studies as per ICH recommendations Q1A(R2) by the developed analytical method.
- To perform impurity profiling of the selected cardiovascular drugs by validated analytical methods.
- To identify the impurities/degradants and elucidate the possible structure of the degradants by Tandem Mass Spectroscopy.
- To develop suitable analytical methods for the in vitro drug-drug interaction studies of selected cardiovascular drugs with the co-administered drugs.

The objective of the research work is described below,

The impurity profiling of the selected drugs is very essential to monitor the safety in use of the drug product and drug substance. It is also vital during manufacturing and till the formulation of final dosage form. The impurities selected for the drug rivaroxaban and dofetilide are the process related impurities with structural similarities with the parent drugs. The formation of these impurities are possible due to the molecular structure containing the functional group present.

The present research work focuses on the determination of drug substance with the spiked impurity according to the specification limit as per USP 40 for Dofetilide. The drug rivaroxaban in not official in any pharmacopoeia. Hence the limit of impurity selected was calculated based on the AL (allowable limit) according to European standards and ICH Q6A guidelines based on the potency of the drug and the repeatability of assay values in the tablet dosage form.

The degradation studies were according to ICH Q1A (R2) stress testing under selected conditions of acid, base, oxidation, light and elevated temperature. The level of degradants were quantified for the allowable limit upto 10%, above which can cause decrease in availability of the drug for its action or cause untoward effects.

The rate of adverse drug reaction increases in patients taking more medications under various categories such as cardiovascular drugs. They are under the risk of drug drug interactions leading to changes in the properties of drugs. A metabolism based *in vitro* interaction studies with co-administered drugs are to be carried out for Ibutilide fumarate which will find the usefulness in the assessment of clinical drug drug interaction. The rat liver microsomes are used in the interaction studies to assess the effect of co-administered drugon the selected cardioavascular drugs.

3. LITERATURE REVIEW

3.1 Apixaban

Dudhe PB., et al(2017) [8]has reported two UV spectrometric method for the estimation of apixaban in bulk drug and tablet dosage form. Methanol was used as the solvent to prepare the standard and sample solutions. For quantitative measurement of apixaban by first method, the area under curve between 269- 289 nm was measured.The second method was based on first order derivative spectroscopy at zero crossing wavelength 279.99nm. Calibration curve were observed between 5-30 mcg/ml for both methods.

Rambabu K., et al (2015) [9] has developed two novel methods having precision, accuracy, specificity and robustness and validated the method fulfilling the ICH guidelines for the quantitative determination of apixaban in pharmaceutical dosage forms. The first method was based on isocratic reverse phase liquid chromatography using C18 column and mobi buffer:acetonitrile (60:40)%v/v at a flow rate of 1ml/min and dete y photodiode aray detector set at 280 nm. The response was linear n 5-50 mcg/ml. The second method was photometric method inv 280 nm with calibration range of 5-50 mcg/ml.

Ibutilide fumarate

Shashikant B., et al (2015)[10] has described a reverse-phase HPLC method for the quantitative determination of process related and degradation impurities of Apixaban. The chromatographic separation was achieved on a Sigma-Aldrich's Ascentis Express® C18 (4.6 mm × 100 mm, 2.7 µ) HPLC column with a runtime of 40 min. Mobile phase-A and mobile phase-B were phosphate buffer and acetonitrile respectively. The column oven temperature was set at 35˚C and photodiode array detector at 225 nm. Nine process related impurities have been detected in test sample of Apixaban. Forced degradation study was carried out under acidic, alkaline,oxidative, photolytic and thermal conditions to demonstrate the stability-indicating nature of the developed RP-HPLC method. The developed method was validated as per ICH guidelines.

Hemant KJ., et al (2017) [11] has reported a HPLC method for the immediate release tablets that were formulated by direct compression method. The chromatographic separation was performed on Purospher Star RP-18e(5µm, 250 x 4.6 mm) column. The mobile phase consisted of water:acetonitrile (60:40)% v/vat a flow rate of 1 ml/min

detected at 280 nm. The linearity was found between 3-30 mcg/ml of the drug. The stabllity of drug was studied at thermal, photolytic, acidic, basic and oxidative conditions.

3.2. Rivaroxaban

Nageswara RR., et al (2014)[12] has developed and validated a stability indicating LC-PDA-MS/MS method was developed and validated for separation, identification and characterization of process related and stress degradation products of Rivaroxaban in bulk drugs. Rivaroxaban was subjected to hydrolysis (acidic, alkaline and neutral), photolysis, thermolysis, and oxidation as per ICH guidelines and found susceptible to acid and base hydrolytic stress conditions. In total, three DPs (DP-1, DP-2, and DP-3) were formed. All the DPs, PR and RVR were separated on a Kinetex C18 (150 _ 4.6 mm, 5 mm) column using a mobile phase consisted of 20 mM ammonium acetate and acetonitrile (65 : 35 v/v) at a flow rate of 1.0 ml min. The structures of PR and DPs were elucidated by the molecular ion fragmentation of RVR, PR and DPs.

Rohde G., (2008)[13]has reported method of High-Performance Liquid Chromatography-Tandem Mass Spectrometryfor the determination of Rivaroxaban in human plasmaallowing the sensitive and specific quantification of rivaroxaban (BAY 59-7939), a Factor Xa inhibitor in advanced development for the prevention and treatment of thromboembolic disorders, in human plasma. After precipitation of plasma proteins with methanol containing the internal standard followed by centrifugation, the plasma supernatant was injected directly onto the HPLC–MS/MS system. Concentrations could be determined between 0.50 and 500 mcg/L. Inter-assay precision was ≤7.4% and inter-assay accuracy was between 96.3 and 102.9% throughout the entire working range.

Schmitz EMH., et al (2014)[14]has developed and validated an UPLC-MS/MS method for the coagulation assays to quantify three novel direct oral anticoagulants such as dabigatran, rivaroxaban, and apixaban in plasma. Agreement between the various coagulation assays and UPLC-MS/MS was determined with random samples from patients using dabigatran or rivaroxaban. Statistically significant differences were observed between the various coagulation assays as compared with the UPLC-MS/MS reference method.

3.3 Ibutilide

Babu MS., et al (2011)[15]has described the spectrophotometric method for Ibutilide Fumarate in Bulk Drug substance form**.** The method is based on complex formation`under acidic conditions with acid dye. The coloured species has absorption maxima at 485 nm and obeys Beer's law in the concentration range of 2.5-20 mcg/ml with a correlation coefficient of 0.9995. The molar absortivity and Sandell's sensitivity are 3.611 x 10^4 l mole cm^{-1} and 0.024 $g/cm^{-2.}$ The method was validated and statistically analyzed.

Satya Babu M., et al (2009) [16] has developed a rapid and sensitive RP-HPLC method with UV detection at 227 nm for routine analysis of washed mother liquors from equipment after manufacturing and thereby cleaning of ibutilide fumarate active pharmaceutical ingredient. Chromatography was performed with mobile phase containing a mixture of aqueous 0.01M potassiumdihydrogen phosphate (KH_2PO_4) and acetonitrile. The gradient elution was developed for better and optimized results. The developed method was validated for precision and finally linearity studies from0.4 to 150%. The method is ascertained to be having good repeatability and reproducibility. The precision, accuracy and linearity were studied and reported to be within the limits.

3.4 Dofetilide

Nisrin K., et al(2013) [17]has described a sensitive and specific HPLC method using a tandem of UV/fluorescence detection was described for the analysis of amlodipine and dofetilide in plasma. The within-day and between-day precision studies showed good reproducibility with coefficients of variation less than 10%. The limits of detection were 0.5 ng/ml and 0.25 ng/ml and the limit of quantification were 1.7 ng/ml and 0.8 ng/ml for dofetilide and amlodipine respectively.

4. SCOPE AND PLAN OF THE STUDY

An extensive literature survey was carried out on the analytical method reported for the selected cardiovascular drugs.

There are few HPLC methods reported for the determination of Apixaban and Rivaroxaban. These methods were applied for dosage forms, degradation studies and impurity detection. A spectroscopy and HPLC methods were reported for the analysis of Ibutilide from the formulation.

For Dofetilide there is one HPLC method reported for its estimation from plasma. Quantification of these drugs are carried by using either UV detector or fluorescence detector. However, no analytical method is reported for degradation studies of Ibutilide and Dofetilide till date.

For the selected drugs there is a need of conducting in vitro interaction studies with co-administered drugs which will be useful in the assessment of clinical drug-drug interaction. Metabolism based drug-drug interaction studies using rat liver microsomes is one of the most specific means in bringing clinical significance of the study. Development of newer analytical method developed will be beneficial in the in vitro interaction studies of the selected drugs.

PLAN OF THE STUDY

Phase I:

Development of analytical method for the selected drugs:

UV spectroscopic method: It involves fixation of various parameters like selection of solvent, λ_{max} instrumental parameters and validation of the method.

HPLC and HPTLC methods: The steps involved in the chromatographic method development are selection of stationary phase (plate/column), mobile phase, detection wavelength, optimization, assessment of chromatographic parameters and validation.

Phase II:

Application of the developed methods for the determination of selected drugs from dosage forms/admixtures.

Phase III:

Application of the developed methods for the degradation of selected drugs. The drugs will be subjected to various degradation conditions and chromatograms will be observed for the presence of additional peak and % reduction of the drugs.

Phase IV:

Impurity profiling: Application of the developed methods to analyze known impurities as well as unknown, unspecified impurities in presence of the selected cardiovascular drugs.

Identification of structures of the possible degradants/impurities by LC-MS/MS methods.

Statistical Analysis:

The data obtained experimentally will be analyzed through appropriate statistical parameters such as student t-test, least square regression analysis, ANOVA etc inorder to ascertain the results obtained.

5. MATERIALS AND INSTRUMENTS

Chemicals

The chemicals potassium dihydrogen orthophosphate, Hydrochloric acid, Sodium hydroxide pellets, Hydrogen Peroxide and reagents like chromotropic acid, acetic anhydride were procured fromQualigensFineChemicals Ltd and SD Fine Chemicals Ltd, Mumbai.

The organic solvents like methanol, toluene, acetone, petroleum ether, carbon tetrachloride, chloroform, ethanol, acetonitrile, tetrahydrofuran and DMSO used for the research work were of AR grade were procured from SD Fine Chemicals Ltd, Mumbai. The HPLC grade methanol and water were procured from Thermo Fisher Scientific India Pvt Ltd/ Merck Specialties Pvt Ltd/ Sigma Aldrich, Mumbai.

Active pharmaceutical ingredients

The drug Dofetilide was a gift sample from Par Formulations Ltd, Chennai. of Ibutilide fumarate and Apixaban was procured from suppliers like Sigma Aldrich, Germany and Manus Akkutteva Biopharma Ltd, Gujarat, India respectively. The formulations of the drugs were procured from local market.

Instruments

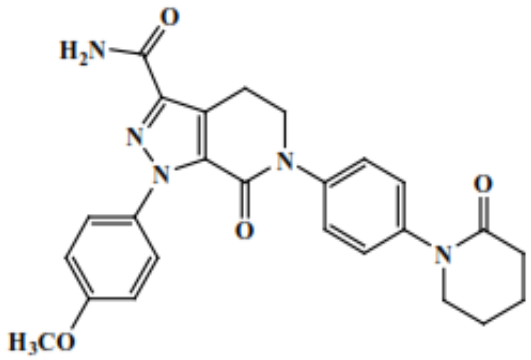
- The weighing of the samples done with Shimadzu Balance BL-22H with a sensitivity of 0.001 g.
- The Digital pH meter MK VI was used for the pH measurement of the buffer and drug solutions.
- The Thermo Fisher freezer was used to store the rat liver microsomes maintained at a temperature of -70^{o}C. The thaw cycle of microsomes for the interaction studies was done using a thermostat waterbath maintained at a temperature of 37^{o}C.

- The UV spectroscopy method was performed using Jasco V-630 Spectrophotometer with 1 cm matched quartz cuvettes using deuterium lamp with a scanning speed of 20 nm/sec.
- The HPTLC method was developed using precoated silica gel plate $G_{60}F_{254,}$ Camag UV chamber for spot identification, Camaglinomat 5 applicator for sample application, 20 x 20 cm and 10 x 10 cm chamber for plate development and Camag TLC Scanner for spot detection with WinCats Software.
- The RP-HPLC method was studied using Shimadzu 2030 *i-prominence* system inbuilt with a degasser, autosampler and four mobile phase pump.
- The identification and characterization of degradation products were performed using API 3200 LC/MS/MS AB.SCIEX system operated in Multiple Reaction Monitoring by Turbo spray mode.

DRUG PROFILE

GENERIC NAME - APIXABAN (ELIQUIS)[18,19]

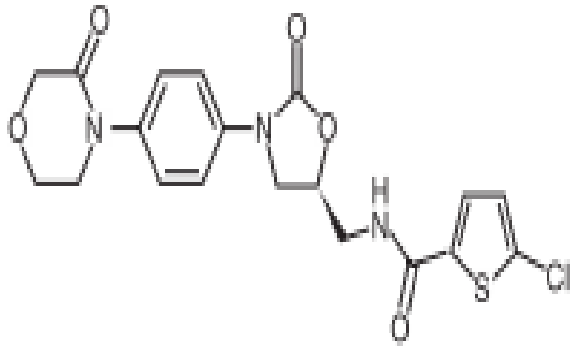
CHEMICAL STRUCTURE -



MOLECULAR FORMULA	-	$C_{25}H_{25}N_5O_4$
MOLECULAR WEIGHT	-	459.5
IUPAC NAME	-	1-(4-Methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1- yl)phenyl]-4,5,6,7-tetrahydro-1*H*pyrazolo[3,4-*c*]pyridine-3-carboxamide[17,18]
APPEARANCE	-	White to pale yellow powder, Amorphous
PHYSICAL STATE	-	powder
SOLUBILITY	-	Soluble in methanol, insoluble in water and ethanol.
AVAILABLE DOSAGE FORM	-	2.5 mg Film-coated tablet
CATEGORY	-	Selective inhibitor of the coagulation factor Xa (FXa)
CDSO APPROVAL DATE	-	August 3rd2012

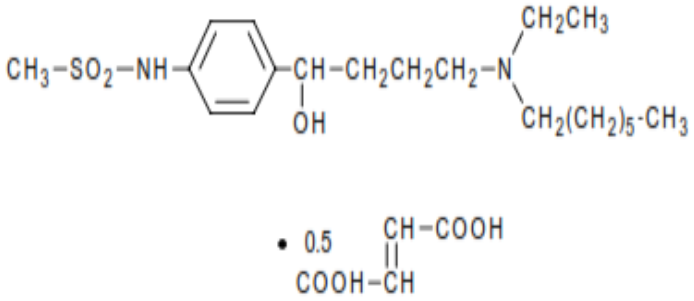
GENERIC NAME -RIVAROXABAN (XARELTO)[20,21]

CHEMICAL STRUCTURE -



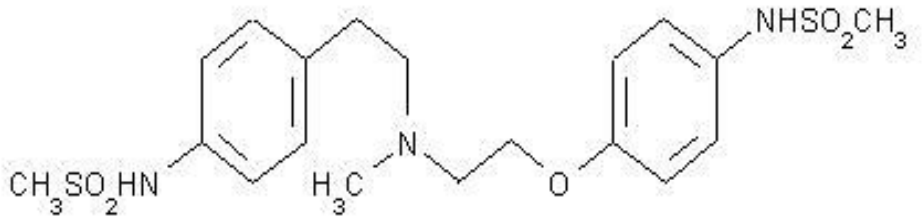
MOLECULAR FORMULA	-	$C_{19}H_{18}ClN_3O_5S$
MOLECULAR WEIGHT	-	435.89.
IUPAC NAME	-	5-Chloro-N-({(5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl)phenyl]-1,3-oxazolidin-5-yl}methyl)-2-thiophenecarboxamide
APPEARANCE	-	odorless, non-hygroscopic, white to yellowish powder.
PHYSICAL STATE	-	Powder
SOLUBILITY	-	Slightly soluble in organic solvents (eg., acetone, polyethylene glycol 400) and insoluble in water and aqueous media.
AVAILABLE DOSAGE FORM	-	10 mg Tablet
CATEGORY	-	Factor Xa inhibitor
CDSO APPROVAL DATE	-	January 30th 2010

GENERIC NAME	-	IBUTILIDE FUMARATE (CORVERT INJECTION) [22,23]
CHEMICAL STRUCTURE	-	



MOLECULAR FORMULA	-	$C_{22}H_{38}N_2O_5S$
MOLECULAR WEIGHT	-	442.62
IUPAC NAME	-	Methanesulfonamide, N-{4-{4-(ethylheptylamino)-1-Hydroxybutyl}phenyl}, (+)(-), (E)-2-butenediote(1:0.5) (hemifumarate)
APPEARANCE	-	white to off-white powder
PHYSICAL STATE	-	Powder
SOLUBILITY	-	Aqueous
AVAILABLE DOSAGE FORM	-	0.1 mg/ml Injection
CATEGORY	-	Antiarrhythmic drug
CDSCO APPROVAL DATE	-	October 6th 2016

GENERIC NAME	-	DOFETILIDE (TIKOSYN) [24,25]
CHEMICAL STRUCTURE	-	



MOLECULAR FORMULA	-	$C_{19}H_{27}N_3O_5S_2$
MOLECULAR WEIGHT	-	441.6
IUPAC NAME	-	Methanesulfonamide, *N*-[4-[2-[methyl[2-[4-[(methylsulfonyl) amino] phenoxy] ethyl]amino]ethyl]phenyl]
APPEARANCE	-	white to off-white powder
PHYSICAL STATE	-	Powder
SOLUBILITY	-	slightly soluble in water and propan-2-ol and is soluble in 0.1M aqueous sodium hydroxide, acetone, and aqueous 0.1M hydrochloric acid.
AVAILABLE DOSAGE FORM	-	0.125 mg - orange and white capsules 0.250 mg-peach capsules0.500 mg – peach and white capsules
CATEGORY	-	Antiarrhythmic drug

6. EXPERIMENTAL METHODS

6.1 APIXABAN

6.1.1 DEVELOPMENT OF VALIDATED UV SPECTROSCOPY METHOD FOR APIXABAN

6.1.1.1 Initial Experimental conditions

Selection of solvent

The drug was checked for the solubility in various aqueous and organic solvents. The solvent in which apixaban showing smooth spectra and completely soluble was selected for the UV method.

Selection of wavelength

The suitable wavelength for the UV analysis was determined by recording the spectrum of apixaban in the range of 200-400 nm. The wavelength at which the absorbance was found to be maximum was choosen for the study.

Preparation of solutions

The stock solution of 1 mg/ml of apixaban was prepared in methanol. The working stock solution was prepared in the concentration of 100mcg/ml with methanol. The further dilution of stock solution was done using methanol.

Fixation of instrumental parameters

To obtain maximum absorbance for the drug solution, the instrumental parameters were optimized by the selection in slit width, the spectral band pass and scanning speed.

6.1.1.2 Validation of UV spectroscopy method

Linearity and range

The serial solution of apixaban was prepared in the range of 2-20 mcg/ml. The individual spectrum of each solution was recorded in the range of 200-400 nm. A

calibration graph was obtained by plotting absorbance of standards versus concentration.The regression analysis of the calibration graph was evaluated by correlation coefficient, intercept and slope.

Precision

The repeatability of measurements was studied with six replicate solution prepared on the same day for the intraday precision. The interday precision was checked on three consecutive days. The average of the six measurements for the interday precision and the precentage relative standard deviations was calculated and presented in the table.

Accuracy

Accuracy for the method developed was performed by the standard addition technique. The standard drug apixaban was added to the tablet powder equivalent to 10 mg of apixaban at two different levels of 50% and 100%. Few ml of methanol was added and solution was kept in the sonicator for 5 minutes. Then the volume was made up to the volume 10 ml. Whatmann filter paper of 0.2 μ pore size was used to filter the solution. The absorbance of prepared working sample solution was measured against methanol as blank. The procedure was repeated in triplicate and the amount of standard recovered at each level was calculated.

Robustnessand Ruggedness

The robustness of the method was evaluated by changing the measurement wavelength 287 nm by ± 0.1 nm in the continuous mode of operation. The ruggedness of the method was evaluated by the simultaneous measurements of the absorbance by two different analysts under similar operating conditions on three different days.

Limit of detection and limit of Quantification

Amount of drug detectable and quantifiable were computed from the formula 3.3σ/S and 10σ/S. The σ indicates the standard deviation of the y-intercept and S denotes the slope of the curve.

Solution stability

A solution of 10mcg/ml of apixaban was prepared and it was checked periodically for the stability at room temperature and in the refrigerator upto four days. The change in absorbance of solution was noted.

Specificity of the method

In order to prove the specificity of the method an admixture consisted of tablet excipients was prepared and used. The tablet formulation of apixaban consists of the excipients such as lactose, sodium lauryl sulphate and glycerol triacetate. An admixture of excipients was prepared and dissolved in methanol. This solution was diluted and scanned between 200 to 400 nm. The spectrum was compared with standard apixaban spectrum to observe any interference due to the matrix and the solvent methanol.

Optical parameters for the method

The optical parameters such as molar absorptivity, Sandell's sensitivity and E(1%, 1cm) were calculated and tabulated.

6.1.1.3 Application of UV spectroscopy method for the analysis of formulation

Twenty tablets of apixaban eacg contained 2.5 mg were weighed individually and the average weight was calculated. They were pulverized and the weight equivalent to 10 mg of apixaban was transferred into a 10 ml volumetric flask. A few ml of methanol was added and sonicated for 5 minutes to dissolve the drug, then made upto the volume. The sample solution was filtered using whatmann filter paper to exclude the undissolved excipients and further diluted to a concentration of 10mcg/ml with the same. The spectrum was recorded for the six replicate samples prepared similarly. The absorbance at the selected wavelength was observed. The assay value was calculated for the six samples and depicted in the table.

6.1.2 DEVELOPMENT OF VALIDATED HPTLC METHOD FOR APIXABAN

6.1.2.1 Chromatographic conditions

Selection of stationary phase

The TLC plate made up of G_{60} F_{254}coated on aluminium support was selected for the analysis of apixaban. The size of silica gel particles was 2µ and thickness of sorbent layer was 0.2mm. The plates were supplied in 20 x 20cm size, which was cut into appropriate size for the method development and optimization.

Selection of mobile phase

The mobile phase system was chosen based on the solubility and polarity of the drug. The solution of the drug was prepared in methanol and used for spotting. Methanol gets vapourized by passing a stream of nitrogen gas. The solvent system made up of PET:THF:ACN, MeOH:ACN:PET and TLN:DCM:THF were used in different proportions to retain apixaban and the results are noted. After different trials of mobile phase system, an ideal system was fixed for the drug based on the peak shape and R_f value.

Optimization of variants in TLC

The composition of mobile phase, chamber saturation time (equilibration time), the distance of solvent development and bandwidth of the spots were thevariants that affect the R_f value, hence they were optimized as below,

Composition of solvents in mobile phase

The volume of each solvent constituting the mobile phase was varied in order to study the effects on the R_f value and peak shape. The suitable composition selected for the analysis was toluene: dichloromethane: tetrahydrofuran in the proportion of 2:2:6 v/v/v.

Chamber saturation time

The mobile phase was placed on one side of the twin trough chamber and shaken well. Different saturation times were maintained from 5 to 30 minutes. The plates were spotted with apixaban and developed to obtain the densitogram. The effect of chamber saturation time on peak shape, development pattern and R_f value were studied.The suitable saturation time was fixed for the method.

Distance of solvent front

The distance travelled by the solvent front affects the Rf value of the drug. The mobile phase was allowed to run on the plate to various distances from 7cm to 9 cm. The ideal R_f value was achieved with the distance of 8cm of the solvent front and hence fixed for the studies.

Band width

The samples were spotted as bands on the TLC plate. The size of the band width determines the compactness, R_f and peak area of the spot. For the ideal band width the samples were spotted on the plate having different sizes of 2 to 6 mm, which was kept 10 mm apart from each other. When the band width was 5mm the peak was compact with acceptable R_f value.

6.1.2.2 Validation of HPTLC method for Apixaban

Preparation of standard stock solution

A quantity of 10mg of apixaban was weighed accurately and transferred into a 100 ml volumetric flask. To this 25 ml methanol was added and sonicated for 5 minutes. The volume was made upto the mark with methanol.

Linearity and range

The sampling of spots were carried out with linear increase in the volume applied on the precoated plate from 2 µl to 20 µl corresponding to the concentration of 200ng/band to 2000 ng/band. A linear graph was plotted with peak area Vs concentration of apixaban.The peak area were noted and subjected to regression analysis.

Precision studies

Six replicate injections of 1000 ng/band were spotted on the plate to perform the intraday precision. The peak area was assessed for the standard deviation and relative standard deviation. Interday precision was determined by spotting the sample on three consecutive days with the 1000ng/band of drug. The SD and % RSD were tabulated.

System precision

The repeatability of injection was studied for the concentration of 1000 ng/band with six replicate injections. The repeatability of detection for the developed plate was checked by scanning the same plate of 1000 ng/band for six times. The data relevant to the values of peak area were calculated for SD and %RSD and presented.

Accuracy

The tablet powder equivalent to 10mg was weighed accurately and transferred to a 10ml volumetric flask. The standard drug of apixaban was added to the tablet powder at the levels of 50% and 100%. The accuracy of the method was determined by calculating percent recovery of the drug by standard addition method. Percent recovery of apixaban was determinedin triplicate at the two different levels. The results of accuracy study were calculated.

Robustness and ruggedness

The robustness was evaluated by deliberate variations in the saturation time by ± 2min, mobile phase ratio by ± 0.1ml for 2:2:6 (v/v/v) ratio of TLN:DCM:THF. The change observed in the peak area and the R_f value was noted. Analyses of apixaban by two analysts with the similar operating conditions were conducted.

LOD and LOQ

The LOD and LOQ of the method developed were acquired by applying 0.1µl to 1µl of standard drug solution of 100mcg/ml. The lowest limit that can be detected and quantified were identified from the responses of peak area were observed and noted.

Stability of developed plate

The volume spotted for the stability study of plate was 10µl of 100 mcg/ml of the working standard. The stability of plate was checked periodically upto 10 hours. The time at which a 10% reduction in the peak area occurred was noted and considered for stating the stability of the drug band on the plate.

System suitability parameters

The HPTLC method was studied for the suitability of various parameters in order to analyze the formulation. The tailing factor, asymmetric factor and efficiency were calculated and shown in the table.

6.1.2.3 Application of HPTLC method for theanalysis of formulation

Twenty apixaban tablets of strength 2.5 mg were weighed individually and the average weight was calculated. The weight equivalent to 10mg of apixaban was taken and transferred into a 25 ml volumetric flask. Methanol was added to make up the volume after which it was placed in the sonicator for 5 minutes for solubulizing the drug. The sample solution was filtered using whatmann filter paper to exclude the excipients present.

The aliquot was applied for six replicate times to obtain 1000 ng/band. The densitogram was evaluated for the R_f value and the peak area. From the response of the sample, the amount of drug present as per tablet and the label claim was calculated.

6.1.3 DEVELOPMENT OF VALIDATED HPLC METHOD FOR APIXABAN

6.1.3.1 Chromatographic conditions

Selection of wavelength

Freshly prepared apixaban stock solution was diluted appropriately in methanol to give a concentration of 10 mcg/ml. This solution was scanned in the UV region and the wavelength of maximum absorbance was identified at 287nm. The wavelength selected was applied for the detection of apixaban in HPLC method.

Selection of initial chromatographic conditions

The solvent system with composition of acetonitrile and water was employed for the trials and the chromatograms recorded were monitored for the peak area, retention time, tailing factor and asymmetrical factor. To improve the peak shape, methanol in different proportions with water were run and after column stabilization, the sample was injected. The ratio from 50:50 (H_2O:MeOH) was initially run with sequential changes in the methanol ratio and the ideal one was chosen for the optimization and procedure.

Optimization of the chromatographic conditions

The HPLC method was optimized with the mobile phase in the composition of methanol and water at selected wavelength, flow rate and injection volume.The chromatograms were inspected for the peak shape and chromatographic characters at 287nm. The mobile phase optimized was done to obtain symmetrical peaks with good characters. The optimized conditions were applied for the analysis of apixaban.

Preparation of mobile phase solvent system

The solvents were filtered through the 0.45µm membrane filter and the filtered solvent was transferred in the glass bottle with the respective pumps.

6.1.3.2 Validation of RP-HPLC Method for Apixaban

Preparation of standard stock solution

A quantity of 10 mg of drug was weighed accurately and transfer to 100 ml volumetric flask. About 10ml of methanol was added to it and sonicated for 5minutes. Then the solution was made up to get a concentration of 100 mcg/ml. This solution was used for the preparation of working standard solution using the same solvent.

Linearity and range

The aliquots of the stock solution were transferred into 10 ml volumetric flask and the volume was made up with methanol to give the concentration ranging from 2 to 20 mcg/ml. The solution was mixed well. The peak area and the retention time of individual concentration were noted. The standard curve with the line of best fit using linear regression analysis was applied for the correlation coefficient, slope of the curve and y intercept in the graph.

Precision studies

The intraday and interday precision studies were carried out with the selected drug concentration of 10mcg/ml as per the ICH guidelines. The chromatograms were evaluated for similarity of the response of peak area and retention time. The standard deviation and relative standard deviation for the peak area were calculated and tabulated.

Accuracy

The tablet powder quantity equivalent to 10 mg was weighed and to this 5 mg of standard drug apixaban was added for 50% accuracy studies. Methanol was added and sonicated for 5 minutes. The solution was filtered with whatmann filter paper. The mixture prepared was diluted with methanol and injected. The accuracy of the method was determined by calculating percent recovery of the drug by standard addition method. For 100% recovery studies, 10 mg equivalent powder was added to 10 mg of drug apixaban and dissolved in methanol, filtered and the sufficient dilutions were

performed and filtered in a 0.2µ syringe filter and placed in the autosampler. The samples prepared were injected and chromatograms were recorded. The results of accuracy was calculated from triplicate samples.

Robustness and Ruggedness

The robustness of the method was evaluated by the minor variations in the flow rate of ± 0.1ml, mobile phase ratio by ± 0.1ml and injection volume ± 0.1 µl . The ruggedness was investigated by different analyst under similar experimental conditions on the same day.

Limit of detection (LOD) and limit of Quantification (LOQ)

The determination of the limit of detection and limit of quantification for apixaban were executed from the linearity curve of the standard solution established between 2 to 20mcg/ml. The slope of the curve and standard deviation of a series of measurements were used for the calculation and the values are presented in the table.

Stability of solution

Apixaban solution was freshly prepared and stored at room temperature and refrigerator to study the stability. The samples in both the conditions were injected immediately for 0 hour sample. For the stability at room temperature the sample was tested at 0, 2, 4, 8, 12, 24 and 36 hours. Sample stored in the refrigerator at 8^{o}C was tested at 0,6,12, 24, 48, 72 and 84 hours. The time at which the response observed varied more than 10% was noted for the sample at room temperature and refrigerator.

System suitability parameters

The values for the parameters were compared with the standard acceptance criteria according to the USP specification and tabulated.

6.1.3.3 Application of RP-HPLC method for theanalysis of formulation

Twenty tablets of apixaban of strength 2.5 mg were weighed individually and the average weight was calculated. The weight equivalent to 10 mg of apixaban was

taken and transferred into a 10ml volumetric flask. The drug was extracted with methanol, filtered with whatmann filter paper and diluted to a concentration of 10mcg/ml with the same. The solution was injected to get chromatogram and the amount present in the formulation was calculated. The procedure was repeated for six times and the average calculated. The % label claim was found and presented in the table.

6.1.3.4 Application of RP-HPLC method for the degradation studies

An isocratic reverse phase high performance liquid chromatography method was developed for apixaban and validated. The method was explored to degradation studies of apixaban. The eluents were monitored for the drug peak and degradant peaks. The forced degradation conditions applied were the acid and base hydrolysis, oxidation, photolysis and thermolysis as per the ICH guidelines. The degradant peaks identified in the chromatogram were quantified for the level of degradation with a time interval of 4 hours till 8 hours. The % degradation was calculated.

Acid hydrolysis

The stock solution prepared freshly with methanol was diluted further to get a concentration of 100 mcg/ml with 0.1M HCl and 25 ml of the solution was placed on the thermostat at 30^{o}C. The zero hour sample was withdrawn and diluted to give a concentration of 10mcg/ml with methanol. The sample was filtered using 0.2µ sterile PTFE filter and injected. The changes in peak area were calculated for the % degradation. The study was conducted upto 8 hours.

Base hydrolysis

A 100 mcg/ml of solution was treated with the 0.1M NaOH and kept over the thermostat maintained at a temperature of 30^{o}C. The sample was collected at 0, 4 and 8hours and diluted with methanol to get a concentration of 10 mcg/ml. After filtration through the 0.2µ filter the solution was injected and analysed. The chromatogram were noted for the changes in the peak area and presented.

Oxidation

The standard solution of 100 mcg/ml was treated with 3% v/v hydrogen peroxide solution on a thermostat maintained at 30^{o}C. Further dilution of the stress sample was done with methanol to get a concentration of 10 mcg/ml and filtered and injected into the column and the peaks were eluted. The presence of additional peaks was also noted. Three samples at an interval of 4hours were studied.

Thermal degradation 40^{o}C and 80^{o}C

The drug solution of 100mcg/ml was prepared in methanol. The controlled temperature of 40^{o}C and 80^{o}C was maintained in separate thermostat. The sample from the flask was diluted to 10mcg/ml and the chromatogram was recorded. The change in peak area and the additional peaks eluted were analyzed for the level of degradation and values are tabulated.

Photolysis 220 V lamp

A lamp of 220 volts was chosen as the source of light and placed in a closed chamber for the photolytic degradation studies. The 100mcg/ml of standard solution was exposed to the light emitted from the selected source. The 0hour sample after dilution with methanol was filtered through a 0.2 µ PTFE filter and injected, followed by the 4hour and 8hour sample prepared similarly. The stability of the drug solution was noted for the peak area.

6.2 RIVAROXABAN

6.2.1 DEVELOPMENT OF VALIDATED HPTLC METHOD FOR RIVAROXABAN

6.2.1.1 Chromatographic conditions

Selection of stationary phase

The readymade plates G_{60} F_{254} used for the HPTLC method were made up of aluminium sheets coated with silica gel particles of 2 μ and the coating thickness of 0.2 mm. The standard dimension of the plate is 20 x 20 cm (length x breadth). Based on the requirements for the analysis, the plate was cut into 10 x 10 cm or 10 x 20 cm and used for the method.

Selection of mobile phase

The solution of rivaroxaban was prepared in 1:1.5 ratio of methanol:acetonitrile. Rivaroxaban is retained on plate by using combination of solvents in which it is sparingly soluble as well as in few solvents in which it is completely. The trials were conducted with MeOH:EA:H_2O and TLN:EA:MeOH. Finally the mobile phase made up of TLN:EA:MeOH (5:3:2 v/v/v) was selected since the Rf value, peak shape and peak area were found to be ideal. The wavelength for the method was 249 nm, it was selected by scanning in the UV region.

Optimization of variants in HPTLC

Selection of the suitable conditions for the method development is the primary necessary as it determines the R_f value, peak shape, area, asymmetric factor etc., The mobile phase system, saturation time of the chamber, the distance of solvent front and spot bandwidth are the variants to be optimized. The aforesaid variants were optimized with numerous trials to obtain the best results.

Composition of solvents in mobile phase

The composition of TLN:EA:MeOH was differentiated in the order of increasing ratio of toulene from 2 to 5 and the correct proportion was found to be 5:3:2 v/v/v. The selected composition was utilized for the analysis of rivaroxaban.

Chamber saturation time

The chambers required for the development of plate were utilized to study the chamber saturation time.The time were varied between 5 to 30 minutes and tested for the acceptable criterion of peak shape, Rf value and uniformity in the spot identified after development. A fixed ratio of mobile phase was transferred and kept aside without any disturbance. Plate spotted with the rivaroxaban standard drug was placed inside. The suitable saturation time that satisfy the peak characteristics was selected for the separation of rivaroxaban.

Distance of solvent front

The sample was spotted at 1 cm from the bottom of the plate as the origin line. The plate was placed in the chamber saturated with the selected mobile phase. The distance of the solvent front was tested at three different levels from 7 cm to 8.5 cm. The capillary action of the silica particles decreases as the solvent moves up against gravity. The physical properties of solvent used for mobile phase are one of the important cause for the change in the capillary action. The most suitable distance of solvent front was identified as 8 cm as the results of peak area, peak symmetry and Rf value were acceptable.

Band width

Automatic sampler was used for spotting the samples as bands from 2 mm to 6 mm. The band width that gave compact spots with constant R_f value were selected for the study.

Preparation of standard stock solution

A quantity of 10 mg rivaroxaban was weighed and transferred into a 10 ml volumetric flask. To this flask methanol was added and sonicated for 5 minutes. The volume was made upto the mark with methanol.

6.2.1.2 Validation of HPTLC method

Linearity and range

The stock solution of drug rivaroxaban was applied on the 10 x 20 cm plate by proportional increase in the volume applied between the range of 0.3 to 2.5µl of 400 mcg/ml of solution corresponding to 120 ng/band and 1000ng/band. The plate was scanned and the spots were detected at 249nm with an R_f value of 0.38± 0.02. The results of calibration data were subjected to regression analysis.

Precision studies

Intraday precision studies have been carried out by applying the sample six times and interday precision was performed by analyzing the reproducibility on three days. The peak area was assessed for the standard deviation and relative standard deviation.

System precision

System precision of the method developed was evaluated by six replicate injection of the standard solution of 600ng/band. Reading the densitogram for six consecutive scans of standard sample of 600ng/band was executed for the system precision studies. The data collected were summarized and tabulated.

Accuracy

Tablet powder equivalent to 10 mg of rivaroxaban was accurately weighed and transferred to a 10 ml volumetric flask. To the tablet powder the standard drug of 5 mg rivaroxaban was added for 50% recovery study. For the 100% recovery studies, a 10 mg of standard drug was added to 10 mg equivalent powder. The accuracy of the method was determined by calculating percentage recovery of the drug by standard addition method. The amount of standard drug recovered was determined in triplicate at the two different levels. The results of accuracy study are noted.

Robustness and ruggedness

Sample solutions were applied on the plate in the concentration of 600ng/band. The plates were subjected to the minor changes of experimental parameters of chamber saturation time and mobile phase ratio to study the robustness. The deliberate variations in the saturation time by ± 2 min, mobile phase ratio by ± 0.1 and 0.2ml for 5:3:2 ratio of TLN:EA:MeOH (v/v/v) were adopted. The peak area and the Rf value were observed for any change and noted.

LOD and LOQ

The standard stock solution was diluted with the same ratio of solvent to obtain a solution of 40mcg/ml. From this 0.9µl, 0.7µl and 0.5µl and 0.2µl were applied in triplicate for the LOD and LOQ.

Stability of the developed plate

The volume spotted for the stability study was 1.5 µl of 400 mcg/ml of the working standard solution of rivaroxaban. The plates were scanned periodically till 8 hours.

System suitability parameters

The tailing factor, asymmetric factor and efficiency were computed with the results of the peak area of the standard drug and shown in the table.

6.2.1.3 Application for the analysis of formulation

Twenty tablets of rivaroxaban in the dosage of 10 mg were weighed individually and the average weight was calculated. Using a mortar the tablets were crushed into a homogeneous powder and mixed well. The weight of tablet powder equivalent to 10 mg of rivaroxaban was taken and transferred into a volumetric flask. Then MeOH and acetonitrile (1:1.5 v/v) was added and sonicated. Then solution was made upto 25ml with same solvents. The solution was filtered and 1.5 µl was spotted six times. After the chromatographic development the peak areas were noted and labelled amount was calculated.

6.2.2 DEVELOPMENT OF VALIDATED RP-HPLC METHOD FOR RIVAROXABAN

6.2.2.1 Chromatographic conditions

Selection of wavelength

A quantity of 10 mg of rivaroxaban was weighed into a standard flask. To this a minimum volume of DMSO was added and shaken well to dissolve the drug. The volume was made up with acetonitrile. The final dilutions were performed to get a solution of 10 mcg/ml using 10 mM orthophosphoric acid as the diluent. The solution was scanned between 200-400 nm and the λmax identified as 249 nm. The measurements were performed at the selected wavelength for the optimization and validation of the method.

Selection of initial chromatographic conditions and its optimization

The detection wavelength was 249 nm which was chosen from the UV spectrum of rivaroxaban. The stock solution of rivaroxaban was prepared using DMSO and acetonitrile. The suitable mobile phase for the analysis of rivaroxaban was based on the solubility. The trial solvents as methanol, acetonitrile and water were placed in three individual pumps. Various ratios of mobile phase in the pump were run and the chromatograms were checked for the responses like retention time, peak shape, tailing factor and peak symmetry. The mobile phase composition was selected based on the retention, peak area and peak characters.

Preparation of standard stock solution

Based on the solubility of the drug, 10mg of rivaroxaban was weighed and dissolved in little quantity of DMSO and volume was made upto the mark with acetonitrile in a 10ml volumetric flask. The further dilution was done with the solvent acetonitrile. The working standard solution was prepared using 10mM ortho phosphoric acid.

Preparation of mobile phase solvent system

Organic solvents methanol and acetonitrile was used for the study in combination with water. These solvents for the HPLC method were filtered through the 0.45µm membrane filter using the suitable filter to remove the particles and fibers present.

Preparation of 10 mM Ortho Phosphoric acid

An accurately measured volume of 0.68ml of Ortho Phosphoric acid was added to 1000ml of previously filtered HPLC water and stored in a tightly closed container.

6.2.2.2 Validation of RP-HPLC Method for Rivaroxaban

Linearity and range

Using acetonitrile as the solvent, a 100 mcg/ml of working standard solution was prepared from the stock solution. Serial dilution of the standard solution in the range of 5 to 30mcg/ml were prepared with 10mM OPA. The samples were injected and chromatograms recorded. The results of the calibration graph were analyzed from the distribution of the data between the x-y axis and by the regression equation.

Precision studies

The intraday and interday precision studies were carried out with concentration of 10mcg/ml as per the guidelines. The chromatogram was evaluated for similarity of the response of peak area and retention time. The standard deviation and relative standard deviation were calculated and tabulated.

Accuracy

Tablet powder of rivaroxaban equivalent to 10mg was weighed. To this 5mg of standard drug rivaroxaban was added for 50% recovery studies. A few ml of DMSO was added to the mixture and sonicated for 5 minutes. The volume was made up with acetonitrile, shaken well and filtered through whatmann filter paper. This solution was diluted further to a concentration of 15mcg/ml and the final dilution was carried out using 10mM Orthophosphoric acid. The sample was filtered with a 0.2µ syringe filter

and injected in the column. The procedure was repeated for the mixture of 10mg equivalent tablet powder with 10 mg of standard drug for the 100% recovery studies. The amount of standard added to each level was calculated for the accuracy of standard rivaroxaban added of the target concentration in triplicate. The results of accuracy study are average of three values and they are depicted in the table.

Robustness and Ruggedness

The sample of standard solution was prepared by diluting stock solution of 1mg/ml with acetonitrile to get a working standard solution of 0.1mg/ml and used for the study. The slight modifications in the experimental parameters were observed for flow rate by ± 0.1 ml, mobile phase ratio ± 0.1 ml and injection volume ± 0.1µl. The ruggedness was investigated with subsequent analysis of standard solution by different analyst under similar experimental conditions on the three different days.

Limit of Detection and limit of quantification

LOD and LOQ for rivaroxaban were determined from the calibration data. The formula applied was 3.3σ/S and 10σ/S where σ represents the standard deviation of the standard response and S is the slope of the selected range of linearity and the values were calculated.

Stability of solution

Working standard solution of rivaroxaban was prepared using 10 mM OPA to attain a concentration of 10mcg/ml. The solution was divided into two portions and one half was kept at the room temperature whereas the other portion was stored in the fridge at 8^{o}C. The solution was filtered in 0.2µ and chromatogram recorded for the 0 hour study. The solution was checked for the stability at room temperature at regular intervals and in the refrigerator for every fixed time period in hours. The changes in peak area in the respective chromatogram were noted. The time at which the change in response more than 10% were observed and tabulated.

System suitability parameters

The system suitability was evaluated for the asymmetric factor, tailing factor at 10% height of peak and presented in the table.

6.2.2.3 Application for the analysis of formulation

Twenty tablets of rivaroxaban in the dosage of 10mg were weighed individually and the average weight was calculated. Using a mortar the tablets were crushed into a homogeneous powder and mixed well. The weight of tablet powder equivalent to 10 mg of rivaroxaban was taken and transferred into a volumetric flask. Then the solvents DMSO and acetonitrile were added. The drug was solubilized by sonicating for 5 minutes. Then the volume was made up using acetonitrile and shaken well. This solution was filtered using a 2µ PTFE filter after which it was diluted sufficiently to obtain a concentration of 10mcg/ml with 10mM OPA as the final diluent.

6.2.2.4 Application for degradation studies of rivaroxaban

To study the degradation of rivaroxaban, it was treated under various stress condition of acid hydrolysis, base hydrolysis, oxidation, elevated temperature and light. Each treated sample of acid, base and oxidation was placed at a controlled temperature of 30^{o}C with the flask connected to the reflux condenser. The zero hour sample was withdrawn and volume made up with 10 mM OPA to get a final working concentration of 10 mcg/ml. The solution was filtered through 0.2 µ syringe nylon filter. The steps of sampling were repeated for every one hour till four hours. The chromatographic conditions developed and validated for the analysis of Rivaroxaban was adopted for degradation studies.

Acid hydrolysis (0.1 M HCl)

Rivaroxaban stock solution was prepared by dissolving 10 mg in a few ml of DMSO and diluted upto 10 ml with acetonitrile. Then 2.5 ml of the solution was made upto 25 ml with 0.1 M HCl(100 mcg/ml), thereafter placed over the thermostat maintained at 30^{o}C. The zero hour sample was withdrawn and diluted to give a concentration of 10 mcg/ml with 10 mM Orthophosphoric acid. The sample was filtered using 0.2 µ sterile PTFE filter. The sample was injected and the changes in peak area were calculated for the % degradation.

Base hydrolysis (0.1 M NaOH)

A 100mcg/ml of working standard solution of rivaroxaban was prepared with the 0.1 M NaOH and the study was conducted is mentioned earlier. The sample was collected every hour till 4 hours and diluted with 10 mM Orthophosphoric acid to a concentration of 10mcg/ml. The solution was filtered and injected. The chromatograms were noted for the peak area.

Oxidation (3 % v/v H_2O_2)

A volume of 2.5 ml of the stock solution was made upto 25 ml with 3% v/v of hydrogen peroxide. Further dilution of the stress sample was done with 10 mM Orthophosphoric acid to a concentration of 10mcg/ml and filtered. After injecting the samples in chromatographic system the presence of additional peaks were noted.

Thermal degradation (80^{o}C)

The drug solution of 100mcg/ml was prepared in water. The controlled temperature of 80^{o}C was maintained in separate thermostat for the solution. The sample from the flask was diluted to 10mcg/ml with 10 mM Orthophosphoric acid and the chromatogram recorded. Retention of the drug and the degradants formed were analyzed for the level of degradation and values are tabulated.

Photolysis (220 V lamp)

A lamp of 220 volts enclosed in a chamber was used for the photolysis of rivaroxaban .The 100mcg/ml of standard solution prepared from the stock solution was exposed to the light emitted from the selected source. Sample was withdrawn at 0hr and diluted with 10mM Orthophosphoric acid and filtered through 2µ PTFE filter and analysed. Changes in the responses were observed and shown in the table.

6.2.2.5 Application of developed RP-HPLC method for the impurity profiling of rivaroxaban

Separation of process impurity of rivaroxaban

The process impurity of rivaroxaban used in the current study is 5-chloro-N-({5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl)phenyl-1,3-oxazolidin-5yl}methyl)-2-thiophenecarboxamide. It is soluble in DMSO. A stock solution of 100 µg/ml was prepared in DMSO and further dilutions were carried out using OPA. The concentration of rivaroxaban used of 100 mcg/ml concentration

The chromatograms were recorded using optimized conditions. The RP-HPLC method consisting of water, acetonitrile and methanol in the ratio of 45:10:45 %v/v/v with the flow rate of 1ml/min was used for the separation of process impurities of rivaroxaban from the API rivaroxaban. The detection wavelength was 249 nm.

Identification of impurities/degradants by LC-MS/MS technique

Preparation of drug/process impurity solution for LC-MS/MS analysis

A 100 µg of rivaroxaban was prepared and subjected to acid hydrolysis, base hydrolysis, oxidation, thermal degradation and photolysis upto a period of 4 hours. The 0 hour and 4 hours sample were analysed by LC-MS/MS to obtain a mass spectra.

The additional peaks obtained in all the degradation studies were monitored by Q_1 and Q_3 scanning to study the possible m/z of degradants. They were also observed for the process impurities at m/z of 291. The mass spectrawas observed for the peaks at the selective RT of degradants.

The LC-MS/MS method was performed using the batch processing of samples in the specifiedOrder in the Multiple Reaction Monitoring Mode with the Q1 and Q3 tuning of the scanning mode of TIC(Total Ion Concentration) for the pure drug and the selected impurity. The intensity of the peaks were reported in cps (cycles per second) at a collisional energy of 60V with polarity positive. The set point source temperature was 500^{o}C.

The turbospray mode operated for the mass spectrometer produced numerous molecular ions in each selected stress condition. The selection of the molecular peaks was scrutinized by identification of the impurity by the clear separation in the LC

system. The MRM mode operation brings out the abundance of the impurity corresponding to the selected m/z of impurity and degradants.

6.3 IBUTILIDE FUMARATE

6.3.1 DEVELOPMENT OF UV SPECTROSCOPY METHOD FOR IBUTILIDEFUMARATE

6.3.1.1 Initial experimental conditions

Selection of solvent

Various organic solvents like methanol, DMSO, acetonitrile, chloroform, dichloromethane and methanol were taken individually and solubility of Ibutilide fumarate was checked for complete solubility. Methanol was selected as ibutilide fumarate was completely soluble and smooth spectra was obtained.

Selection of wavelength

The drug solution of ibutilide fumarate prepared in methanol was scanned at the UV region of 200-400nm. The wavelength selected for the studies was 228 nm as the acceptable absorbance was found.

Preparation of solutions

The accurately weighed quantity of 10 mg of ibutilidefumarate was transferred into a 10ml volumetric flask. A few ml of methanol was added and shaken well to dissolve the drug. The volume was made up to give a concentration of 1mg/ml. Further dilutions of the sample were performed with the same solvent.

Fixation of experimental parameters

Methanol was the suitable solvent selected for the analysis of Ibutilide fumarate as the drug was completely soluble in it. The blank used was methanol. The wavelength of maximum absorbance was observed at 228 nm for the methanolic drug solution. The scanning speed selected was 400 nm/minute in the continuous mode operation.

6.3.1.2 Validation of UV spectroscopy method

Linearity and range

The stock solution of 1mg/ml was diluted suitably to study the linear range with methanol as the solvent. This working standard solution was used to prepare the concentration range of drug solution between 2-16 mcg/ml. Individual solution were scanned in the range of 200-400 nm and the spectrum recorded. The values were calibrated and subjected to regression analysis.

Precision

The methanol was studied for the repeatability of values and reproducibility of values by the intraday precision and interday precision. The intraday (on the same day) and interday (on three successive days) precision was carried out on six different determinations.

Robustness and ruggedness

The robustness was determined by variation in the selected wavelength of study at which maximum absorbance was found for the drug. The λ_{max} was observed at 228 nm. The absorbance was noted at ± 0.1 nm of the selected wavelength. The experiment was performed by two analyst under similar conditions and the responses are recorded and noted.

Limit of detection and limit of Quantification

The formula 3.3 σ/S and 10σ/S was used to the calculate the detectable limit and quantification limit from the calibration curve. The σ represents standard deviation and S is the slope of the curve

Solution stability

The absorbance of the drug solution in methanol was measured at room temperature and in the refrigerator. At room temperature, every 1 hour reading was noted till 5 hours. In the refrigerator the solution was checked for stability at regular interval of 6hours. The results obtained were analysed for change in response and tabulated.

Specificity of the method

The interference of the solvent used for the solution preparation was studied by recording the spectrum of methanol against water as blank. The overlay spectrum of methanol blank and the drug solution of ibutilide fumarate in methanol are presented in the figures.

Optical parameters for the method

The optical parameters evaluated for the UV spectroscopy method for ibutilide fumarate was molar absorptivity, sandell's sensitivity and E(1%, 1 cm).

6.3.1.3 Application to Injection drug solution

A solution of ibutilide fumarate was prepared by dissolving 0.1mg of drug, 0.189mg of sodium acetate and 8.9g of sodium chloride. The pH of solution was adjusted to 4.6 with sodium hydroxide and used for the analysis. A suitable value from the above was diluted with methanol ad UV spectrum was recorded from which amount found was calculated.

6.3.2 DEVELOPMENT OF VALIDATED HPTLC METHOD FOR IBUTILIDE FUMARATE

6.3.2.1 Chromatographic conditions

Selection of stationary phase

Precoated aluminium plate G_{60} F_{254} with 2µ particle size of silica gel and thickness of 0.2mm layer was used for the HPTLC analysis of ibutilide fumarate. The size of the plate was 20 x 20 cm, which was cut into appropriate size for the method establishment and validation.

Selection of mobile phase

The solution of the drug were prepared in methanol and used for spotting. A stream of nitrogen gas is used to evaporate the solvent methanol. Different proportions of ACN:EA: MeOH in increasing ratio of acetonitrile were tried but the peak of the drug had fronting at appreciable R_f value. So the isopropyl alcohol was included in the mobile phase instead of acetonitrile in the composition of MeOH:EA:IPA. The increase in the ratio of methanol shifted the R_f to higher value and resulted in good resolution between ibutilide fumarate and fumaric acid. The selected system resulted in proper peak shape, R_f value and complete resolution between the drug ibutilide and fumaric acid. The mobile phase with MeOH:EA:IPA in the composition of 8:1:1 v/v/v was selected for the study. The plate was scanned at 228 nm after the development.

Optimization of variants in TLC

Chromatographic variants of the method like composition of mobile phase, chamber saturation time (equilibration time), the distance of solvent development and bandwidth of the spots were fixed after selecting the appropriately suitable condition.

Composition of solvents in mobile phase

The solvent selected for the mobile phase was tested in different ratio to obtain the acceptable R_f value and resolution between ibutilide peak and the fumaric acid peak. It was observed that the ratio of methanol affects the retention of ibutilide peak and the

fumaric acid peak. The composition of methanol was varied and the densitograms were observed. The suitable composition of MeOH:EA:IPA selected for the analysis was 8:1:1v/v/v which gave good resolution between the two peaks and acceptable densitometric criteria.

Chamber saturation time

The chamber saturation time has an accountable affect on the R_f value, peak symmetry and spot development. The time in minutes from 5 to 30min were modified for the saturation of the selected mobile phase placed inside the chamber. The optimum chamber saturation time was found to be 20 minutes

Distance of solvent front

The Rf value of the drug is influenced by the distance travelled between the application line and the solvent front. The mobile phase was allowed to run on the plate to various distances from 7cm to 9cm. The ideal R_f value was achieved with the distance of 8cm of the solvent front and hence fixed for the studies.

Band width

The band width for the sample application is of concern for the proper peak shape, area and uniform spot development. For the selection of suitable band width the samples were spotted on the plate having different sizes of 1 to 6mm, which were separated by 10 mm apart from each other. When the band width was 5mm the peak were compact with acceptable R_f value and appeared symmetric.

Preparation of standard stock solution

10 mg of ibutilide fumarate was weighed and transferred into a 10ml volumetric flask. To this flask methanol was added and sonicated for 5minutes. The volume was made upto the mark with methanol. Then the solution was diluted with the same for the working concentration of 100mcg/ml.

6.3.2.2 Validation of HPTLC method

Linearity and range

Spots application of pure drug Ibutilide fumarate was performed by proportional increase in the volume applied between the range of 1 to 9µl. The concentration of drug was in the range of 1000 to 9000ng/band. The responses observed after scanning the plate were subjected to regression analysis. The peak area was noted.

Precision studies

Intraday precision studies have been carried out by applying the sample six times repeatedly and interday precision was performed by analyzing the reproducibility on three days. The peak area was assessed for the standard deviation and relative standard deviation and represented in the table.

System precision

Plate spotted with the standard drug and developed under the fixed chromatographic conditions was scanned for six times and the peak area were noted for a concentration of 3000ng/band. The same concentration was applied for six replicate times simultaneously and the peak area noted. The SD and %RSD was calculated for the values obtained and presented in the table.

Robustness and ruggedness

The robustness was evaluated by deliberate variations in the saturation time by ± 2min, mobile phase ratio by ± 0.1 ml for 8:1:1 (v/v/v) ratio of MeOH:EA:IPA. Any observable change in the peak area and the R_f value were noted with reference to a value of 10% as the acceptable criteria.

LOD and LOQ

The detectable limit and quantifiable limit for the method of ibutilide fumarate were determined by applying sample bands with the volume from 0.1 to 0.7µl of the standard solution (1000 mcg/ml).The concentration in which ibutilide was detectable and quantifiable were noted from the peak area obtained.

Stability of developed plate

The volume spotted for the stability studies was 3µl of 1000mcg/ml of the working standard. The plate was scanned periodically to analyze the changes in the peak area up to 5hours. The effect on the R_f value and peak shape was also evaluated. Time at which the peak area reduction with more than 10% was noted.

System suitability parameters

The HPTLC method was studied for the suitability of various parameters for the analysis of formulation. The tailing factor, asymmetric factor and efficiency were calculated and shown in the table.

6.3.2.3 Application to Injection drug solution

Ibutilide fumarate drug solution was prepared by adding 0.1mg of drug, 0.189mg of sodium acetate and 8.9g of sodium chloride in HPLC water. The pH of solution was adjusted with sodium hydroxide to 4.6.The solution was diluted, filtered and spotted as bands on the TLC plate. Then the chromatogram was studied for the amount present and % label claim calculated.

6.3.3 DEVELOPMENT OF VALIDATED HPLC METHOD FOR IBUTILIDE FUMARATE

6.3.3.1 Chromatographic conditions

Selection of wavelength

Ibutilide fumarate stock solution was prepared using methanol and diluted appropriately with the same to give a concentration of 10 mcg/ml. This solution was scanned in the UV region and the wavelength of maximum absorbance i.e., λ_{max} was observed. The identified λ_{max} of 228 nm was used for the detection of ibutilide fumarate by the RP- HPLC method.

Selection of Initial Chromatographic Condition

The method for the analysis of Ibutilide fumarate was carried out with trials using two solvent system. The water and methanol in the composition of 50:50 v/v was initially tried with variation in the flow rate of 0.8, 0.9 and 1.0 ml/min. The injection volume was varied was 10 µl and 15 µl. After considering the chromatographic parameters like peak resolution and tailing factor the ratio tried was 60:50 v/v with injection vol. of 10, 15 and 20µl. As the resolution was acceptable but gave split peaks, the solvent system was not considered.

Optimization of Chromatographic condition

The peak shape, peak area, resolution between fumaric acid and Ibutilide peak was taken into account. The mobile phase composition of water and methanol (55:45 v/v) with a flow rate of 0.9 ml and an injection volume of 20µl was selected for performing the work. The peak area were measured at 228 nm where Ibutilide fumarate was found to give maximum absorbance in the UV spectrum.

Preparation of standard stock solution

The primary stock solution of Ibutilide fumarate was prepared by adding an accurately weighed samples of 10 mg in an 10 ml volumetric flask and adding a few ml of methanol. The flask was shaken well to dissolve the drug and volume made up with methanol. The working standard solution of 100mcg/ml was prepared with methanol by further diluting the stock solution.

Preparation of mobile phase solvent system

The solvents used for the HPLC method for the analysis of Ibutilide fumarate was filtered through 0.45 μm membrane filter. The solvent was transferred to the respective bottle holding the mobile phase pump.

6.3.3.2 Validation of RP-HPLC Method for ibutilide fumarate

Linearity and range

The stock solution of Ibutilide fumarate was diluted in sequential manner for the preparation of linearity graph. The linearity of the method was established across 8 different concentrations starting from 2 μg/ml to 16 μg/ml. The solutions prepared were mixed well and filtered using a 0.2 μ syringe filter. The responses obtained for these chromatograms has been plotted and subjected to the calculation of correlation co-efficient, slope and y-intercept of the regression equation.

Precision studies

Standard solution suitably diluted in methanol to provide a solution of 10 mcg/ml was prepared and filtered in a 0.2 μ syringe filter. The intraday and interday precision studies were carried out with the selected drug concentration as per the ICH guidelines. The chromatograms were evaluated for similarity of the response of peak area and retention time. Six replicate injections were run for the intraday precision studies. For the interday precision study, the method of sampling was performed on three consecutive days. The standard deviation and relative standard deviation for the peak area were calculated and tabulated.

Robustness and Ruggedness

Ibutilide fumarate stock solution prepared with methanol was diluted to working standard solution of 10 mcg/ml. The solution was filtered in a 0.2 μ syringe filter and injected individually for each variation adopted. The robustness of the method was evaluated by the minor variations in the flow rate of ± 0.1 ml, mobile phase ratio by ± 0.1 ml and injection volume ± 0.1 μl . The ruggedness was investigated by different analyst under similar experimental conditions on three consecutive days.

Limit of detection and limit of Quantification

The determination of the limit of detection and limit of quantification for ibutilide fumarate were executed from the calibration data based on the signal to noise ratio.. The slope of the curve and standard deviation of a series of measurements were used for the calculation and the values are presented in the table.

Stability of solution

Ibutilide fumarate solution was freshly prepared by weighing 10 mg in a requisite volume of methanol. The drug was dissolved in methanol and then volume made up with the same solvent. Drug solution in the concentration of 10 mcg/ml was filtered through 0.2 µ PTFE filter. The sample solution prepared were stored at room temperature and refrigerator. The sample in both the conditions were injected immediately for 0 hour sample and the run time was 7 minutes. For the stability at room temperature the sample was tested at 0, 2, 4, 8, 12, 24 and 36 hours. Sample stored in the refrigerator at 8^{o}C was tested at 0,6,12, 24, 48, 72 and 84 hours. The time at which the response observed varied more than 10% was noted for the sample at room temperature and refrigerator to determine the stability of the drug.

System suitability parameters

The system suitability was evaluated for the asymmetric factor, tailing factor at 10% height of peak, resolution between ibutilide and fumaric acid and number of theoretical plates. The values for the parameters were compared with the standard acceptance criteria and tabulated.

6.3.3.3 Application to injection drug solution

Drug solution of ibutilide fumarate was prepared in the composition as mentioned in the earlier sections. The solution was diluted sufficiently and filtered. The chromatogram was recorded and the values calculated for the amount present.

6.3.3.4 Application of RP-HPLC method for the degradation studies

RP-HPLC method developed and validated according to guidelines was adopted for the degradation studies of ibutilide fumarate drug substance. Chromatograms recorded at 228 nm were studied for the drug peak and degradant peaks. The parameters observed were retention time, resolution and tailing factor. Ibutilide fumarate was subjected to the acid and base hydrolysis, oxidation, photolysis and thermolysis. The % degradation was calculated and tabulated for each stress condition.

Acid hydrolysis

A concentration of 100 mcg/ml of ibutilide fumarate was prepared with 0.1 M HCl and 25 ml of the solution was placed on the thermostat at 30^{o}C. The zero hour sample was withdrawn and diluted to give a concentration of 10 mcg/ml with methanol. The study was conducted for every 30minutes upto 4 hours. The sample was filtered using 0.2 µ sterile PTFE filter, injected in the column using the fixed chromatographic conditions. The change in peak area were observed to find out the % degradation.

Base hydrolysis

Working standard of 100 mcg/ml of the drug ibutilide fumarate solution was treated with the 0.1 M NaOH, maintained at a temperature of 30^{o}C using a thermostat. The sample was collected at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4 hours and diluted with methanol to obtain the concentration of 10mcg/ml. The syringe filter with a pore size of 0.2 µ was used to get a clear solution, after which the sample was injected. The chromatogram were noted for the peak area, analysed for the level of degradation and tabulated.

Oxidation

A solution of 3% v/v hydrogen peroxide was used in oxidative degradation study of ibutilide fumarate. The 100 mcg/ml in 3% v/v hydrogen peroxide of the oxidising mixture was placed on a thermostat maintained at 30^{o}C. Further dilution of the stress sample was done with methanol to a concentration of 10mcg/ml and filtered in a 0.2 µ filter. The collected clear solution was injected in the column and the peaks eluted were noted for the area. The presence of additional peaks were also noted.

Thermal degradation 40^{o}C and 60^{o}C

The working standard 100 mcg/ml of drug solution was prepared using methanol. The controlled temperature of 40^{o}C and 60^{o}C were cautiously maintained in separate thermostat with the solution. The sample from the flask was diluted to get a final solution of 10mcg/ml, filtered and the chromatogram recorded. The peak area and the additional peaks eluted were studied for the change. The results were analyzed for the % of degradation and values are summarized from 0 hour till 4 hours.

Photolysis

A closed chamber with a 220 watts lamp was used for the photolysis of ibutilide fumarate. Standard solution 100 mcg/ml of drug was exposed to the light emitted from the selected source. The 0 hour sample was withdrawn and diluted to 10 ml with methanol, filtered in a 0.2 µ nylon syringe filter. The sample after every half an hour were analysed for a period of 4 hours. The degree of degradation was studied and values shown in table.

6.3.3.5 Application of RP-HPLC for *in-vitro* drug-drug interaction studies of ibutilide fumarate with selected cardiovascular drug

In vitro drug-drug interaction study of ibutilide fumarate was carried out with selected anti hypertensive drug verapamil. A RP-HPLC method was developed to give acceptable resolution between the drug ibutilide fumarate and verapamil with acceptable chromatographic characteristics and adopted for the *in vitro* drug-drug interaction study.

The diluted solutions of two drugs were scanned in UV region to record the spectra individually. A suitable wavelength for interaction study was selected from overlay spectra of two drugs.

Preparation of buffer pH 7.4[(26)]

The buffer was prepared by weighing accurately 0.6g of potassium dihydrogen ortho-phosphate, 6.4g of disodium hydrogen orthophosphate and 5.85g of sodium chloride in 1000ml of HPLC grade water. Final adjustment to the required pH was carried out with a solution of 0.1M HCl.

Preparation of enzyme solution[(27)]

The rat liver microsome stored at -70^{o}Cwas placed in a thermostat maintained at 37^{o}C to thaw. The stock enzyme procured consists 20mg/ml of protein concentration. From this 0.05ml of enzyme solution was diluted to 5 ml with buffer pH 7.4 to give a concentration of 0.2mg/ml of protein by placing on an ice bath.

Preparation of solvent system for HPLC

Aqueous and organic solventsused for the work such as methanol, water and acetonitrile were filtered through the membrane filter of pore size 0.45µ. The solvents were placed in the respective pumps and used for the analysis.

Initial chromatographic conditions

Initial conditions include the selection of the mobile phase system to obtain acceptable resolution between ibutilide and the interacting drug, verapamil. Various ratio of the mobile phase ratio using methanol, water and acetonitrile were carried out. The chromatographic parameters of concern were good peak shape, acceptable resolution and symmetry of the drug peak to select mobile phase ratio.

Optimization of the chromatographic conditions

The mobile phase composition ofmethanol: acetonitrile: water in the ratio of 50:25:25 v/v/v was found to give acceptable peak characteristics and resolution between the drug peaks. The analysis was carried out with a flow rate of the solvent system was 1ml/min. The validation and system suitability parameters are tabulated.

***In-vitro* drug – drug interaction studies**

Study in simulated Conditions

Ibutilide fumarate (10mg) and verapamil (10mg) were weighed and transfered into a 10ml volumetric flask. The stock solution of the mixture was prepared using methanol. The mixture solution was further diluted to give a concentration 100mcg/ml of each drug. From this solution 2.5ml was pipetted out and diluted to 25ml using buffer pH 7.4, mixed well and placed over a thermostat at a constant temperature of 37^{o}C (10mcg/ml of each drug). The flask was swirled well to mix the drug solution and buffer. From this reaction mixture, a volume of 2ml was withdrawn for the 0hour sample and diluted 10times with methanol. The diluted solution was filtered through 0.2µm PTFE sterile filter and injected in the column. Thesampling was repeated for

every half an hour till 4hours.The blank buffer treated similarly was injected to observe for any interference at the RT of two drugs. Change in the peak area of the drug ibutilide fumarate and verapamil and also any additional peaks elutedwere noted at each time interval till the end of the study.

***Invitro*studies of Ibutilide with rat liver microsomes**

Ibutilide fumarate/ Verapamil with Enzyme

A2.5ml of 100mcg/ml of ibutilide fumarate/Verapamil drug solution was kept individually in a round bottom flask on a thermostat. The diluted enzyme solution (0.2mg/ml protein) was added to the drug solution in the flask. Volume was made upto 25ml with the buffer. The reaction mixture was shaken well and the sampling was done at every half an hour from zero hour. They were diluted with methanol and filtered through a 2µm syringe filter and injected in the system. The prominent change in the peak area of ibutilide fumarate/verapamil was tabulated at regular intervals and at end of study amount of ibutilide remained was calculated.

Ibutilide fumarate and Verapamil with Enzyme

The 2.5ml of the solution containing the mixture ofIbutilide fumarate and Verapamil in the concentration of 100mcg/ml of each was transferred into the flask kept over a thermostat maintained at 37^{o}C. A volume of 5ml enzyme solution in the concentration of 0.02 mg/ml of protein was added to the mixture immediately and volume was made upto 25ml with the buffer solution (10 mcg/ml of each drug). After mixing the reaction bath, the 0 hour sampling was withdrawn and diluted with methanol to give a concentration of 2 mcg/ml of each drug. The sampling procedure was repeated for every 30 minutes. The sample was filtered and the chromatogram was recorded. The peak area of the drug and any additional peak formed were noted.

6.4 DOFETILIDE

6.4.1 DEVELOPMENT OF VALIDATED UV SPECTROSCOPY METHOD FOR DOFETILIDE

6.4.1.1 Experimental conditions

Selection of solvent

The solubility of dofetilide in various aqueous solvents was checked. The solvent in which it is completely soluble and give smooth spectrum was chosen for the study. Methanol was selected as solvent for Dofetilide.

Selection of wavelength

Analysis of dofetilide by UV spectroscopy was determined by recording the spectrum in the range of 200-400 nm. The methanol solution was used for the study.A wavelength 231 nm was chosen as the absorbance was found maximum.

Preparation of solution

The stock solution of 1 mg/ml of dofetilide was prepared in methanol. The stock solution was further diluted for the analysis using methanol.

Fixed experimental parameters

The ultra violet spectra of dofetilide in methanol was sufficiently smooth, showna λmax of 231nm. The instrumental parameters like bandwidth 0.5mm, scan speed 400nm/min were found optimum for the of dofetilide.

6.4.1.2 Validation of UV spectroscopy method

Linearity and range

The solutions of dofetilide in the range of 2-18 mcg/ml were prepared in methanol. The solution was scanned and the spectrum recorded between 200-400 nm. The calibration graph was evaluated for correlation coefficient, intercept and slope and the values are tabulated.

Precision

The solution of 10 mcg/ml concentration was freshly prepared for the precision studies. The intraday precision was analyzed by repeating the measurements for six replicate times on the same day and interday precision was checked on three consecutive days. The absorbance values was subjected to statistical treatment of SD and % RSD. The results are tabulated.

Accuracy

Accuracy studies are performed bythe standard addition method by adding known amount of the standard dofetilide was added to admixture to the powder equivalent to 0.50mcg in a flask. Few ml of methanol was added to this mixture and sonicated for 5 minutes. Final volume was made up with the same and filtered using whatmann filter paper. The recovery studies were performed at two different levels of 50% and 100%. The procedure was repeated in triplicate and the standard drug recovered at each level was calculated and presented.

Robustness and Ruggedness

Deliberate variations in the parameter of wavelength by ± 0.1 nm from 231 was observed from the spectrum recorded for the robustness of the method. The ruggedness of the method was evaluated by the simultaneous measurements of the absorbance by two different analyst under similar operating conditions on three different days with the same instrument.

Limit of detection and limit of quantification

The amount of substance that can be detected by the method adopted was calculated by applying the formula of $3.3\sigma/S$. The minimum amount that can be quantified was obtained from the formula $10\ \sigma/S$. The σ represents the standard deviation of the measurements and S represents the slope of the y-intercept in the calibration graph.

Solution stability

The solution was checked for the stability at room temperature at regular intervals till 36 hours at room temperature and in the refrigerator periodically for 6 days. The change in absorbance of solution was noted and tabulated.

Specificity of the method

The specificity of the developed method was assessed with the recording of spectrum forsolvent methanol against water in the reference cell. The absorbance of methanol at the λ_{max} of the drug was noted to check the specificity of the method. An admixture of following inactive ingredients, microcrystalline cellulose, corn starch, colloidal silicon dioxide and magnesium stearate was prepared in the methanol diluted suitably and the spectrum was recorded for the absorbance of the admixture at the selected wavelength was observed.

Optical parameters for the method

The optical parameters such as molar absorptivity, Sandell's sensitivity and E (1%1cm) were calculated from the responses recorded for the standard dofetilide and tabulated.

6.4.1.3 Application of UV spectroscopy method for the analysis of admixture

The admixture of the excipients containing magnesium stearate, starch and microcrystalline cellulose were weighed and added to 0.5mg of dofetilide in a mortar. The mixture was triturated well and transferred to the 10ml standard flask. To this 5ml of methanol was added and sonicated for 5minutes. The volume was made up to the mark and mixed well. The solution prepared was further diluted with methanol to produce a concentration of 10mcg/ml. The solution was scanned in the UV region and the spectrum was recorded.

6.4.2 DEVELOPMENT OF VALIDATED HPTLC METHOD FOR DOFETILIDE

6.4.2.1 Initial chromatographic condition

Selection of stationary phase

Dofetilide was analysed by using TLC plate made up of G_{60} F_{254} coated on aluminium support with the particle size of 2 µ and sorbent layer thickness of 0.2 mm. The plates procured as 20 x 20 cm size, which was cut into required size of 10 x 10 cm and 10 x 20 cm for the HPTLC method for dofetilide.

Selection of mobile phase

The drug dofetilide was basic in nature and found soluble in chloroform, 0.1 M HCl, 0.1 M NaOH and methanol. Based on the solubility and polarity of the drug the mobile phase system was selected. Methanol was used for the solution preparation and sample application was performed with an automatic sampler fitted with nitrogen gas supply. The detection wavelength was 233 nm.The trials involved a number of mobile phase system from which the most appropriate solvent system was fixed for the drug. The mobile phase consisting of methanol: toluene: acetonitrile in the composition of 4:2:4 v/v/v was selected for the study. The plate was scanned at 233 nm after the development.

Optimization of variants in TLC

The variants like the composition of mobile phase, chamber saturation time (equilibration time), the distance of solvent front and bandwidth of the applied spots, were suitably selected for the drug.

Chamber saturation time

The mobile phase was placed on one side of the twin trough chamber after proper mixing in the ratio mentioned. The saturation times tried were from 5 to 30 minutes. The plates were spotted with dofetilide solution and developed. The effect of chamber saturation time on spot development, peak shape and R_f value were studied.

Composition of solvents in mobile phase

The volume of each solvent constituting the mobile phase was varied in order to study the effects on the R_f value and peak shape. The suitable composition selected for the analysis of dofetilide was methanol:toluene: acetonitrile in the ratio of 4:2:4 v/v/v.

Distance of solvent front

The R_f value of the drug depends on the distance travelled by the solvent front. The mobile phase was allowed to run to on the plate to various distances from 7cm to 9cm. The suitable R_f value was achieved with the distance of 8.5 cm of the solvent front and hence fixed for the studies.

Band width

The size of the band width determines the compactness, R_f and peak area of the spot. Different sizes of band width from 1 to 5 mm were spotted and scanned for the chromatographic characters. When the band width was 5 mm, the peak shape were compact with R_f value minimum and peak area was good.

Preparation of standard stock solution

Stock solution of dofetilide was prepared by weighing 10mg and transferred into a 10 standard flask. A few ml of methanol was added and shaken well to dissolve the drug. The volume was made upto the mark with methanol.

6.4.4.2 Validation of HPTLC method for Dofetilide

Linearity and range

The spotting of sample was carried out with linear increase in the volume applied on the precoated plate. Linearity was establishedby spotting different volume from 0.5 to 5 µl of 100 mcg/ml solution corresponding to the concentration of 50 ng/band to 500 ng/band. The Rf value was found to be 0.53. The values of linearity was subjected to statistical treatment and presented in the table.

Precision studies

Six replicate injections of 400ng/band were spotted on the plate to perform the intraday precision. The peak area was assessed for the standard deviation and relative standard deviation. Interday precision was determined by spotting the sample for six times on three consecutive days with the same concentration of drug. The SD and % RSD was evaluated and tabulated.

System precision

The scanning of the spotted plate for six times was executed and spotting the same volume of injection of the dofetilide solution was studied to ascertain the system precision. The samples were spotted for the same concentration and scanned repeatedly for six times. The SD and % RSD were calculated and tabulated.

Accuracy

The accuracy of the method was determined by calculating percent recovery of the drug. The accuracy study was carried out at 50% and 100% levels of assay concentration of doefitide. The % recovery was calculated from the formula and results are shown in table

Robustness and ruggedness

The experimental condition fixed for the HPTLC method were varied intentionally to check the robustness of the method. Parameters like saturation time variation by ± 2 min, mobile phase ratio variation by ± 0.1 ml were carried out. Any significant change in the peak area and the Rf value were noted to prove robustness. Ruggedness proven by analysis of standard by two different analysts.

LOD and LOQ

A volume of 0.1 to 1µl (10 – 100ng/band) of stock solution were spotted on the plate and the method developed was carried out. The concentration of drug that can be quantified with acceptable peak area and the lowest concentration that can be detected with a symmetrical peak in the band spotted were verified and noted.

Stability of developed plate

The drug solution of 400ng/band was spotted to check the stability of drug on the plate. The peak area was noted at fixed intervals in hour by placing the plate at room temperature till 18 hours. The time where the peak area difference was found to be more than 10% was observed and presented.

System suitability studies

The suitability of the method for the analysis under the fixed conditions of operation was ascertained from the tailing factor, asymmetric factor and efficiency.

6.4.4.3 Application of HPTLC for the analysis of admixture

The admixture of the excipients were prepared based on the product monograph of the dofetilide capsules. Inactive components such as magnesium stearate, starch and microcrystalline cellulose were weighed and added to 500mcg of drug substance in a mortar. The mixture was triturated well and transferred to the 10ml standard flask. To this 5ml of methanol was added and sonicated for 5minutes to extract the drug. The volume was made up to the mark and mixed well. The solution prepared was further diluted with methanol to produce a solution of 100mcg/ml. It was spotted six replicate times from which the amount present was calculated.

6.4.3 DEVELOPMENT OF VALIDATED HPLC METHOD FOR DOFETILIDE

6.4.3.1 Chromatographic conditions

Selection of wavelength

A stock solution of 1mg/ml further diluted with 10mM Orthophosphoric acid to get 10mcg/ml. The solution was scanned in the UV region and the spectrum recorded. The λ_{max} was found at 233 nm, which was selected for the analysis.

Selection of initial chromatographic conditions

TheShimadzu Shim pack GWS (250 mm x 4.6 µ id x 5 µm particle size) was used as the stationary phase for the chromatographic analysis of dofetilide. Initially a mobile phase consist of methanol and water used for separation of doefitilide. To improve peak shapes a three mobile phase system consisting of ACN, methanol and water was tried. Various ratio of the solvent system in the increasing order of %B ratio for methanol was tried and the peaks eluted were monitored for the acceptable criteria of peak shape, retention time, tailing factor and peak symmetry.

Optimization of the chromatographic conditions

Suitable conditions selected for the HPLC method analysis of dofetilide involves the mobile phase composition of methanol, acetonitrile and water with fixed flow rate and injection volume. The ratio of methanol was kept constant and the composition of acetonitrile was gradually increased with decrease in the ratio of water. The mobile phase consisting of methanol, acetonitrile and water in the ratio of 10:35:55 v/v/v with a flow rate of 1ml and injection volume of 20µl was found to give acceptable chromatographic characters.

Preparation of standard stock solution

Dofetilide stock solution of concentration of 1 mg/ml was prepared in methanol. The working standard solution was also prepared in methanol to get a solution of 100mcg/ml.

Preparation of 10 mM Orthophosphoric acid

A 10 mM Orthophosphoric acid was prepared by adding 0.68 ml of the acid to 1000 ml of HPLC grade water. The solution was mixed well and filterd through 0.45 μm membrane filter. This solution was used as the final diluent for all the solutions in the established RP-HPLC method.

Preparation of mobile phase solvent system

The solvents fixed for the HPLC method were methanol, acetonitrile and water. Selected solvents were filtered through the 0.45 μ membrane for the removal of the fine particles and fibers present. The filtered solvents were transferred to the respective reservoirs and pumps.

6.4.3.2 Validation of RP-HPLC Method for dofetilide

Linearity and range

Stock solution of dofetilide was diluted further to prepare a solution of 100 mcg/ml using methanol. Working standard solution of dofetilide prepared in methanol was diluted with 10 mM ortho phosphoric acid to give serial dilution of 5 concentrations of 10, 20, 30, 40 and 50 mcg/ml. The chromatogram of the standard solutions shows a linear increase in the peak area.

Precision studies

Six replicated injections of the standard solution in the concentration of 10 mcg/ml were run on the same day for studying the intraday precision and once in a day for three consecutive days for investigating the interday precision. The chromatograms were studied for the responses and tabulated.

Accuracy

The accuracy for the HPLC method was determined at two different levels at 50 % and 100 % of the standard solution added to the previously weighed capsule powder. The results were expressed in terms of % recovery of the standard drug added and % relative standard deviation of the measurements. The method was adopted for the admixtures contain 0.5 mg of dofetilide.

Specificity

The specificity of the method was studied by recording the chromatogram of 10mM OPA (Blank) and excipients solution (Matrix of admixtures) in fixed condition and observing for any interference.

Robustness and Ruggedness

Intentional variation of the experimental parameters of flow rate, column temperature and wavelength were investigated. The change in flow rate of ± 0.1 ml, mobile phase of ± 0.1 nm and injection volume ± 0.1 µl were applied to study the robustness of the method. The ruggedness was evaluated by two analysts analyzing the standard sample under similar experimental conditions using the same instrument.

Limit of detection (LOD) and limit of Quantification (LOQ)

LOD and LOQ values were calculated from the data obtained in the linearity graph for the standard solution. The slope of the linearity plot was determined. They were calculated as 3.3σ/S and 10σ/S respectively.

Stability of the solution

Chromatograms of the standard solution for a selected concentration of 10mcg/ml was recorded at fixed interval and checked for the responses. The solution stored at room temperature and refrigerator were analysed for the variation in the peak area upto the acceptable limit of 10%.

System suitability parameters

Selected parameters for the HPLC system includes the number of theoretical plates, tailing factor and asymmetrical factorfor the peaks recorded were computed.

6.4.3.3 Application to admixture of dofetilide

The admixture of the excipients was prepared based on the product monograph of the dofetilide capsules. Inactive components such as magnesium stearate, starch and

microcrystalline cellulose were weighed and added to 500mcg of drug substance in a mortar. The mixture was triturated well and transferred to the 10 ml standard flask. To this 5 ml of methanol was added and sonicated for 5minutes to extract the drug. The volume was made up to the mark and mixed well. The solution prepared was further diluted with 10 mM ortho phosphoric acid to produce a solution of 10mcg/ml. Finally the solution was filtered in a 0.2 µ sterile PTFE filtered and injected for six replicate times. The amount present was calculated and tabulated.

6.4.3.4 Application of RP-HPLC for the degradation studies of dofetilide

HPLC method developed and validated with the mobile phase made up methanol, Acetonitrile and water in the ratio of 45:10:45 %v/v/v with the flow rate of 1ml/min used for separation of dofetilide related compound from the API dofetilide measured at a wavelength of 233nm.The forced degradation of dofetilide was carried out as described below.

Acid hydrolysis

The working standard solution of dofetilide was placed in the reflux flask consisting of 0.1 M HCl. After proper mixing the solution was placed on the thermostat maintained at 30^{o}C. The sample at zero was collected and diluted with 10 mM Orthophosphoric acid. The final solution was filtered with 2 µ sterile PTFE filter. The recorded Chromatogram were noted for change in responses at 0, 4 and 8 hours.

Base hydrolysis

Adequately diluted solution of dofetilide was treated with the 0.1 M NaOH and kept over the thermostat maintained at a temperature of 30^{o}C. The sample was collected at 0,4 and 8 hours and diluted with 10 mM Orthophosphoric acid to a concentration of 10 mcg/ml. The solution was filtered with 2 µ sterile PTFE filter and sample were injected.

Oxidation

The standard solution of dofetilide was treated with 3% v/v hydrogen peroxide solution on a thermostat maintained at 30ºC. The filtered sample was diluted with 10 mM Orthophosphoric acid, injected in the column and the peaks eluted were noted for the area. The presence of additional peaks were also noted. Three samples collected at 0,4 and 8 hours were studied and the results were completed.

Thermal degradation 80ºC

The solution preparation for thermal degradation of 80 ºC was carried out using methanol to give a concentration of 100mcg/ml. The sample withdrawn at 0, 4 and 8 hours were diluted using 10 mM Orthophosphoric acid and filtered before the analysis. The peak area of the drug were noted

Photolysis 220 Volts lamp

As per the photo stability guidelines the stress applied to the drug was a closed light chamber fitted with a 220 V lamp. The 100 mcg/ml of standard solution was exposed to the light. The 0 hour sample was injected followed with the 4 hour and 8 hour sample. The change in responses were noted.

6.4.3.5 Application of RP-HPLC to study the Impurity of Dofetilide

Separation of process impurity of dofetilide

The stock solution of dofetilide and its impurities were individually prepared in a concentration of 1mg/ml. A quantity of 10 mg of them were transferred in individual 100ml volumetric flask and 1ml of methanol was added to dissolve. The volume was made up with the same. A mixture of dofetilide and impurity also was prepared in same method and used.. Finally further dilution were made with OPA before analysis. The chromatograms of dofetilide and impurities were recorded and the peak characters were observed.

The capsule admixture containing 0.500mg of dofetilide was spiked with impurity 0.0025mg (50% level), 0.005mg (100%level), 0.0075mg (150% level) as stated by USP. This solution was dissolved, diluted as mentioned earlier. The chromatograms were recorded and the peak characters were noted. The method was validated for prescribed parameters and applied to analyze impurity spiked into capsule admixture. The peak area were noted and % level was calculated.

7. RESULTS

7.1 APIXABAN

7.1.1 DEVELOPMENT OF VALIDATED UV SPECTROSCOPY METHOD FOR APIXABAN

7.1.1.1 Selection of experimental conditions

Methanol was selected as the solvent for apixaban as it was completely soluble and gave a smooth spectrum in the 200-400 nm range. From the absorption spectrum a wavelength of 287 nm was chosen as apixaban exhibited higher absorbance (λ_{max}) at this wavelength. The UV spectrum of apixaban in methanol (10 mcg/ml) is shown in Figure 1.The method was carried out at a scan speed of 400 nm/min, a band width of 0.5 nm and slit width of 0.1 mm which were found optimum for the analysis of apixaban.

7.1.1.2 Validation of UV Spectroscopy Method

Linearity and range

The selected range of concentration that obey the Beer's law was found between 2 to 20mcg/ml. The absorbances of the standard solution are shown in table 1. The overlay spectra of standard solution for the selected range are shown in figure 2. The linearity graph is presented in figure 3. The slope, correlation coefficient and intercept were derived for the linearity curve. The regression equation was found to be y=0.0239*x+0.0062 (y=a*x+b) with the correlation coefficient (r^2) of 0.998 and standard error of 0.3939, respectively.

Precision studies

The intraday precision studies were carried out by six replicate readings for the standard solution on the same day as shown in table 2. Interday precision studies were determined by recording the spectrum on three days and calculating the average of six responses for the same concentration of drug solution. The values of intraday precision are presented in table 2 and the interday precision are given in table 3.

Accuracy

The percent recovery of the standard solution at the two levels of 50% and 100% signifies that the method is accurate with the amount of recovery close to 100% of the standard drug added with the formulation. The percentages of standard drug recovered at two levels are shown in table 4.

Robustness and Ruggedness

Robustness of the method was studied with deliberate variations in the wavelength parameter by ±0.1 nm from the selected wavelength of 287 nm.It was observed that the absorbance didn't have a significant variation.The measurement of the standard solutions by two analyst shows that the method was rugged as there was no significant change in the absorbance measured.

Limit of detection and limit of Quantification

The mathematical calculation of the detectable limit and quantified limit for the UV method was performed with the responses obtained. The detectable limit for apixaban was found to be 0.53mcg/ml and the limit which can be quantified was found to be 1.62mcg/ml.

Stability of solution

Freshly prepared solution of apixaban in the concentration of 10mcg/ml was stored at room temperature and in the refrigerator. The 0 hour sample was scanned immediately after preparation. The solution was found to be stable for 12 hours at room temperature and 72 hours in the refrigerated condition. The values are shown in table 5.

Specificity of the method

The method was found to be highly specific without any interference from the solvent selected as shown by the overlay spectra of solvent methanol and the drug solution in Figure 4. The interference due to the excipients added in the formulation of tablet dosage was evaluated . The overlay spectrum of apixaban and admixture is shown in figure 5.

Optical parameters for the method

The calculated values of molar absorptivity, Sandell's sensitivity and E (1%,1cm) are presented in the table 6.

7.1.1.3 Application of UV spectroscopy method for theanalysis of formulation

The spectrum recorded for the assay sample of the tablet was observed for the absorbance at the selected wavelength of 287 nm. Six replicate determinations were carried out for the analysis of formulation. The assay value was calculated for each sample and noted. The SD and %RSD were computed for the values of label claim and shown in the table 7.

Fig.1. UV Absorption spectrum of apixaban (10 mcg/ml)

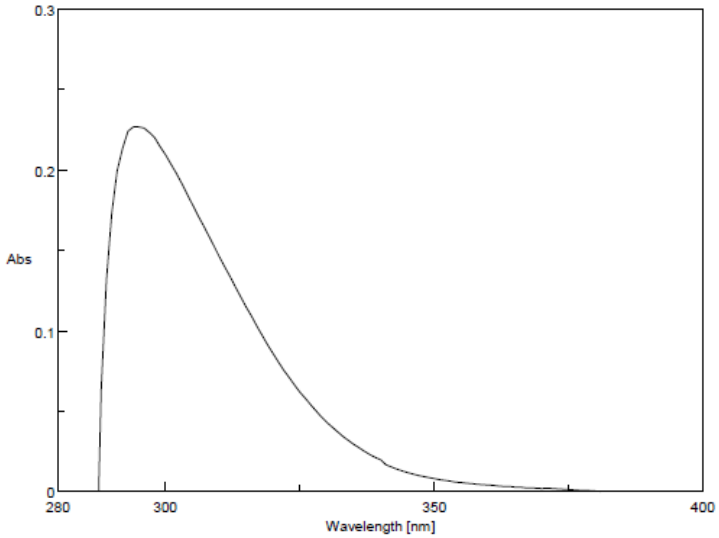


Fig.2. Overlay Spectrum of Apixaban

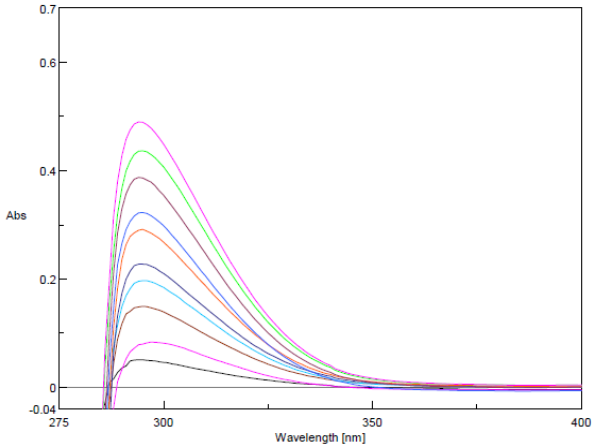


Table 1. Calibration data for Apixaban

Concentration mcg/ml	Absorbance
2	0.0506
4	0.0793
6	0.1492
8	0.1965
10	0.2267
12	0.2909
14	0.3222
16	0.3856
18	0.4359
20	0.4888

Fig. 3. Calibration graph of Apixaban

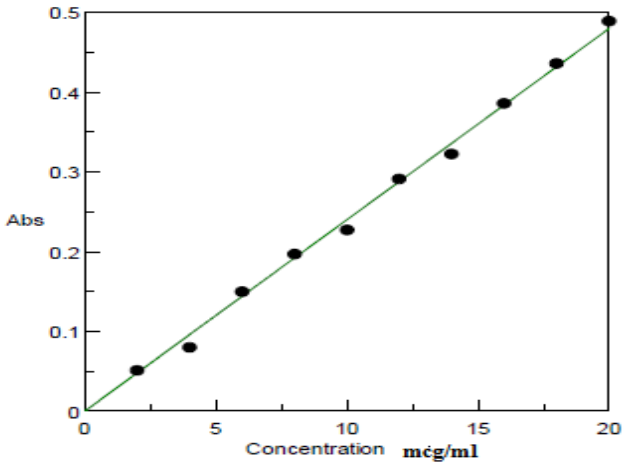


Table 2. Intraday Precision Studies of apixaban

Precision	Absorbance*	SD	%RSD
Intraday 1	0.2233	0.0006	0.51
Intraday 2	0.2231		
Intraday 3	0.2248		
Intraday 4	0.2237		
Intraday 5	0.2240		
Intraday 6	0.2239		

Table 3. Interday Precision Studies of apixaban

Interday Precision	Absorbance*	SD	%RSD
Day 1	0.2225	0.0016	0.69
Day 2	0.2189	0.0009	0.88
Day 3	0.2220	0.0012	0.74

*****Mean of six observations

Table 4. Accuracy studies of apixaban

Level	Amount of Std added (mg)	Amount of Std recovered (mg)	% Recovery of the Std*	SD	% RSD
50%	5	5.13	102.62	0.012	0.006
100%	10	10.17	101.73	0.031	0.0015

*****Mean of six observations

Table 5. Stability of Apixaban solution

Room Temperature		Refrigerator	
Time in Hours	**Absorbance**	**Time in Hours**	**Absorbance**
0	0.3818	0	0.3825
2	0.3803	6	0.3823
4	0.3720	12	0.3795
8	0.3658	24	0.3732
12	0.3649*	48	0.3699
24	0.2974	72	0.3607*
36	0.2253	84	0.3441

* Maximum time of stability

Fig. 4. Overlay spectrum of apixaban and methanol

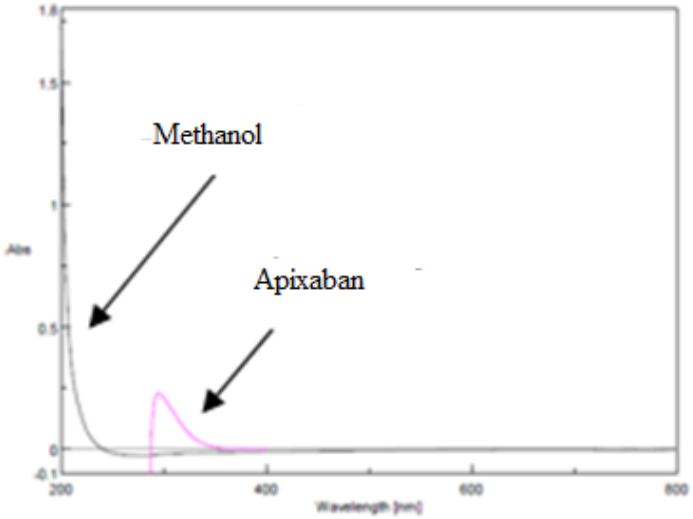


Fig.5. Overlay spectrum of Apixaban and Excipients

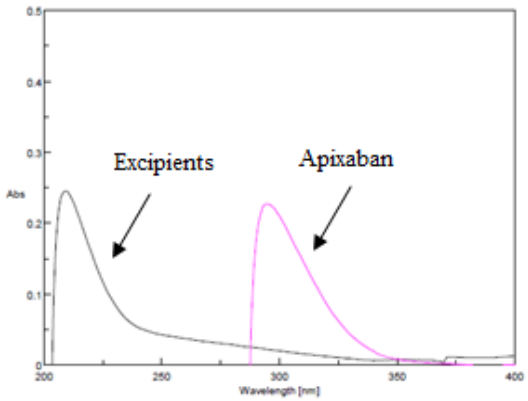


Table 6. Optical parameters of Apixaban

Parameter	Result
Molar absorptivity (L mol^{-1} cm^{-1})	1.041 X 10^4
Sandell's sensitivity (mcg/ml)	0.04411
E(1%,1cm)	244.34

Fig.6. Spectrum of the tablet formulation of Apixaban

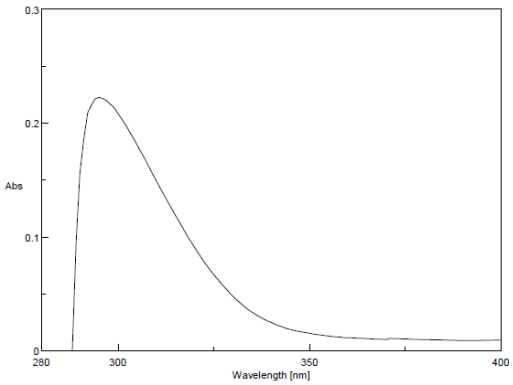


Table 7. Analysis of Apixaban Formulation

Label claim	Amount found	% Label claim*	SD	% RSD
2.5 mg	2.48 mg	99.2	0.003	0.04

*Mean of six observations

7.1.2 DEVELOPMENT OF VALIDATED HPTLC METHOD FOR APIXABAN

7.1.2.1 Chromatographic conditions

Selection of stationary phase

Precoated silica gel G_{60} F_{254} plates were used for the separation of apixaban (20 x 20 cm size). As per the requirement for the method the plate was concisely cut into appropriate size of 10 x 10 cm and 10 x 20 cm.

Selection of mobile phase

The mobile phase system were tried in various combination of petroleum ether, tetrahydrofuran, acetonitrile, methanol, dichloromethane and toluene. The composition tried with PET:THF:ACN, TLN:DCM:MeOH, TLN:DCM:MeOH and DCM:TLN:THF. It was observed that all the solvent systems carried the drug apixaban with the solvent front except the one with DCM:TLN:THF which gave good separation for the drug between the application point and the solvent front and retained at R_f of 0.1(Table 8). Hence further optimization was carried out using the composition and of these solvents.

Optimization of variants in TLC

The mobile phase system, chamber saturation time, the distance of solvent front and bandwidth of the spots are a few factors that affectthe Rf value of drug. The optimization of these factors were carried out to fix these variants for the analysis of apixaban.

Composition of mobile phase system

The composition of Toluene:dichloromethane:Tetrahydrofuran were varied and change in $R_f$value werenoted and shown in table 9. The ratio of 2:2:6 v/v/v of TLN:DCM:THF was fixed as the mobile phase system which resulted in proper separation of apixaban.

Chamber saturation time

Chamber saturation time determines the compaction and R_f value of apixaban. The trial was made from 10-30mins and at 20mins saturation time both R_f value and compaction was found good hence selected for the study.

Distance of solvent front

Distance of solvent front decides the $R_f$value and retention of apixaban. Hence optimization is carried out from 6-9 cm distance, from which 8 cm distance was chosen for the study. The optimized densiometric conditions are shown in table 10.

7.1.2.2 Validation of HPTLC method for Apixaban

Linearity and range

The sample spots developed on the plate were scanned at 287 nm. The linearity was found between 200-2000 ng/band and the values of linearity were subjected to statistical treatment. The data for regression are Y = 3121.620+5.337*X, r^2 = 0.9851 and SE =6.65. The overlay 3D densitogram of apixaban is shown in figure 7. The calibration data are shown in table 11 and the calibration graph obtained for the linearity is shown in figure 8.

Precision studies

The degree of agreement between the results for the six determinations of the standard sample was assessed by the SD and % RSD value for the intraday precision. The interday precision was carried out by repeating the six replicate injections of the standard on three consecutive days. The SD and % RSD are given in the table 12 and 13 and the representative densitogram is shown in figure 9.

System precision

A standard solution of apixaban was injected forsix times to determine the system precision of the method and %RSD was calculated for apixaban and the six replicate scanning of the plate were run and the peak area of apixaban were noted. The SD was calculated for the peak area values and %RSD of system precision was found to be below 1 as stated in the table 14.

Accuracy

The accuracy was determined by spiking the assay sample with the standard drug at 50% and 100% level. The % recovery was calculated for the standard added to the assay sample. The SD and %RSD calculated for the recovered sample was presented in the table 15.

Robustness and ruggedness

The variations in the saturation time by ± 2 min and small changes in the mobile phase ratio by ± 0.1 ml for 2:2:6 ratio of TLN:DCM:THF were studied for the peak area and Rf values. A change less than 10% was observed in the peak area and the Rf value. The results of analyzing apixaban by two different analysts with the similar operating conditions had no significant variation. The %RSD were found to be below 2.

LOD and LOQ

The LOD and LOQ values were determined by spotting lower concentration of apixaban. It was observed from the bands spotted, that the concentration at 50ng/band the peak was detectable with peak area of 256.87 below which the peak shape was not symmetric and 100 ng/band peak was quantified with peak area of 838.92. They are represented in figure 10 and 11.

Stability of developed plate

The volume spotted for the stability studies was 10 µl of 100 mcg/ml of the working standard. The stability of plate was checked periodically till 10 hours and the plate was found to be stable for 8 hours after which the responses had notable change. The drug was stable on the plate till 8hours after which 10% reduction occurred in the peak area (table 16).

System suitability parameters

The tailing factor, asymmetric factor and efficiency were calculated to confirm suitability of the method (Table 17).

7.1.2.3 Analysis of formulation by HPTLC method

An aliquot of tablet formulation was prepared and spotted onto the plate. After the development and scanning, the peak area was noted to calculate the amount present and label claim are shown in table 18 and figure 12.

Table 8.Selection of Mobile Phase System for Apixaban

Mobile phase	Ratio(v/v/v)	Observations
PET:THF:CAN	6:2:2	Spots move with solvent front
MeOH:ACN:PET	2:2:6	Spots move with solvent front
TLN:DCM:MeOH	3:3:4	Spots move with solvent front
DCM:TLN:THF	2:6:2	Rf less than 0.1

Table 9. Composition of Mobile Phase for Apixaban

TLN:DCM:THF (v/v/v)	R_f
5:1:4	0.2
4:4:2	< 0.1
6:3:1	No movement of the Spots
2:6:2	0.21
4:2:4	0.26
3:4:3	0.27
3:2:5	0.33
2:2:6	**0.55**

Table 10. Optimized Densitometric Conditions for Apixaban

Parameter	Fixed Condition
Stationary phase	Precoated silica gel plate G_{60} F_{254}
Mobile phase	TLN:DCM:THF
m/p ratio	2:2:6 v/v/v
Chamber saturation	20 minutes
Distance of solvent front	8 cm
Band width	5 mm
Slit dimensions	5 x 0.45 mm
Scan speed	20 mm/sec
Scan wavelength	287 nm

Fig.7 Overlay 3D Densitogram of standard Apixaban

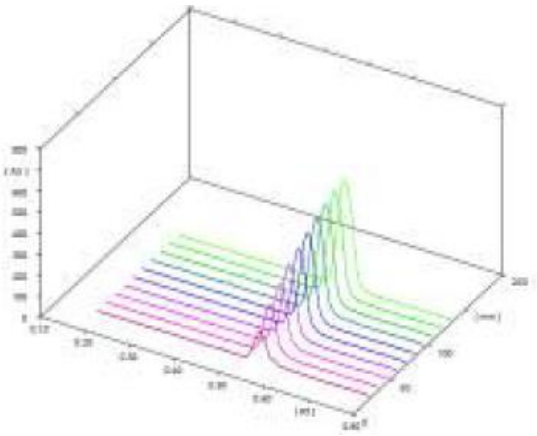


Table 11. Calibration data of Apixaban

Concentration ng/band	R_f value	Peak area
200	0.55	2574
400	0.55	4014
600	0.56	5587
800	0.56	7846
1000	0.56	8996
1200	0.55	10036
1400	0.55	10972
1600	0.55	11747
1800	0.56	12440
2000	0.56	13003

Fig.8 Calibration graph of Apixaban

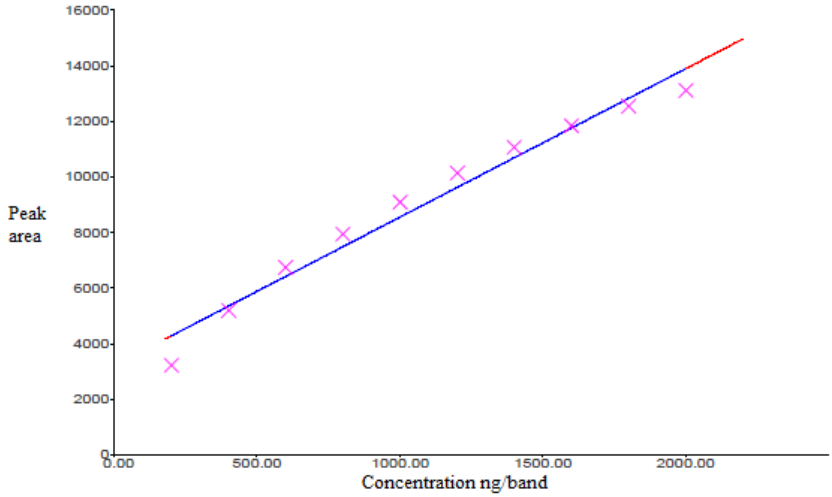


Fig.9 Representative densitogram of precision studies

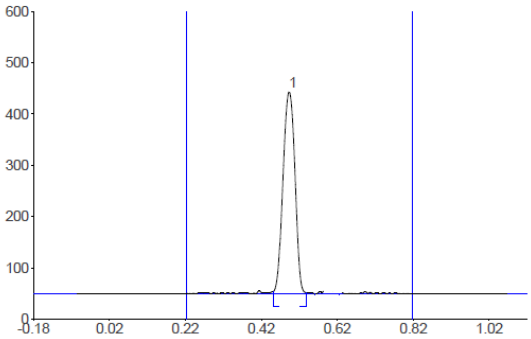


Table 12. Intraday Precision Studies of Apixaban

Precision	Peak area	SD*	%RSD*
Intraday 1	8990	42.95	0.08
Intraday 2	8922		
Intraday 3	8925		
Intraday 4	8914		
Intraday 5	9005		
Intraday 6	8999		

*Average value

Table 13. Interday Precision Studies of Apixaban

Precision	Peak area*	SD	%RSD
Day 1	8943	30.44	0.03
Day 2	8987	29.05	0.01
Day 3	9002	32.98	0.11

*****Mean of six observations

Table 14. System Precision of Apixaban

Parameter	Peak area*	SD	%RSD
Repeatability of injection	8988	23.61	0.07
Repeatability of scanning	8960	41.73	0.14

*****Mean of six observations

Table 15. Accuracy studies of Apixaban

Level	Amount of Std added (mg)	Amount of Std recovered (mg)	% recovery of Std.*	SD	% RSD
50%	5	5.01	100.33	58.21	0.07
100%	10	10.10	101.05	16.42	0.009

*Mean of six observations

Fig.10 LODof Apixaban

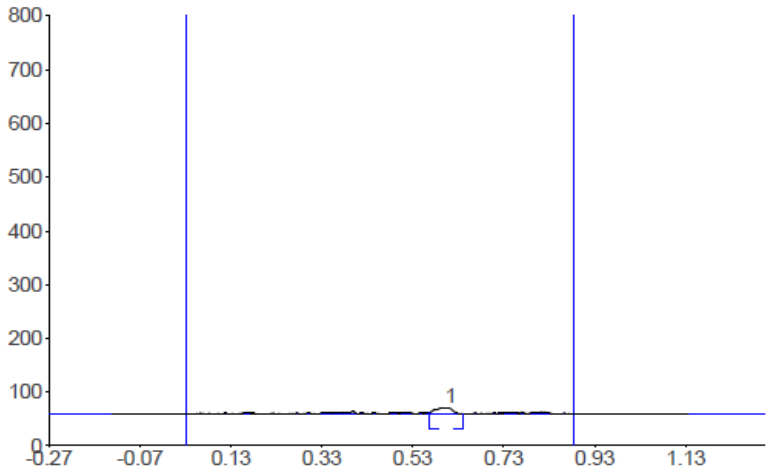


Fig.11. LOQ of Apixaban

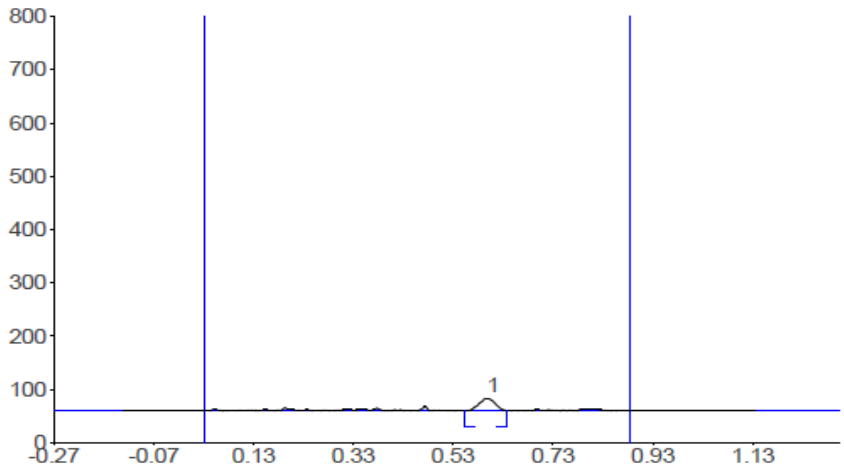


Table 16.Stability of developed plate for Apixaban

Time Hours	Peak area	SD	%RSD
0	8991	39.22	0.43
2	8974		
4	8696		
6	8449		
8	8115*		
10	8037		

*Maximum time of stability

Table 17. System suitability parameters of Apixaban

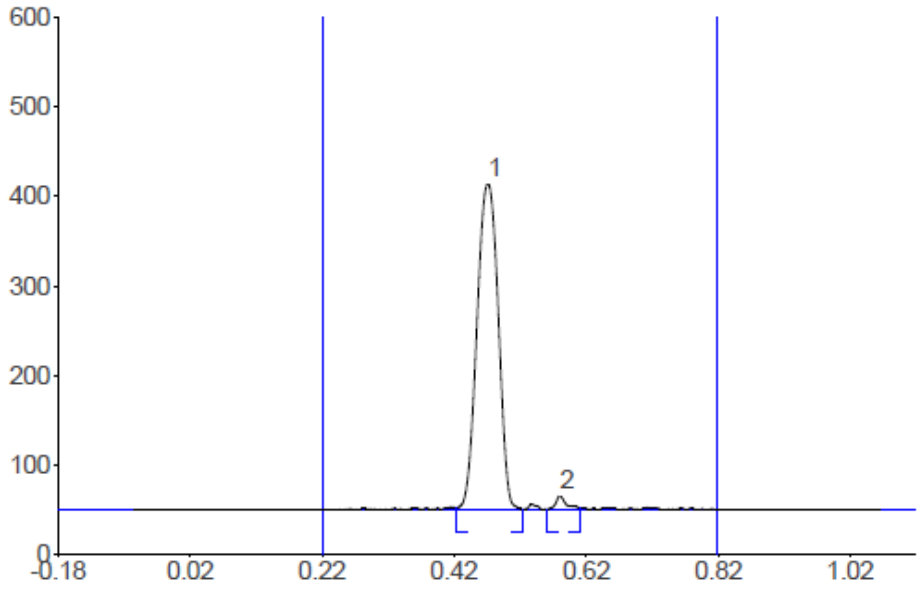
Tailing factor	Asymmetric factor	Efficiency
0.5311	1.1006	1850

Table 18. Analysis of Apixaban Formulation

Label claim	Amount found	% Label claim*	SD	% RSD
2.5 mg	2.482 mg	99.28	3.54	0.50

***Mean of six observations

Fig.12. Densitogram of Apixaban formulation



7.1.3 DEVELOPMENTOF VALIDATED RP-HPLC METHOD FOR APIXABAN

7.1.3.1 Chromatographic conditions

Selection of wavelength

The solution of apixaban was prepared in methanol as it was found to have complete solubility. The scanning of the apixaban solution in the UV region was found to give maximum absorbance at 287 nm, which was selected as the detection wavelength for the RP-HPLC method. (Figure 13)

Initial chromatographic conditions and optimization

The separation of apixaban by RP-HPLC method was carried out on a C18 Phenomenex column (250 mm x 4.6 µ id x 5 µm particle size). The initial chromatographic separation was carried with acetonitrile and water as the mobile phase in different ratio. A 10 mcg/ml of apixaban in methanol was injected and chromatogram recorded. Splitting was observed in the eluted peaks. So methanol and water were tried in different ratio of increasing order of % B pump. The chromatograms recorded are presented in figure 14 to figure 20 and observations trial are shown in table 19.

Optimization of the chromatographic conditions

The HPLC method was optimized with the mobile phase made up of two solvents, water and methanol in the ratio of 10:90 v/v with a flow rate of 1ml/min and injection volume of 10µl. The chromatogram was recorded in the fixed conditions. The study was carried out at ambient temperature. The parameters fixed are summarized in the table 20 and the figure 21 shows the representative chromatogram of the standard solution.

7.1.3.2 Validation of RP-HPLC Method for apixaban

Linearity and range

Linearity of the detector response was examined by analyzing a seriesof seven different concentrations of apixaban. The calibration curve was constructed by diluting the stock solution of apixaban with methanol to give a concentration range of 2-20 mcg/ml. The calibration data are shown in table 21. To assess theappropriateness of using a linear regression model to fit the data,residual plots were produced. The points in the residual plots were distributed around the horizontal axis. This random dispersionof the residuals suggests that the linear model gives a good fit ofthe data. Linear regression analysis shows correlation co-efficient (r) of 0.9998, slope (1798), standard error (0.1033) and y-intercept (686.86). The linear graph (Figure 22) and overlay chromatogram (figure 23) were generated.

Precision studies

Standard solution of apixaban was prepared in methanol to obtain a concentration of 10mcg/ml. Six replicate injection of the solution on the same day was carried out for the intraday precision and three consecutive days for the Interday precision studies. The standard deviation and relative standard deviation values are given in table 22 and 23

Accuracy

The accuracy of the method was determined by calculating percent recovery of apixaban by using standard addition method. Percent recovery of apixaban was determined at two different level 50% and 100% of the target concentration in triplicate. The results of accuracy study are shown in table 24.

Robustness and Ruggedness

Minor variations in the flow rate ± 0.1 ml, injection volume ±0.1µl, mobile phase ratio ± 0.1 ml/min. were studied and the responses were found to be robust. The ruggedness was evaluated by two analysts recording the chromatogram under similar operating conditions. The values are shown in table 25.

Limit of detection and limit of quantification

The signal to noise ratio of the instrument was applied by the responses of the drug in the linear range of 2 to 20mcg/ml. The LOD and LOQ were found to be 0.0013mcg/ml and 0.0039mcg/ml.

Stability of the solution

A 10µg/ml solvent was used for stability assesement. The responses observed at different time intervals are summarized in table 25. The solution of apixaban at room temperature was found to be stable till 12 hours with acceptable change in the detector response below 10% in the peak area. The refrigerator sample of Apixaban protected from light was found to be stable for 72 hours after which there was appreciable decrease in the peak area above 10% of the response compared with the zero hour sample. The responses observed at different time intervals are summarized in table 26.

System suitability parameters

Signal to noise ratio of the instrument was applied for the evaluation of asymmetric factor of the peak at 5% of its height and tailing factor at 10% height of peak. The values for the parameters were compared with the standard acceptance criteria and tabulated (Table 27).

7.1.3.3 Application of RP-HPLC method for the analysis of formulation

Amount of drug present in the tablet formulation was found out by analysing the chromatogram of formulation sample for the peak area and quantifying in comparison with the standard drug peak area. The analysis of commercial formulation by the developed method indicated that the label claim for the tablets were within limits specified for the label claim. The representative chromatogram is shown in figure 24. The amount of drug present and % label claim present in the tablet (table28).

7.1.3.4Application of RP-HPLC method for the degradation studies

RP-HPLC method developed and validated according to the ICH guidelines was employed for the degradation studies. Hydrolytic degradation of apixaban in acid, base, thermal, light and oxidation process was evaluated for the % degradation of the drug till a time period of 8 hours.

Acid Hydrolysis (0.1 M HCl)

The drug solution of apixaban was found to elute at 2.3 minutes. The additional peak for the degradant peak appeared at a retention time of 2.1 minutes. The change in peak area was calculated for the amount of drug present at 8 hours and the % of degradation with 0.1 M HCl as shown in table 29. The chromatograms are shown in figure 25 to 27.

Base hydrolysis (0.1 M NaOH)

The base hydrolysed apixaban sample shows the peak at 2.3 minutes with additional peak at 1.6 minutes. There was gradual increase in the peak area eluted at 1.6 minutes. The calculated amount of degradation is shown in table 29.The chromatogram at 0, 4 and 8 hour sample are present in figure 28 to 30, respectively.

Oxidation (3% v/v H_2O_2)

The oxidation of the drug solution at different time intervals was found to exhibit peaks at 2.3 (drug) and 2.1minutes (degradant). The peak area change was compared with the response at zero minute. The degree of change in the peak area was found to be below 10%. The change in the peak area is noted in the table 29. The figures 31 to 33 are the chromatograms of apixaban after oxidation.

Thermal degradation (40ºC and 80ºC)

The standard apixaban solution prepared in methanol was subjected to 40 ºC and 80 ºC in separate thermostat. The drug peak area at 2.3 minutes was decreasing gradually indicating the degradation of the drug. The changes in the responses were shown in table 29. The degradation at 40 ºC are shown in figures 34 to 36. The changes in the elevated temperature of 80ºC are shown in figures 37 to 39.

Photolysis (220 volts light)

Stability of apixaban was demonstrated using a 100µg/ml solution under the effect of light upto 8 hours. The chromatogram was examined for change in peak area at 2.3 minutes. There was negligible change in the peak area for the drug. Effect of light was studied at the stipulated time intervals and values are shown in table 29. The chromatograms 40 to 42 showed the negligible changes in the responses of apixaban under light.

Fig.13 UV Spectrum of apixaban

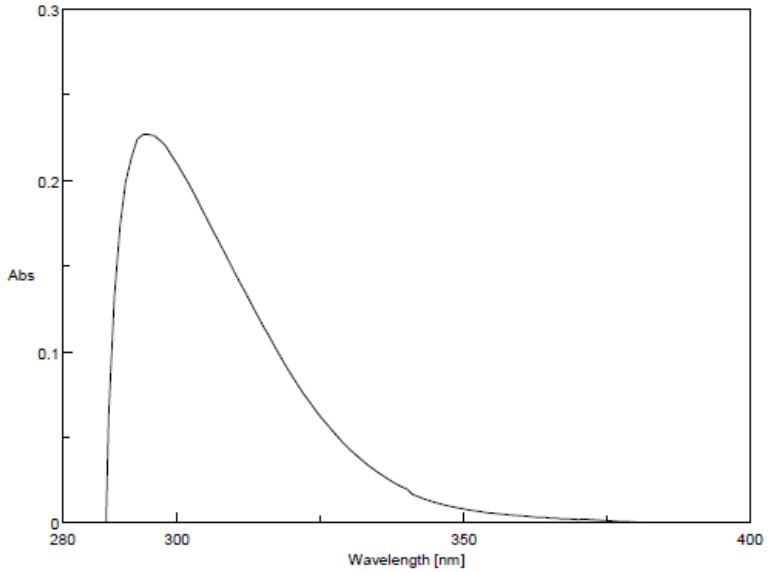


Table 19. Initial Chromatographic Conditions of Apixaban

Trial	Mobile phase ratio(v/v)	RT Min	Observation
Trial 1	50:50	10.842	Rt at 10.8 Min.
Trial 2	60:40	5.308	Fronting
Trial 3	65:35	3.450	Tailing
Trial 4	70:30	3.653	Tailing
Trial 5	75:25	3.136	Tailing
Trial 6	80:20	3.052	Tailing
Trial 7	85:15	2.917	Tailing

Fig. 14 HPLC Chromatogram for Trial 1

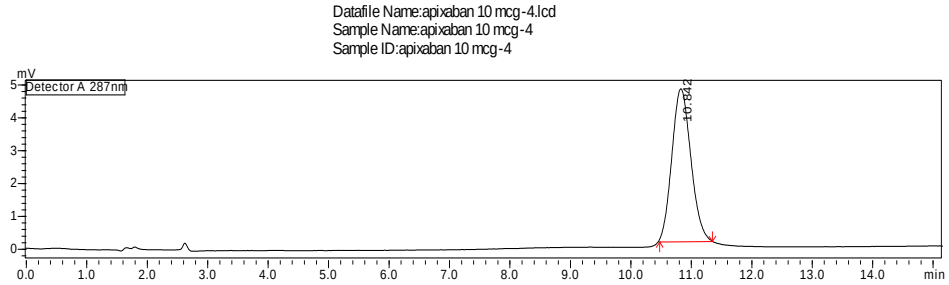


Fig.15 HPLC Chromatogram for Trial 2

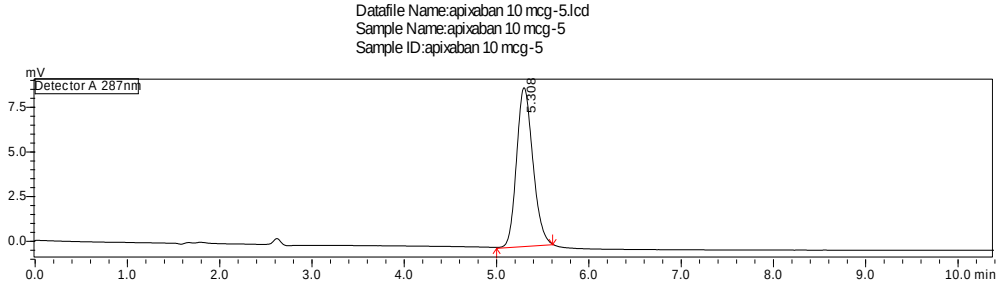


Fig.16 HPLC Chromatogram for Trial 3

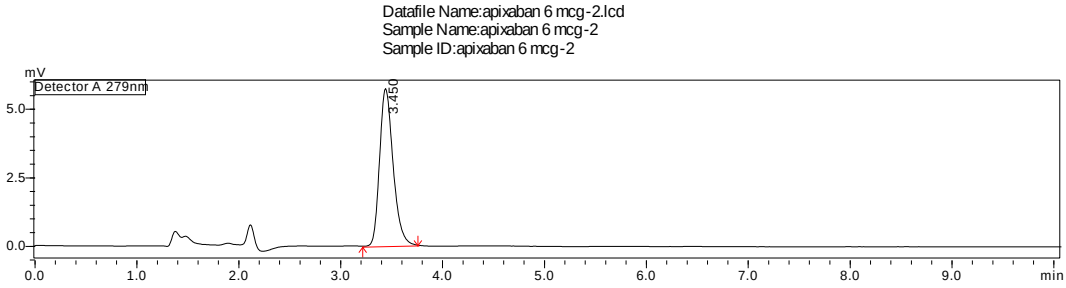


Fig.17 HPLC Chromatogram for Trial 4

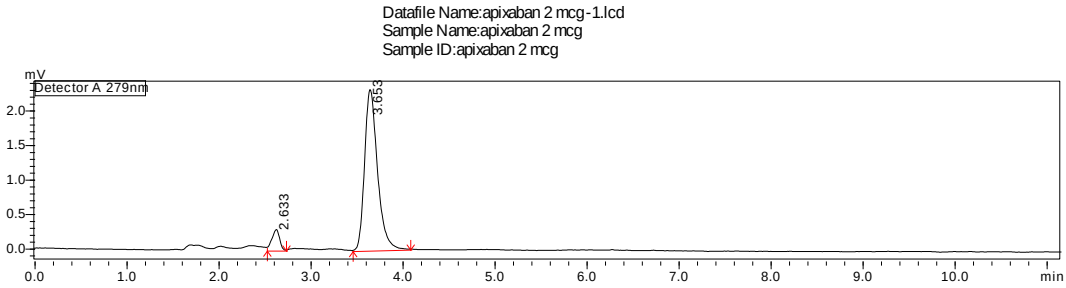


Fig.18 HPLC Chromatogram for Trial 5

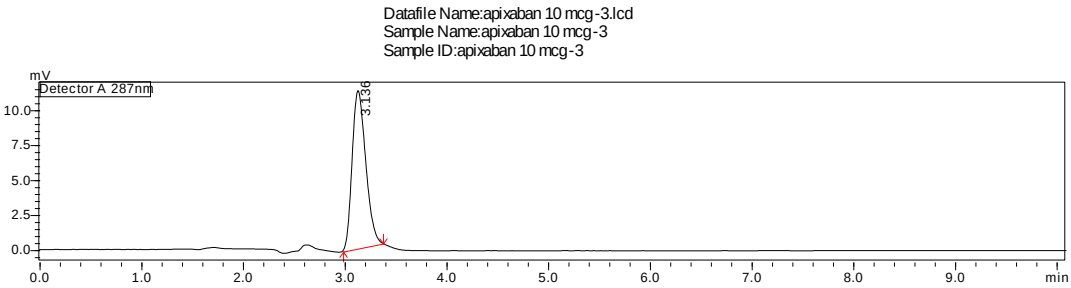


Fig.19 HPLC Chromatogram for Trial 6

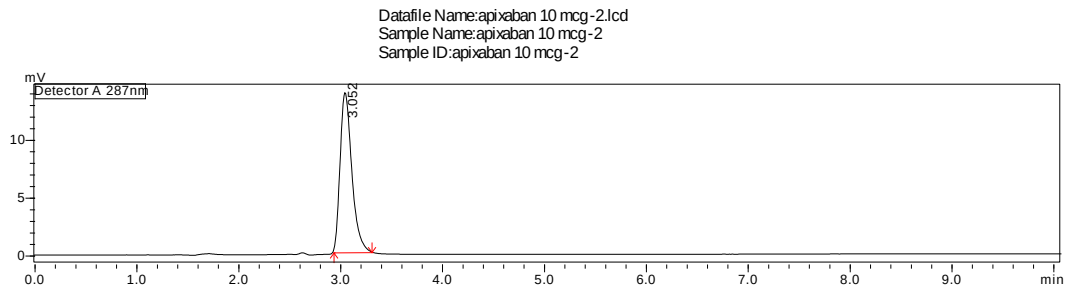


Fig.20 HPLC Chromatogram for Trial 7

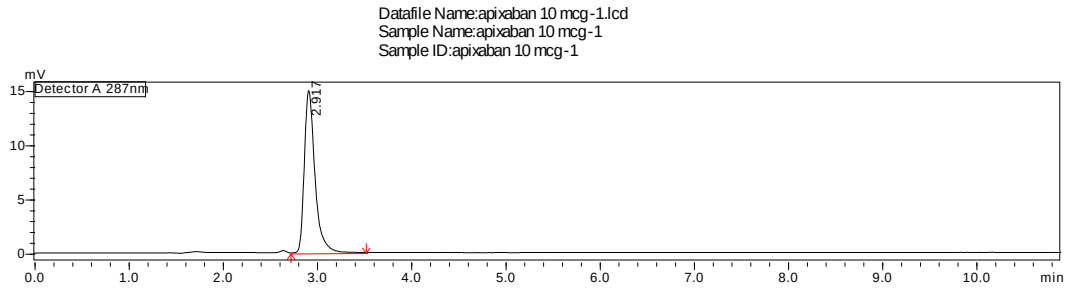


Fig.21 Representative HPLC Chromatogram for apixaban in fixed conditions

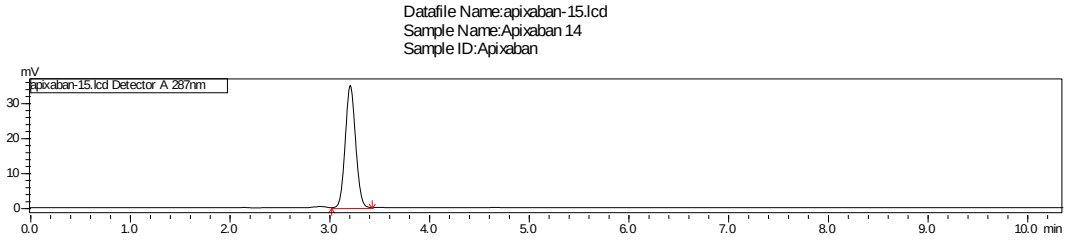


Table 20. Optimized chromatographic conditions for Apixaban

Parameters	Fixed experimental conditions
Column	250 mm x 4.6 id x 5 µ particle size
Wavelength(nm)	287
Mobile phase	H_2O:MeOH
Ratio (v/v)	10:90
Flow rate (ml/min)	1
Injection volume (µl)	10
Temperature of column (^oC)	Ambient temperature

Table 21. Calibration datafor Apixaban

Concentration mcg/ml	RT Min.	Peak area
2	3.199	37066
4	3.201	72930
6	3.211	105776
8	3.210	145550
10	3.211	180575
12	3.210	214180
14	3.214	252069
16	3.218	288872
18	3.216	326228
20	3.212	356833

Fig.22 Calibration graph of apixaban

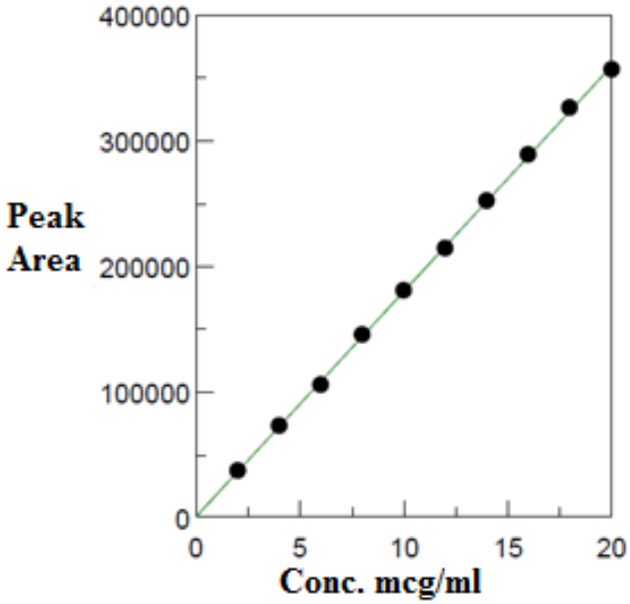


Fig.23 Overlay chromatogram of standards of apixaban

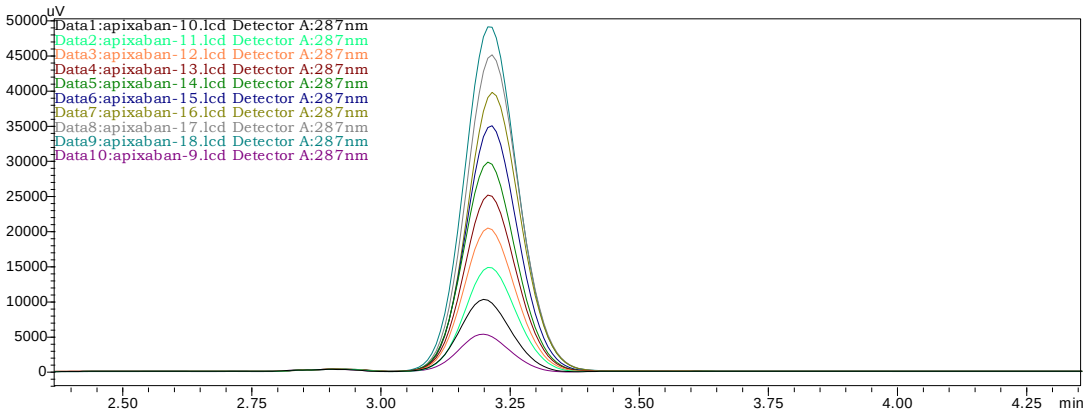


Table 22. Intraday precision of apixaban

Precision	Peak area	SD*	%RSD*
Intraday 1	179288	294.83	0.94
Intraday 2	179943		
Intraday 3	179456		
Intraday 4	179707		
Intraday 5	179665		
Intraday 6	179142		

*Average value

Table 23. Interday precision of apixaban

Precision	Peak area	SD	%RSD
Day 1	180224	477.56	0.27
Day 2	179349	454.49	0.82
Day 3	179868	648.33	0.59

*Mean of six determinations

Table 24. Accuracy studies for apixaban

Level	Amount of Std added Mg	Amount of Std recovered* Mg	% Recovery	SD	% RSD
50%	5	4.98	99.65	496.7	0.006
100%	10	9.88	98.80	277.5	0.001

*Mean of three determinations

Table 25. Robustness and Ruggedness

Parameter	Variation	Peak area	RT
Flow rate	0.9 ml	186575	3.566
	1.1ml	179112	2.936
Injection volume	9.9 µl	178724	3.216
	10.1 µl	181520	3.215
Mobile phase ratio(v/v)	+0.1ml	179949	3.220
	-0.1ml	179904	3.215
Ruggedness	Analyst 1	181121	3.214
	Analyst 2	181054	3.215

Table 26. Stability of Apixaban solution

Room Temperature		Refrigerator	
Time in Hours	Peak Area	Time in Hours	Peak Area
0	180575	0	180572
2	180101	6	180129
4	179660	12	178663
8	175332	24	176990
12	168009*	48	174887
24	159126	72	165997*
36	129114	84	150759

*Maximum time of stability

Table 27. System suitability parameters

Tailing factor	Efficiency (N)	Asymmetric factor
0.51	89002	1.0216

Fig.24 Representative chromatogram of apixaban formulation

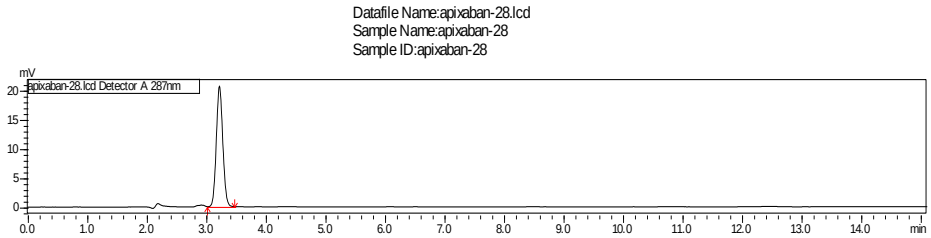


Table 28. Analysis of Apixaban formulation

Label claim	Amount found*	% Label claim	SD	% RSD
2.5 mg	2.49	99.6	98.5	0.62

*mean of six determinations

Fig.25 HPLC chromatogram of apixaban (Acid Hydrolysis – 0 hour)

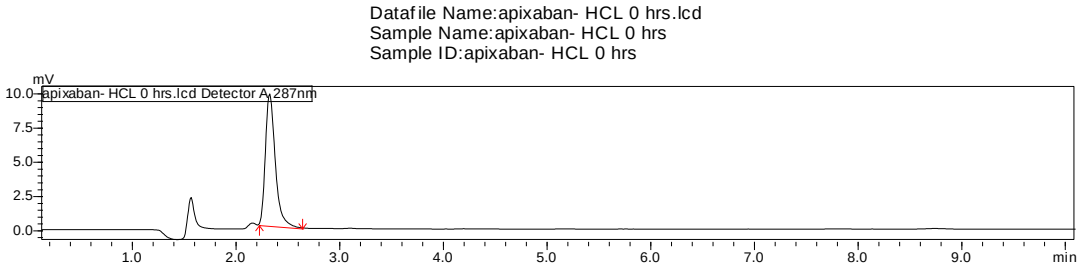


Fig.26 HPLC chromatogram of apixaban (Acid Hydrolysis – 4 hour)

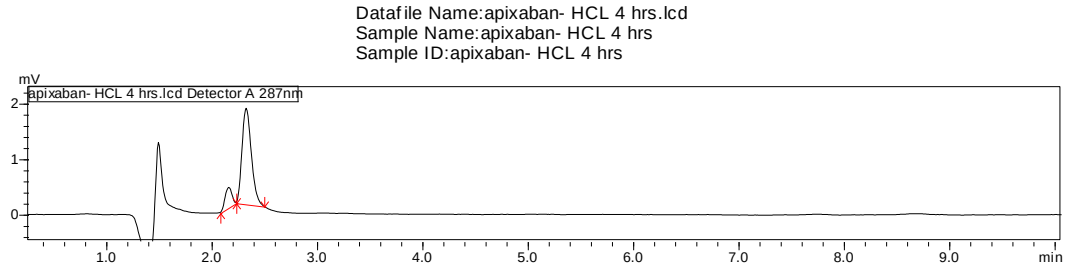


Fig.27 HPLC chromatogram of apixaban (Acid Hydrolysis – 8 hour)

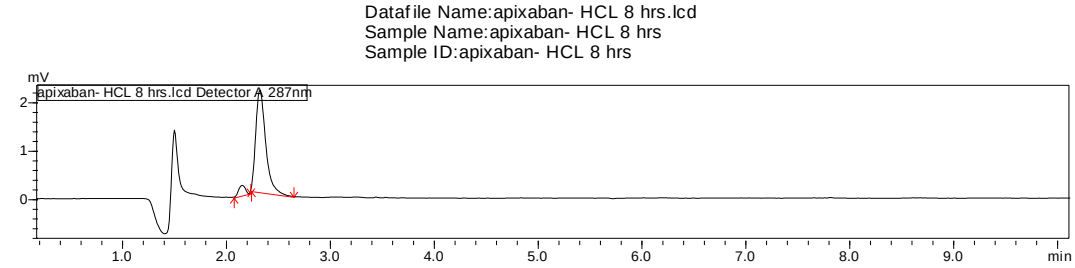


Fig.28 HPLC chromatogram of apixaban (Base Hydrolysis – 0 hour)

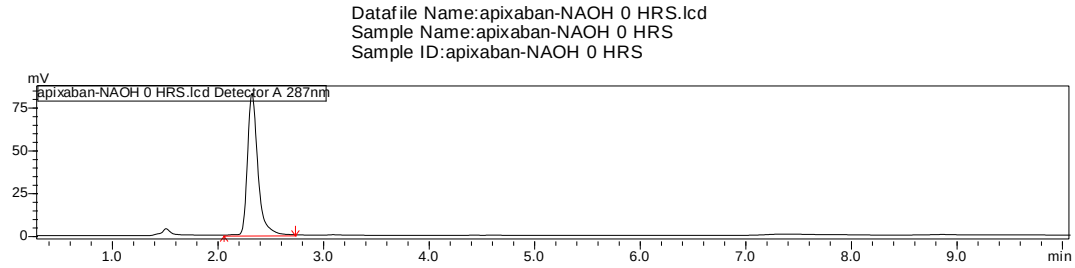


Fig.29 HPLC chromatogram of apixaban (Base Hydrolysis – 4 hour)

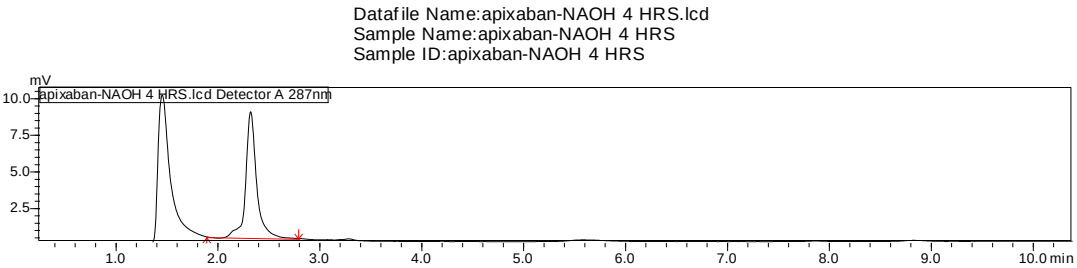


Fig.30 HPLC chromatogram of apixaban (Base Hydrolysis – 8 hour)

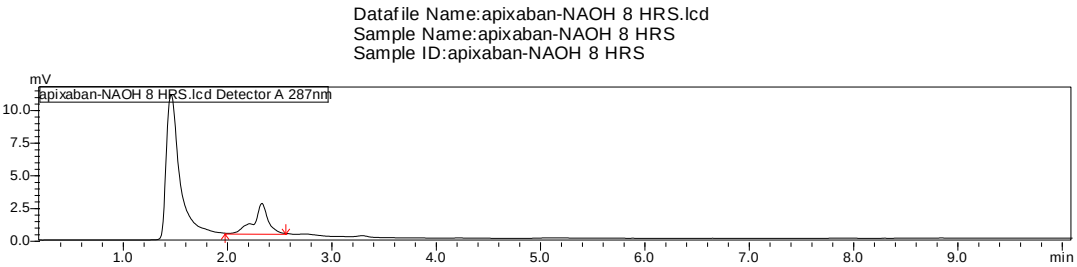


Fig.31 HPLC chromatogram of apixaban (Oxidation – 0 hour)

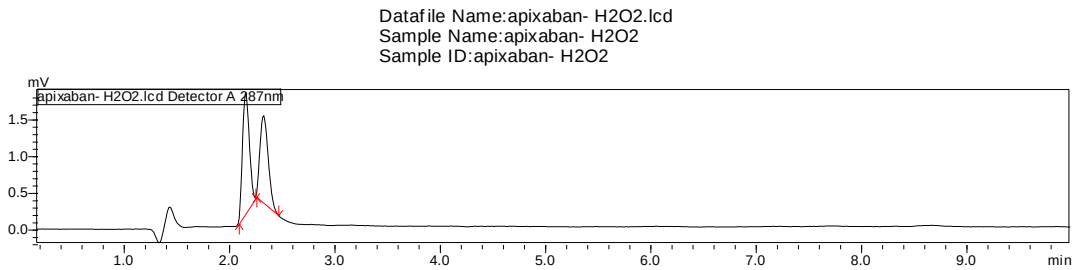


Fig.32 HPLC chromatogram of apixaban (Oxidation – 4 hour)

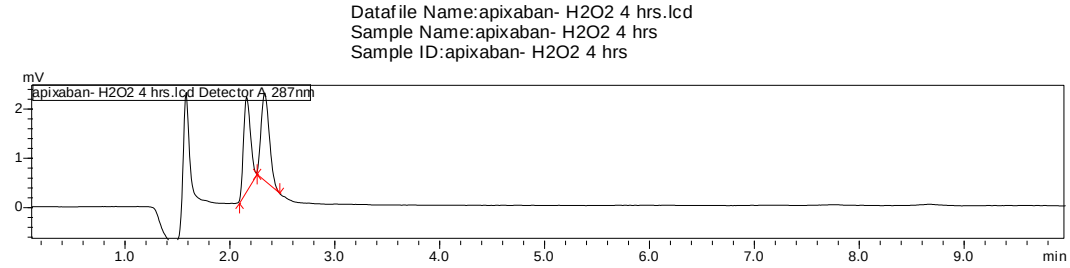


Fig.33 HPLC chromatogram of apixaban (Oxidation – 8 hour)

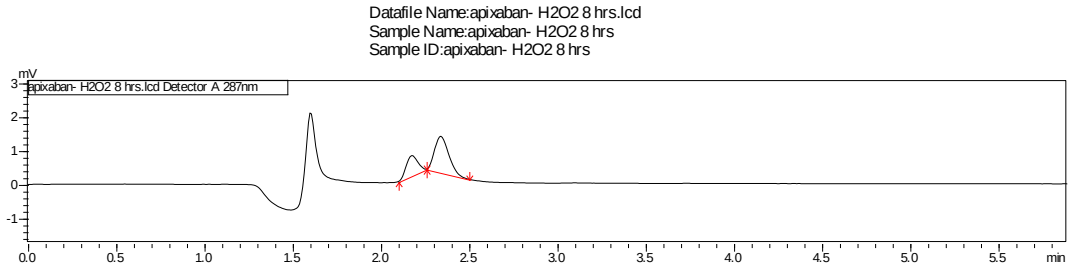


Fig.34 HPLC chromatogram of apixaban (40 ºC – 0 hour)

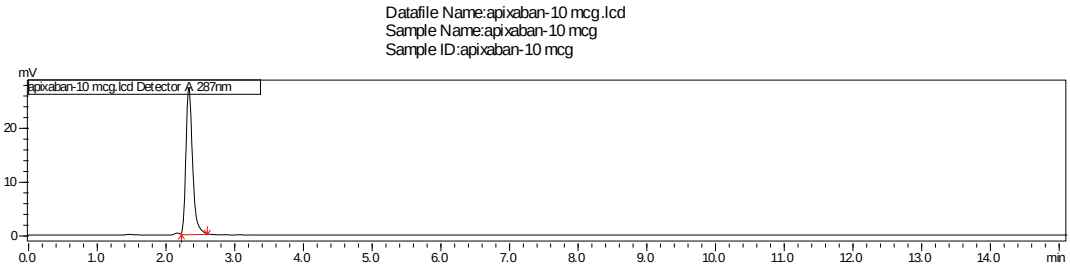


Fig.35 HPLC chromatogram of apixaban (40 ºC – 4 hour)

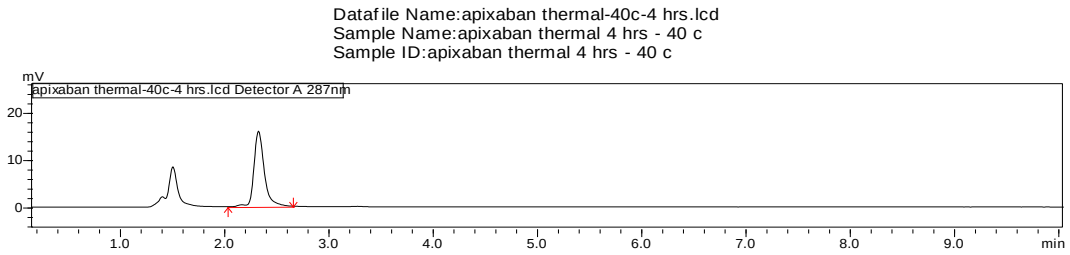


Fig.36 HPLC chromatogram of apixaban (40 ºC – 8 hour)

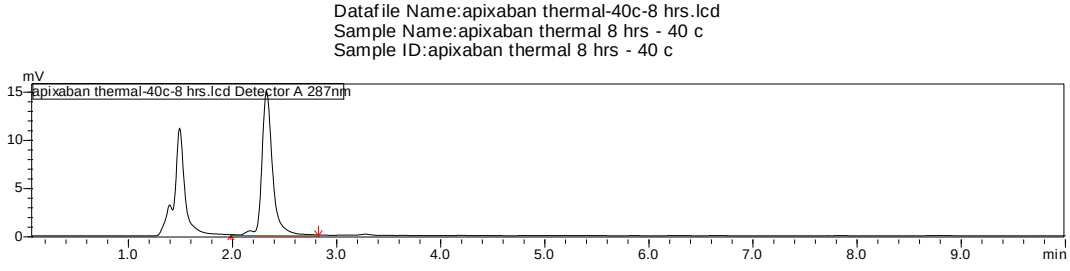


Fig.37 HPLC chromatogram of apixaban (80 ºC – 0 hour)

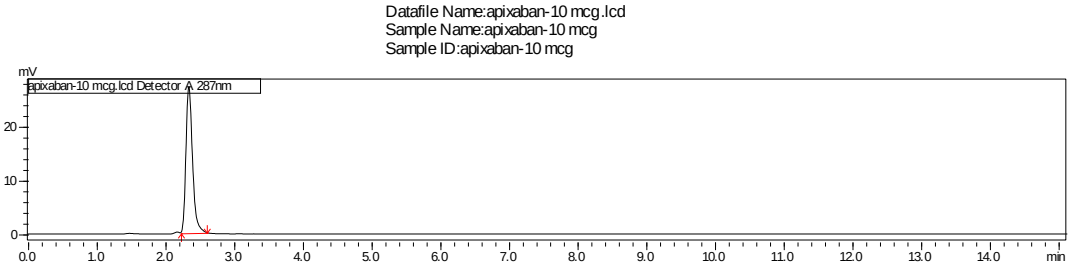


Fig.38 HPLC chromatogram of apixaban (80 ºC – 4 hour)

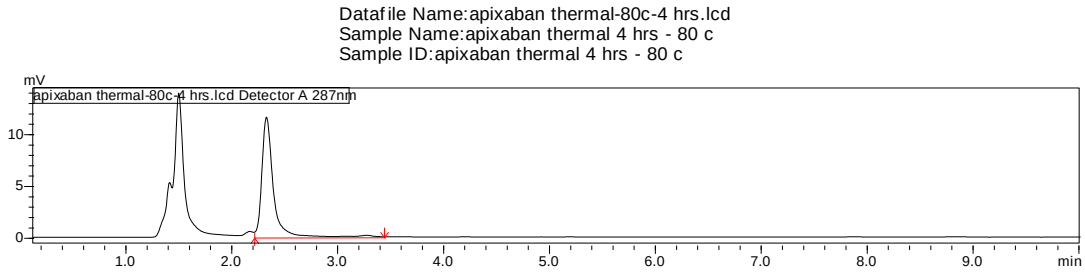


Fig.39 HPLC chromatogram of apixaban (80 ºC – 8 hour)

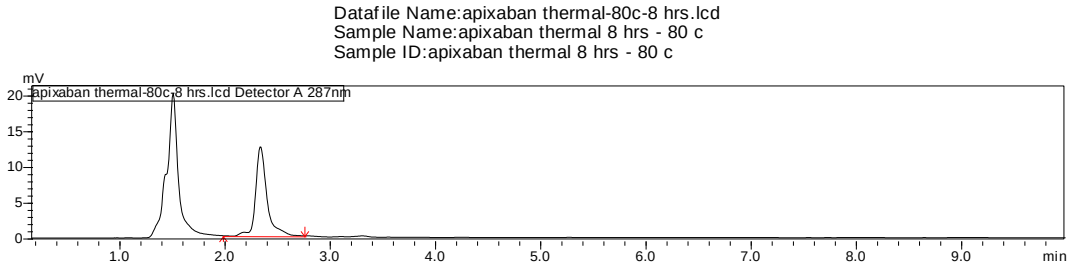


Fig.40 HPLC chromatogram of apixaban (Photolysis – 0 hour)

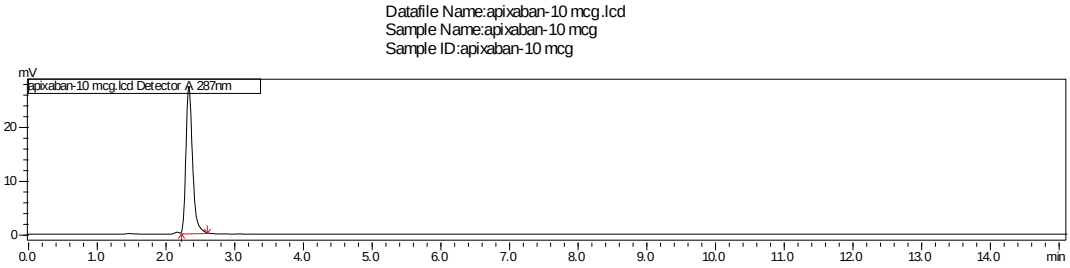


Fig.41 HPLC chromatogram of apixaban (Photolysis – 4 hour)

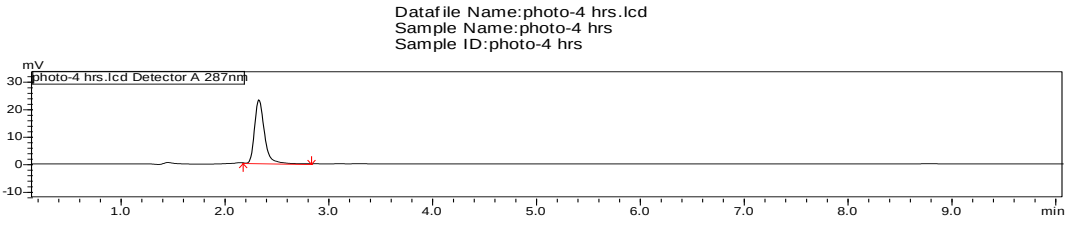


Fig.42 HPLC chromatogram of apixaban (Photolysis – 8 hour)

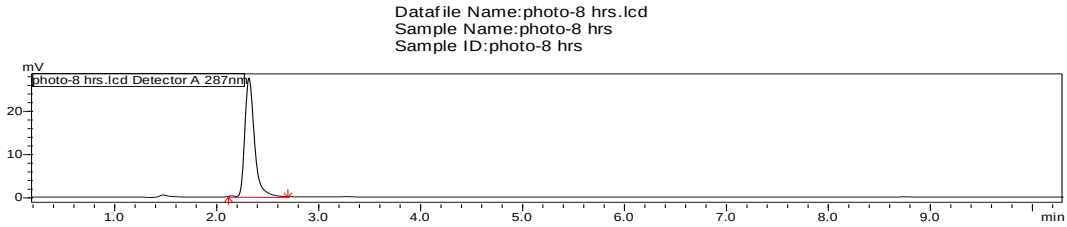


Table 29. Degradation studies of Apixaban by RP-HPLC

Sample name	RT Min.	% Degradation
Acid degradation 0 hrs	2.332	-
Acid degradation 4 hrs	2.168 2.333	- 56.64
Acid degradation 8 hrs	2.162 2.331	- 77.96
Base degradation 0 hrs	2.331	-
Base degradation 4 hrs	2.330	76.83
Base degradation 8 hrs	2.338	88.96
Oxidation 0 hrs	2.162 2.331	- -
Oxidation 4 hrs	2.168 2.340	3.62
Oxidation 8 hrs	2.178 2.341	7.34
Thermal 40 ºC 0 hrs	2.347	-
Thermal 40 ºC 4 hrs	2.337	20.41
Thermal 40 ºC 8 hrs	2.345	42.33
Thermal 80 ºC 0 hrs	2.347	-
Thermal 80 ºC 4 hrs	2.334	27.62
Thermal 80 ºC 8 hrs	2.336	48.81
Photolysis 0 hrs	2.347	-
Photolysis 4 hrs	2.335	0.16
Photolysis 8 hrs	2.326	0.97

7.2 RIVAROXABAN

7.2.1 DEVELOPMENT OF VALIDATED HPTLC METHOD FOR RIVAROXABAN

7.2.1.1 Chromatographic conditions

Selection of stationary phase

The HPTLC method was performed with precoated aluminium plates G_{60} F_{254} coated with silica gel particles.

Selection of mobile phase

A mobile phase in which water was replaced by toluene found good peak shape and R_f valueof rivaroxaban was found. Mobile phase system consisting of MeOH:EA:H_2O and TLN:EA:MeOH were prepared in different composition with increasing ratio of methanol. The sample spotted on the plate was developed. The drug migrated with the solvent front in the ratio of 2:6:2. At the ratio of 4:2:4, the R_f was 0.81 but with tailing of the peak. However in TLN:EA:MeOH, the R_f value was found 0.17, hence used for optimized of steps. The observations are shown in the table 30.

Optimization of variants in TLC

The primary factors which determines the R_f value, peak shape, area, asymmetric factor are mobile phase system, saturation time of the chamber, the distance of solvent front and spot bandwidth. These parameters were selected accordingly to suit the separation of rivaroxaban,the shift in the R_f value are presented in the table 31.

Composition of solvents in mobile phase

The composition of TLN;EA:MeOH was differentiated in the order of increasing ratio of toulene from 2 to 5 and the correct proportion was found to be 5:3:2 v/v/v(table 29). The selected composition was utilized for optimization of other parameters. Table 31 shows the effect of mobile phase ratio on peak R_f value.

Chamber saturation time

The separation of rivaroxaban was influenced by the time of saturation selected for the chamber. When the time below 15 minutes was tried, the peak fronting was seen. Increase in time upto 20 min and 30 min resulted in asymmetric peaks. A saturation time of 15 minutes was selected as the results were found to be appropriate.

Distance of solvent front

When the solvent was migrated to 7 cm in the plate from the R_f value was found 0.22 with tailing in the peak. Whereas the distance increased for the travel of solvent gave significant variation in the R_f value and the peak shape. So a distance of 8 cm was fixed for further studies as the R_f value was found to be 0.38 and tailing was observed to be less than 1.0 at 10% peak height.

Band width

The solution of rivaroxaban standard solution was spotted on the plate and was evaluated for the peak shape, area and integrity of the band. When the band width was 2 to 4mm, peak shape was narrow with considerable change in the peak area. The optimum band width was found to be 5mm which was found to result in compact band and acceptable peak area. The optimized chromatographic conditions are stated in the table 32.

7.2.1.2 Validation of HPTLC method

Linearity and range

The stock solution of drug Rivaroxaban applied on the 10 x 20cm plate by proportional increase in the volume applied between the range of 0.3 to 2.5µl of 400 mcg/ml of solution corresponding to 120ng/band and 1000ng/band. The plate was scanned and the spots detected at an R_f value of 0.37± 0.02. The results were subjected to regression analysis were y = 0.1416A-124.77 with correlation coefficient of 0.9961.Figure 43 shows the 3D densitogram for the linear range. The calibration data and the graph are shown in table 33 and figure 44, respectively.

Precision studies

Precision studies were carried out by replicate of standards and analyzed. Representative densitogram for the precision studies is given in the figure 45. The table 34 and 35 carries the value of 600ng/band of standard drug for the intraday and interday precision studies. Values were subjected to mathematical derivation of SD and %RSD as shown in the corresponding table.

System precision

System precision of the method developed was evaluated by six replicate injection of the standard solution of 600ng/band. Reading the densitogram for six consecutive scans of standard sample of 600ng/band was executed for the system precision studies. The data obtained are presented in the table 36 with the calculated SD and %RSD.

Accuracy

The accuracy was determined by spiking the assay sample with the standard drug at 50% and 100% level. The % recovery was calculated for the standard added to the assay sample. The SD and %RSD calculated for the recovered sample were presented in the table 37.

Robustness and ruggedness

Sample solutions were applied on the plate in the concentration of 600ng/band. The deliberate variations in the saturation time by ±2min, mobile phase ratio by ± 0.1 and 0.2ml for 5:3:2 ratio of TLN:EA:MeOH (v/v/v) were made and found that the change in the peak area and the R_f value was below the limit of 10%.

LOD and LOQ

The standard stock solution was diluted with the same ratio of solvent to obtain a solution of 40mcg/ml. From this 0.9µl, 0.7µl and 0.5µl and 0.2µl were applied in triplicate for the detection of LOD and LOQ. The detectable limit was found to be 8ng/band and quantifiable limit for the method was 28ng/band. Figure 46 and 47 depicts the densitogram of LOD and LOQ respectively.

Stability of developed plate

The volume spotted for the stability studies was 1.5µl (600ng/band) of 400 mcg/ml of the working standard and the stability of plate was checked periodically upto 8hours. The plate was found to be stable for 7hours after which the peak responses had abrupt change as given in table38.

System suitability parameters

The tailing factor, asymmetric factor and efficiency were computed with the results of the peak area of the standard drug and shown in the table 39.

7.2.1.3 Application of HPTLC method for the analysis of formulations

The rivaroxaban tablets were analyzed for the amount present and label claim present. The standard deviation and % relative standard deviation for the six replicate determinations are presented in the table 40. The representative densitogram is given in the figure.

Table 30. Selection of Mobile Phase System for Rivaroxaban

Mobile phase	**Ratio (v/v/v)**	**Observations R_f**
MeOH:EA:H_2O	2:6:2	Spot move with solvent front
	3:5:2	0.89
	4:4:2	0.81 and $T_f > 1$
	5:3:2	0.75 and $T_f > 2$
TLN:EA:MeOH	6:3:1	0.17 and $T_f = 1.0$

Table 31. Effect of composition of Mobile Phase System

TLN:EA:MeOH (v/v/v)	**R_f**
2:4:4	0.84
3:5:2	0.72
4:4:2	0.68
5:3:2	0.38

Table 32. Optimized Densitometric Conditions for Rivaroxaban

Parameter	**Fixed Condition**
Stationary phase	Precoated silica gel plate G_{60} F_{254}
Mobile phase	TLN:EA:MeOH
mobile phase ratio	5:3:2 v/v/v
Chamber saturation	15 minutes
Distance of solvent front	8 cm
Band width	5 mm
Slit dimensions	5 x 0.45 mm
Scan speed	20 mm/sec
Scan wavelength	249 nm

Fig.43 3D densitogram of standard Rivaroxaban

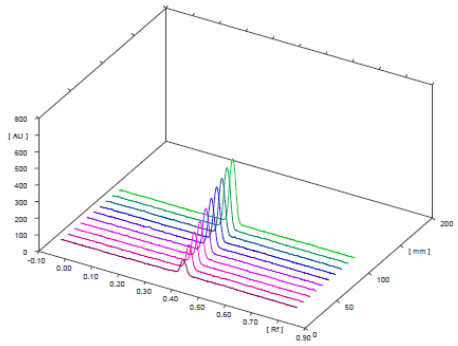


Table 33. Calibration data of Rivaroxaban

Concentration ng/band	**R_f value**	**Peak Area**
120	0.38	1577
200	0.38	2607
320	0.37	2458
400	0.37	4041
520	0.37	4899
600	0.36	5378
720	0.36	6140
800	0.35	6475
920	0.35	7241
1000	0.35	7517

Fig. 44 Calibration graph of Rivaroxaban

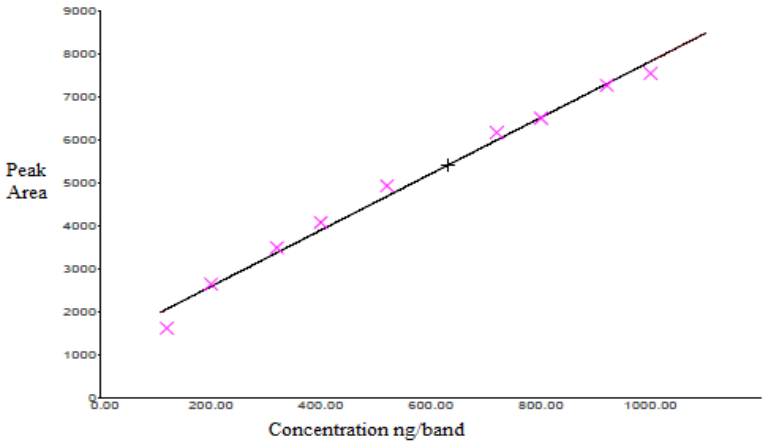


Fig.45 Representative densitogram for precision studies

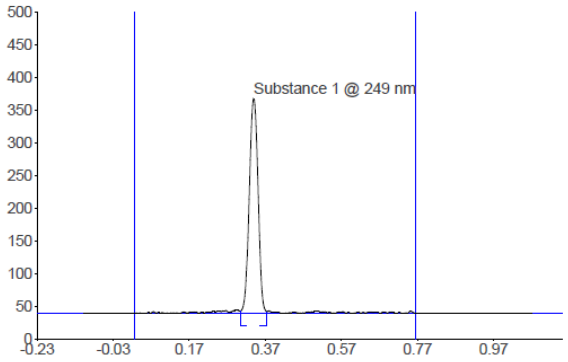


Table 34. Intraday Precision Studies of Rivaroxaban

Precision	Peak area	SD*	%RSD*
Intraday 1	5382	5.819	0.13
Intraday 2	5377		
Intraday 3	5379		
Intraday 4	5365		
Intraday 5	5374		
Intraday 6	5375		

*Average value

Table 35. Interday Precision Studies of Rivaroxaban

Precision	Peak area*	SD	%RSD
Day 1	5377	6.22	0.05
Day 2	5382	10.44	0.33
Day 3	5374	9.32	0.18

***Mean of six observations

Table 36. System precision of Rivaroxaban

Parameter	Peak area*	SD	%RSD
Repeatability of injection	5380	11.73	0.20
Repeatability of scanning	5372	6.44	0.07

***Mean of six observations

Table 37. Accuracy studies of Rivaroxaban

Level	Amount of Std added(mg)	Amount of Std recovered (mg)	% recovery of Std.*	SD	% RSD
50%	5	5.01	99.87	39.13	0.55
100%	10	10.10	99.93	28.09	0.38

Fig.46 Densitogram of LODof rivaroxaban

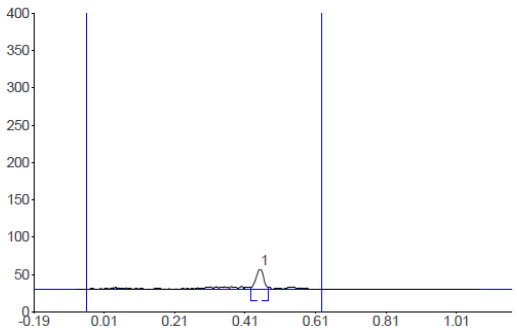


Fig.47 Densitogram of LOQof rivaroxaban

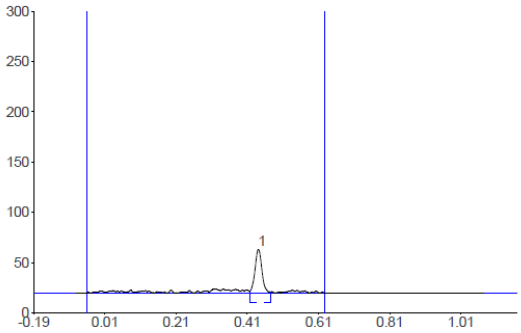


Table 38. Stability of Rivaroxabanon plate

Time in Hours	Peak area	SD	%RSD
0	5382	197.32	1.65
1	5367		
2	5345		
3	5322		
4	5310		
5	5299		
6	5250		
7	4941*		
8	4839		

***Maximum time of stability

Table 39. System suitability parameters for Rivaroxaban

Tailing factor	Asymmetric factor	Efficiency
0.5190	1.0659	1078

Fig.48 Densitogram of Rivaroxaban formulation

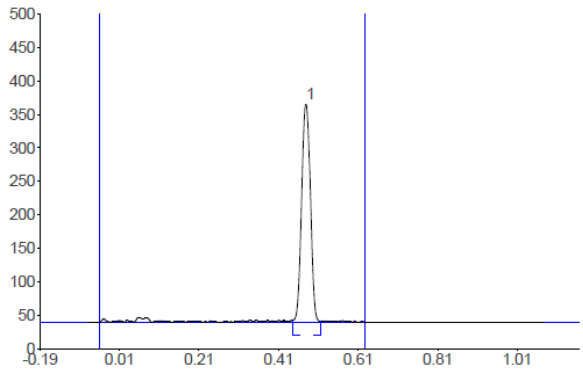


Table 40. Analysis of Rivaroxaban Formulation

Formulation	Label claim	Amount found*	% Label Claim	SD	% RSD
Xarelto tablets	10 mg	10.02 mg	100.2	113.0	0.70

7.2.2 DEVELOPMENT OF VALIDATED RP-HPLC METHOD FOR RIVAROXABAN

7.2.2.1 Chromatographic conditions

Selection of wavelength

Rivaroxaban working standard solution of 100 mcg/ml concentration was diluted to give a final solution of 10 mcg/ml. with the diluent 10mM ortho phosphoric acid. The solution was scanned in the UV region of 200 to 400nm and the λmax was found to be 249nm. The UV spectrum is given in the figure 48.

Optimization chromatographic conditions

The trial chromatograms of different ratio are shown in the figure 49 to 61. The RT of trials are also represented in the table 41. While increasing or decreasing the composition, peak shape got disturbed with either fronting or tailing. Finally a composition of 40:15:45 of Water:ACN: MeOH was optimized for the analysis of rivaroxaban. The optimized chromatographic conditions are shown in the table 42. Selection of the mobile phase system to obtain acceptable experimental responses for the drug is the foremost important criteria of analysis. A mobile phase consisted of Water:ACN:MeOH was tried with different ratio. The optimization was done in concern of the solvent system, injection volume, retention time and peak area of the drug rivaroxaban.

7.2.2.2 Validation of RP-HPLC Method for Rivaroxaban

Linearity and range

Linearity of rivaroxabanwas explored in the concentration range of 5 to 30mcg/ml. It was subjected to regression analysis. Regression data of linearity was computed as correlation co-efficient (r) of r^2> 0.99, slope of 20834.65, standard error of 1.0817 and y-intercept -16809 were derived from the graph. Figure 62 to 67 shows the standard chromatogram and figure 68 shows the linear graph. The calibration data is shown in the table 43.

Precision studies

Chromatogram of the standard solution analysed for six replicate times signifies the results were repeatable. The results of intraday precision values are presented in table 44. The interday precision studies were performed on three consecutive days are summarized in the table 45. The standard deviation and relative standard deviation were within the acceptable criteria.

Accuracy

The accuracy of the method was determined by calculating percent recovery of the drug by standard addition method. Percent recovery of apixaban was determined at two different level 50% and 100% of the target concentration in triplicate. The results of accuracy study are shown in table 46.

Robustness and ruggedness

A 10µl of drug solution of rivaroxaban in the concentration of 10mcg/ml was injected in the column for each condition. Minor variations in the flow rate ±0.1ml, injection volume ±0.1µl, mobile phase ratio ± 0.1ml/min. were studied and the responses were found to be robust i.e., below 10% deviation from the standard drug response. The ruggedness was evaluated by two analysts recording the chromatogram under similar operating conditions. The changes were within the limit specified. The ruggedness of the method was evaluated by assessing the results obtained by two analysts and found to have the %RSD less than 2 (Table 47).

LOD and LOQ

The amount of the drug Rivaroxaban was mathematically derived from the slope of the linearity curve(S) and the standard deviation of the measurements of the drug solution(σ). The amount detectable and quantifiable was 0.001 and 0.003 mcg/ml respectively.

Stability of solution

The standard solution (10µg/ml) was periodically assessed for the responses and it was observed that at stored room temperature it was stable upto 12hours. Periodic analysis of the sample kept in the refrigerator and was found to be stable for 36 hours. After which the solution shows a decrease in the signal by more than 10% of peak area. The corresponding values at each time interval for both the conditions are shown in table 48.

System suitability parameters

The system suitability was evaluated for the asymmetric factor, tailing factor at 10% height of peak. The values are shown in the table 49.

7.2.2.3 Application of RP-HPLC method for the analysis of formulations

A strength of 10mg tablet formulation of rivaroxaban analyzed by investigating the chromatogram of formulation sample for the peak area and quantifying in comparison with the standard drug peak area. The analysis of commercial formulation by the developed method indicated that the label claim for the tablets were within specified limit for tablets and capsules. The chromatogram of the formulation is shown in figure 69. The % label claim, standard deviation and relative standard deviation are presented in table 50.

7.2.2.4 Application for degradation studies of rivaroxaban

Degradation studies under various stress condition was applied for revealing the stability of the drug substance. The hydrolysis, thermolysis, oxidation and photolysis were selected according to the ICH guidelines.

Acid Hydrolysis (0.1 M HCl)

The sample was treated with acid and the 0hour sample was injected immediately after preparing the stress sample and repeated for every one hour till 4hour. Acid treated drug solution of rivaroxaban was identified at the retention time of 7.6min

and the additional peaks at 9.0, 10.8, 11.4 and 14.1min. The change in the peak area of the drug was more than 10% with the degradants eluted at separate retention time (Figure 70&71).The values are shown in the table51.

Base Hydrolysis (0.1 M NaOH)

Rivaroxaban was subjected to the stress condition of 0.1M sodium hydroxide and chromatogram was observed. The chromatograms were observed for reduction in peak area and additional peaks based on which the stability of drug was assessed. The drug was found to elute at 7.8min with additional degradant peaks at 6.6, 9.3, 10.5 and 21.1min. The change in the peak area was mathematically calculated with the response at the zero hour. The % degradation was found to be more than the specified limit (Figure 72&73). The % degradation values are depicted in the table52.

Oxidation (3% v/v Hydrogen peroxide)

Oxidation of the drug rivaroxaban with 3% v/v hydrogen peroxide was inspected for the drug peak at the retention time of 7.5min and degradant peak at 9.6 and 11.5min (Figure 74&75). The drug was prone to oxidation as the level of degradation was more than limit acceptable for the drug substance. The values are listed in the table 53.

Thermal degradation (80 °C)

At the elevated temperature the retention was at 7.6 min with the other peaks at 9.1 and 10.6min (Figure 76&77). Thermal degradation of sample shown that level of degradation was found to be more than 10% denoted by the peak area of the drug rivaroxaban. The values are summarized in the table 54.

Photolysis (light 220 V)

The drug solution was exposed to the light source according to the Q1B photostability testing of new drug substances and products by the ICH guidelines. The limit exposed should be not less than 200 watts. The drug was susceptible to degradation of more than 10%. The drug was found to elute at 7.4 min and degradant peak at 9.1, 9.4 and 11.4min. The distinguished peak area of the degradants were noted (Figure 78&79). The values are presented in the table 55.

7.2.2.5 Application of RP-HPLC method for impurity profiling of rivaroxaban

Separation of process impurity of rivaroxaban

HPLC chromatographic conditions

The method developed and validated for the drug rivaroxaban was implemented for the impurity profiling studies. The final dilution for the mixture of drug and impurity was tried with acetonitrile and the OPA solution for the proper peak shape and acceptable resolution. The buffer solution was found to have better resolution than the acetonitrile solution. Rivaroxaban and the impurity eluted at 8.1 and 11.4minutes, respectively in the buffer solution. The chromatograms are shown in figure 80 to 82 and the peak characters are present in the table 56.

Identification of impurities/degradants by LC-MS/MS technique

Fixed Mass Spectroscopy conditions

Systematic optimization of LC-MRM MS measurements shows that positive mode yielded a better mass spectrometer response than the negative mode. The most sensitive mass transitions of Rivaroxaban and the selected impurity were achieved by the clear separation in the chromatograms run for the individual degradation sample subjected to the stress conditions.

The drug rivaroxaban and its impurities were identified by monitoring the fragmentation of single-charged molecule ions (rivaroxaban + H^+) with m/z transitions of 435.9 into 144.8, 193.0, 205.3, 237.4,273.8, 318.3.

The mass spectra of rivaroxaban (API) at m/z 436 and the process impurity at m/z at 291 are shown in picture 83 and 84 respectively.

Acid hydrolysis

The chromatogram run in LC-MS/MS for the acid hydrolyzed sample at 4 hours is shown in the figure 85. The drug peak at 7.5minute with the impurity peak at 10.8 minute, whereas the degradants formed elute at 9.1 and 13.9 minute. The transitions of the $M+H^+$ for rivaroxaban at the retention time of 7.5 minute was into m/z of 427, 435.5, 436, 440, 441.9, 442.3 and 443 . The 442.3 is the base peak as given in the MS/MS spectrum figure 86.

At the retention of 10.8minute, the m/z transitions identified were 287.5, 292 and 293.8. The selected impurity of rivaroxaban with the m/z of 292 elutes at this retention time. The M+2 peak of m/z value of 294.2 forms as the base peak with most abundance. Spectrum recorded by MS/MS technique is shown in figure 87.

The retention time of 13.9 minute signifies the presence of m/z 273.1, 273.6, 274.5 and 279. The m/z value of 274 occurs as a transition ion in the Q3 scanning of pure drug rivaroxaban. Results obtained for the mass spectroscopy is given in figure 88.

Peaks identified with the corresponding m/z value at selected retention time are given in table 57. The proposed structure for the degradants formed is shown in table 58.

Base hydrolysis

The HPLC chromatogram of base hydrolysis reveals the drug peak at 7.7 minute with the additional peaks at 8.9, 10.7 and 12.9minutes (Figure 89). The transitions of the $M+H^+$ for rivaroxaban at the retention time of 7.7 minute was into m/z of 432.1 as the base peak, 433.1,434.6 and435.8. . The mass spectra of rivaroxaban is given in the Figure 90.The selected impurity of m/z value is 291identified at 10.7 minute. The transition peaks were found as ($M+H^+$) with m/z value of 292.2 and 292.7 are present as shown in the picture 91. The peak at 8.9 minutes was identified for the m/z values of 203.3 as the base peak and fragment peak of m/z value of 205.1. The m/z peak of 205 is one of the daughter ion formed in the Q3 positive mode scan of rivaroxaban. The figure 92 is the MS/MS spectrum at the selected retention time. At the retention of 12.9minutes, the m/z transitions identified were 192.5, 193, 195.3, 195.9, 196.6, 196.9 as the base peak with most abundance and 197.4. Them/z of 193 being one of the daughter ion of rivaroxaban was found at this retention time which is shown in Figure 93. The table 59 and 60 are the content for the identified and unidentified peaks at the selected retention time and proposed structure for the degradants.

Oxidation

Oxidation of rivaroxaban pure drug shows the drug peak at 7.4minute with the degradant peaks at 9.6 and 11.2minute (figure 94). The transitions of the $M+H^+$ for rivaroxaban at the retention time of 7.4 minutes was into m/z of 435.9, 436.6, 438.1, 438.9, 453, 455, 455.8, 458, 459.9, 461.7 as the base peak and 465.5. The corresponding LC-MS/MS results are given in figure 95.

The peak at 9.6 minutes was identified for the m/z values of 229, 230.3, 231, 233.5, 235.8 as the base peak, 237.3, 239.1 and 241.7. The transition ion of 237 is one of the daughter ion found in the fragmentation of the pure drug rivaroxaban as depicted in figure 96.

At the retention of 11.2 minutes, two m/z transitions of equal intensity were found for the mass of 291.7 and 292. The selected impurity of rivaroxaban identified at 11.2 minutes is shown in figure 97.

Identification of the mass peaks at the selected retention time of the HPLC chromatogram is given in table 61 with the proposed structure for the impurities in table 62.

Thermal degradation

The Q1 scanning of the transitions of rivaroxaban pure drug shows the drug peak at 7.6 minute with the degradant peaks at 9.3, 10.7 and 16.4 minutes. The chromatogram recorded is shown in the figure 98.

The peak at 7.6 minutes identified for the drug rivaroxaban presents transitions of m/z of 436, 438, 452.9, 455.5, 458.1 as the base peak, 458.8 and 460.2. The respective mass spectrum is shown in the figure 99.

The peak at 10.4 minutes was identified for the m/z values of 287, 292.1, 301.3, 303 where the m/z of 305.1 was found as the base peak. The MS/MS spectrum relevant to the retention time are shown in figure 100. The two peaks at 9.3 and 16.4 were not identified for any degradants. Identification of the mass peaks at the selected retention time of the HPLC chromatogram is given in table 63 with the proposed structure for the impurities in table 64.

Photolysis

The drug solution of Rivaroxaban under light after four hours shows the peak at 7.4 minute with the degradant peaks at 9.1, 9.4 and 11.4 minutes. The transitions of the $M+H^+$ for rivaroxaban at the retention time of 7.4 minute was into m/z of 435.1, 436.1, 437.5, 439.4, 452.9, 454.8, 456.3, 458.2 as the base peak, 459.2, 459.7, 460.1 and 460.7 . The corresponding chromatogram of LC analysis and MS/MS spectrum are shown in figure 101 and 102.

The peak at 9.1minute was identified for the m/z values 184.4, 192.3, 193.4 as the base peak, 197, 201, 205.1 fragment of the drug and 206.5. The m/z value of 192.3 is the fragment peak of API rivaroxaban in Q_3 screening by MS/MS technique. The figure 103 shows the mass spectrum for the identified impurity at the specific retention time.

At the retention of 9.4 minute, the m/z transitions of 272, 273 transition ion of rivaroxaban, 275, 276,8, 279, 280.2 and 281.1. The selected impurity with the m/z value 291 was identified at 11.4 minutes. The MS/MS analysis is shown in figure 104 and 105.

The list of peaks identified at the selective retention time is given in table 65. The proposed structure for each degradant identified is given in table 66.

Fig.49UV Spectrum of Rivaroxaban

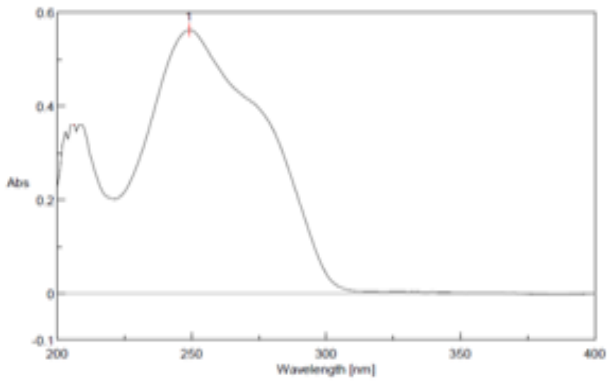


Fig. 50 HPLC Chromatogram of trial 1

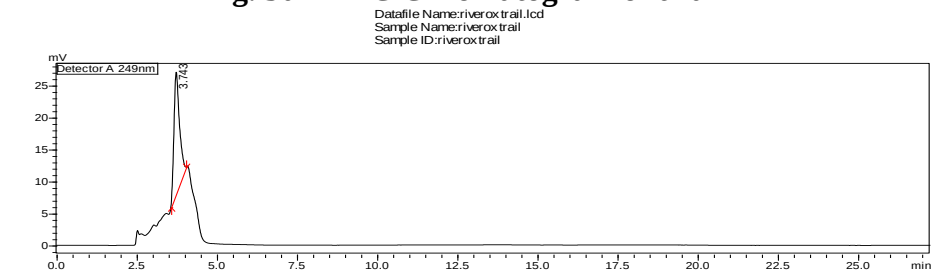


Fig. 51 HPLC Chromatogram of trial 2

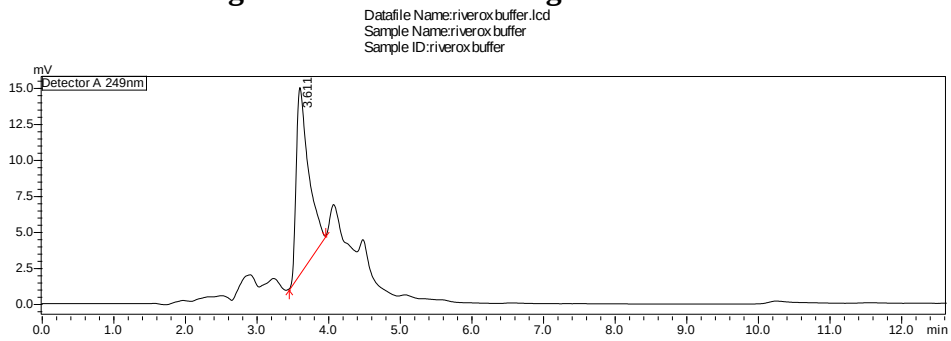


Fig. 52 HPLC Chromatogram of trial 3

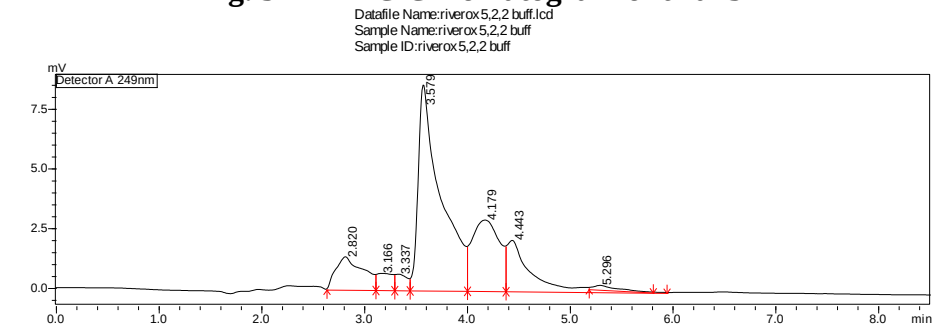


Fig. 53 HPLC Chromatogram of trial 4

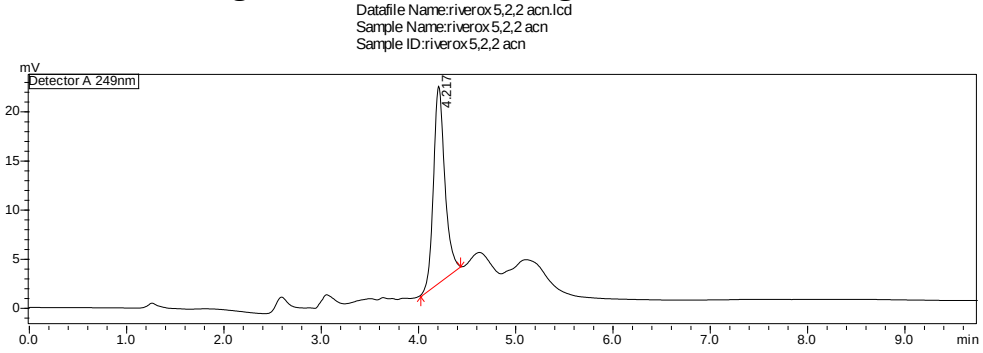


Fig. 54 HPLC Chromatogram of trial 5

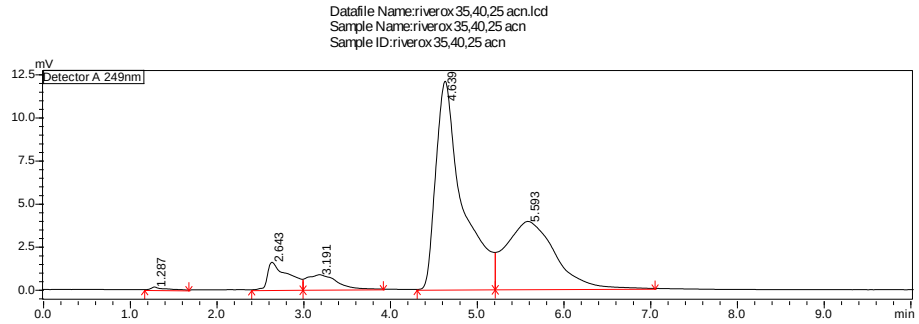


Fig. 55 HPLC Chromatogram of trial 6

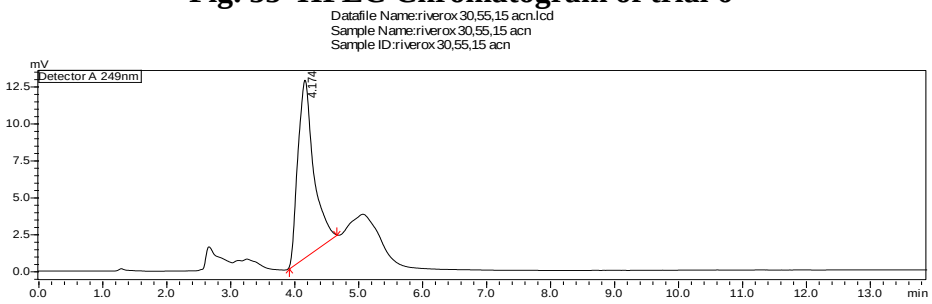


Fig. 56 HPLC Chromatogram of trial 7

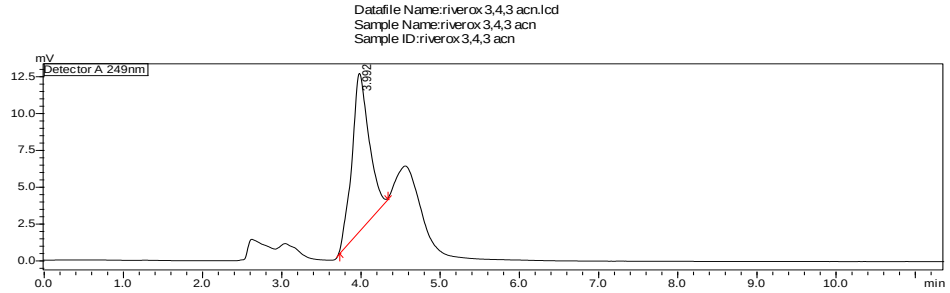


Fig. 57 HPLC Chromatogram of trial 8

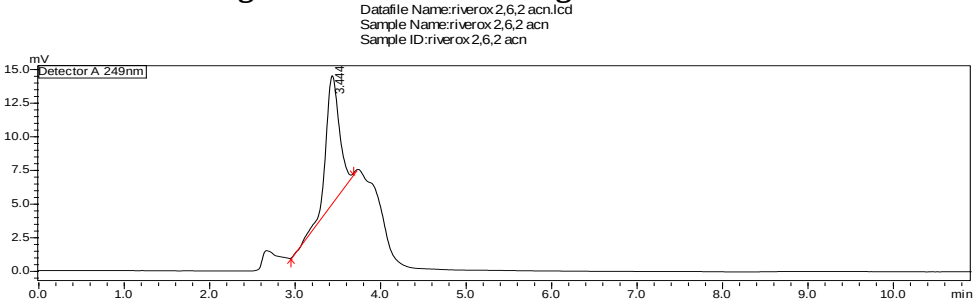


Fig.58 HPLC Chromatogram of trial 9

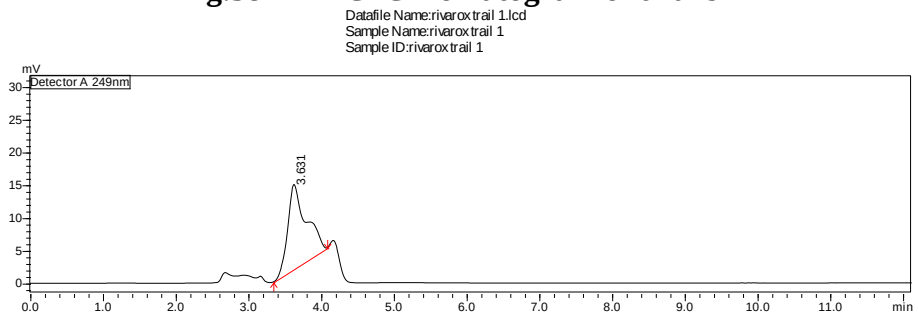


Fig. 59 HPLC Chromatogram of trial 10

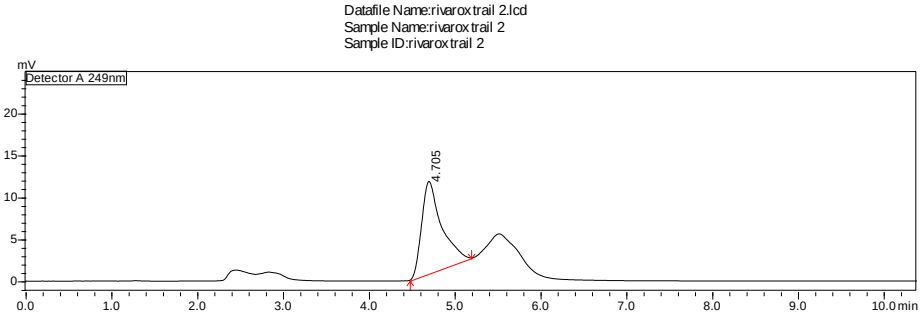


Fig.60 HPLC Chromatogram of trial 11

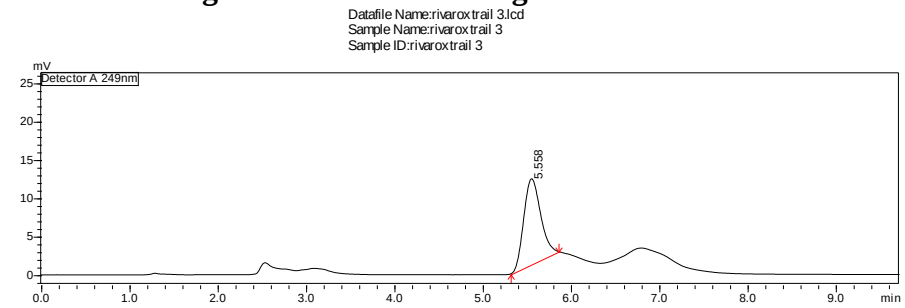


Fig. 61 HPLC Chromatogram of trial 12

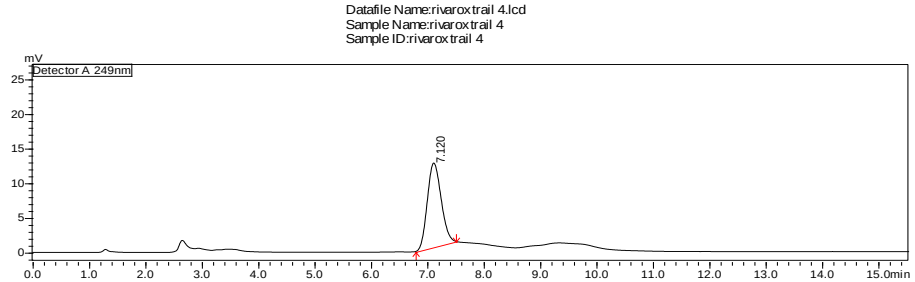


Fig. 62 HPLC Chromatogram of trial 13

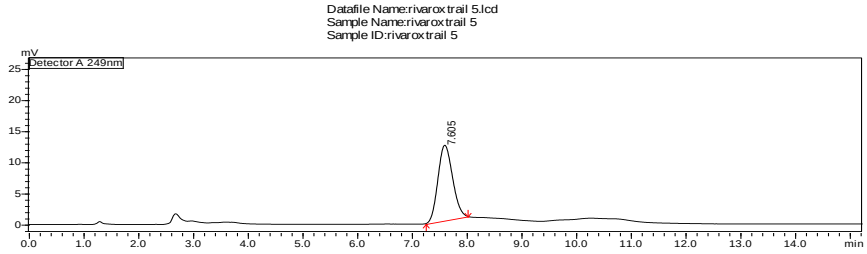


Table 41. Initial Chromatographic Conditions of Rivaroxaban

Trial	mobile phase ratio v/v/v	Injection Volume(µl)	RT Min.	Observations
Trial 1	50:25:25 in ACN	20	3.743	Tailing observed
Trial 2	50:25:25 in OPA	20	3.611	Split peak
Trial 3	40:30:30	10	3.579	Split peak
Trial 4	60:20:20	10	4.217	Split peak
Trial 5	40:25:35	10	4.639	Split peak
Trial 6	40:25:35	10	4.174	Split peak
Trial 7	30:55:15	10	3.992	Split peak
Trial 8	80:10:10	10	3.444	Peak asymmetric
Trial 9	40:35:25	10	3.631	Peak asymmetric
Trial 10	10:45:45	10	4.705	Tailing observed
Trial 11	20:35:45	10	5.558	Tailing observed
Trial 12	35:20:45	10	7.120	Tailing observed
Trial 13	40:15:45	10	7.605	Tailing observed

Table 42. Optimized chromatographic conditions of Rivaroxaban

Parameters	Fixed experimental conditions
Column	250 mm x 4.6 id x 5 µ particle size
Wavelength(nm)	249
Mobile phase	H_2O:ACN: MeOH
Ratio of mobile phase(v/v/v)	45:10:45
Flow rate(ml/min)	1
Injection volume(µl)	10
Temperature of column(^oC)	Ambient temperature

Fig.63 Standard chromatogram of Rivaroxaban (5mcg/ml)

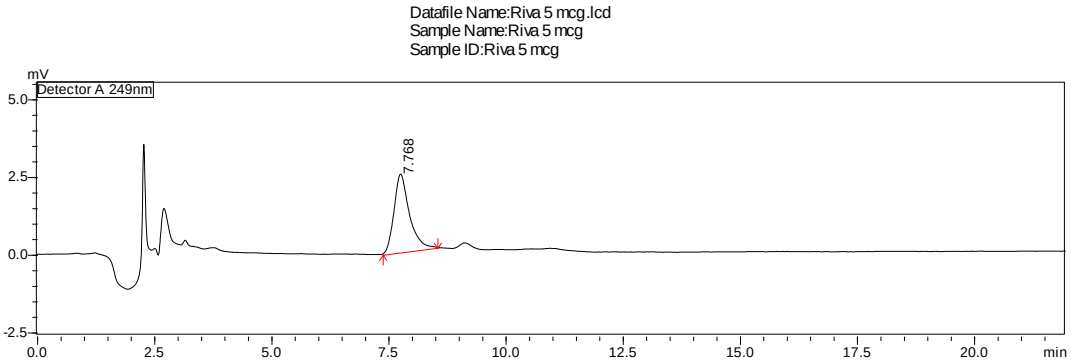


Fig. 64 Standard chromatogram of Rivaroxaban (10mcg/ml)

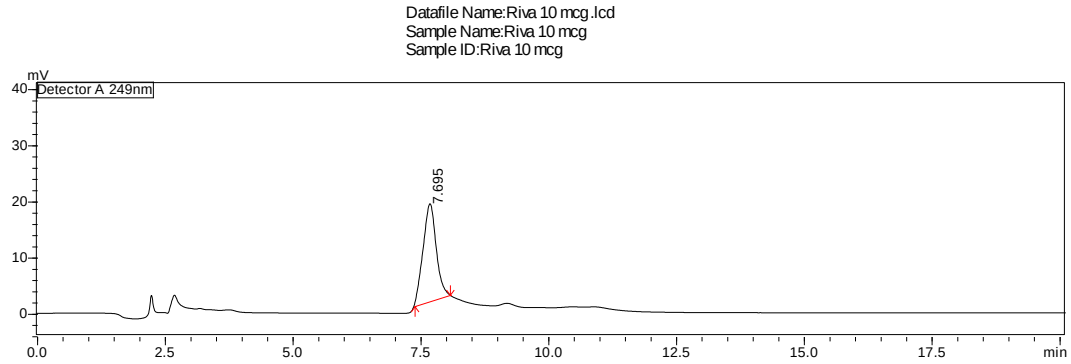


Fig. 65 Standard chromatogram of Rivaroxaban (15mcg/ml)

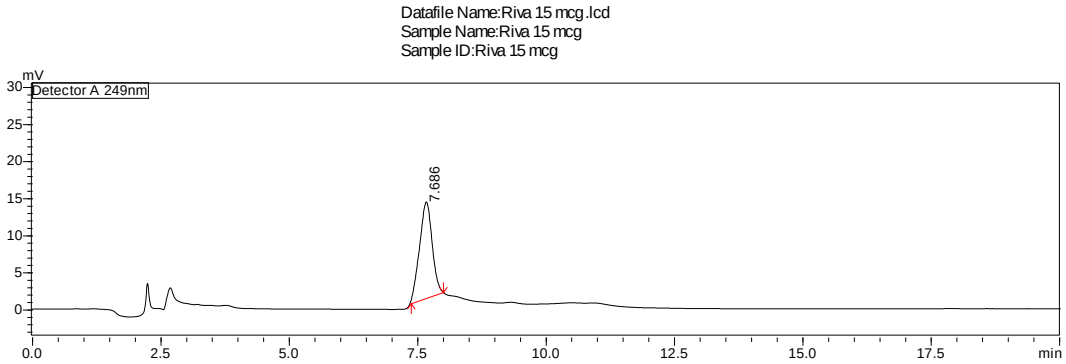


Fig.66 Standard chromatogram of Rivaroxaban (20mcg/ml)

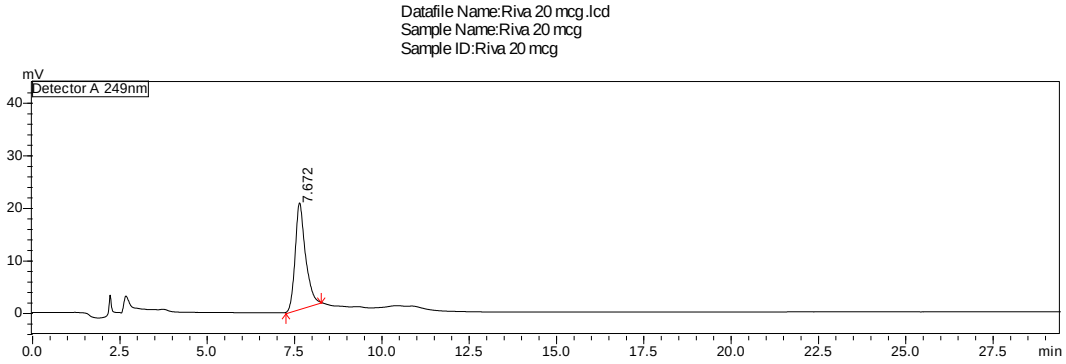


Fig.67 Standard chromatogram of Rivaroxaban (25mcg/ml)

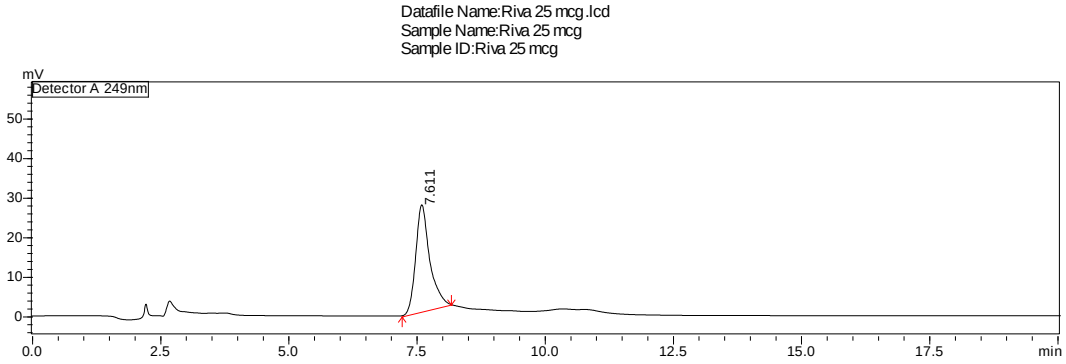


Fig.68 Standard chromatogram of Rivaroxaban (30mcg/ml)

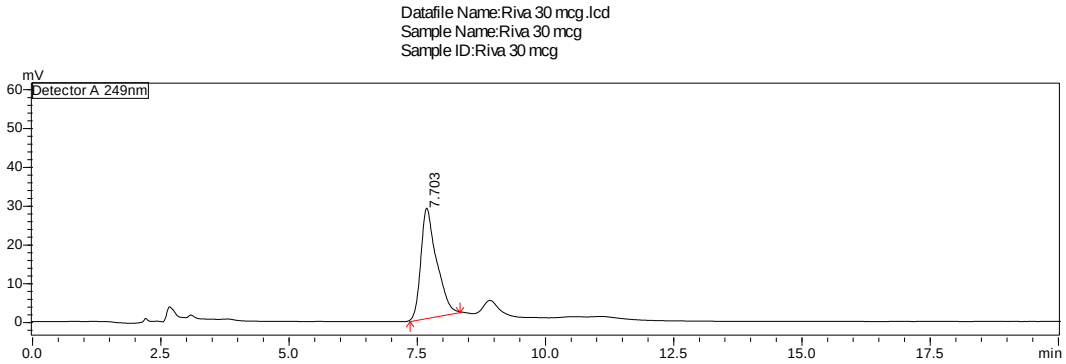


Table 43. Calibration data for Rivaroxaban

Concentration mcg/ml	RTMin.	Peak area
5	7.768	56631
10	7.695	217536
15	7.686	307629
20	7.672	405933
25	7.611	506018
30	7.703	593104

Fig.69- Calibration graph of Rivaroxaban

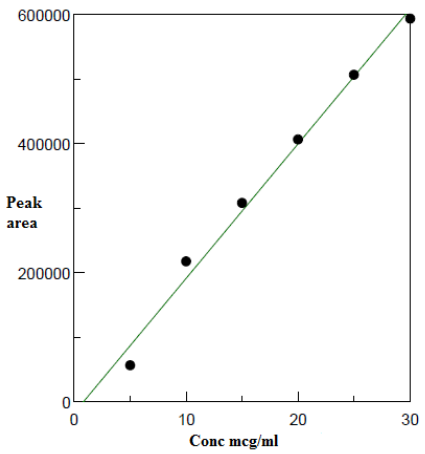


Table 44. Intraday precision studies of Rivaroxaban

Precision	Peak Area	SD*	%RSD*
Intraday 1	218004	203.86	0.84
Intraday 2	217993		
Intraday 3	217588		
Intraday 4	217865		
Intraday 5	217744		
Intraday 6	217521		

*Average value

Table 45. Interday precision studies of Rivaroxaban

Precision	Peak area*	SD	%RSD
Day 1	218291	187.4	0.16
Day 2	217982		
Day 3	217954		

*Mean of Six observations

Table 46. Accuracy studies of Rivaroxaban

Level	Amount of Std added mg/ml	Amount of Std* recoveredmg/ml	% Recovery of the Std.	SD	% RSD
50%	5	4.94	98.8	188.42	0.006
100%	10	10.11	101.1	204.33	0.009

*Mean of three observations

Table 47. Robustness and ruggedness of Rivaroxaban

Parameter	Variation	Peak area	RT
Flow rate	0.9 ml	217510	8.966
	1.1ml	217490	7.451
Injection volume	9.9 µl	217532	8.152
	10.1 µl	217558	8.039
Mobile phase ratio(v/v)	+ 0.1 ml	217506	8.178
	-0.1 ml	217489	8.911
Ruggedness	Analyst 1	217533	7.908
	Analyst 2	217521	7.972

Table 48. Stability of Rivaroxaban solution

Room Temperature		Refrigerator	
Time in Hours	**Peak area**	**Time in Hours**	**Peak area**
0	217604	0	217599
4	212290	12	201198
8	205532	24	198338
12*	199453	36*	196954
24	195805	48	195740
28	193077	60	189005

*Maximum time of stability

Table 49. System suitability parameters for Rivaroxaban

Tailing factor	Efficiency (N)	Asymmetric factor
1.11	77698	0.83

Fig.70 HPLC Chromatogram of Rivaroxaban formulation

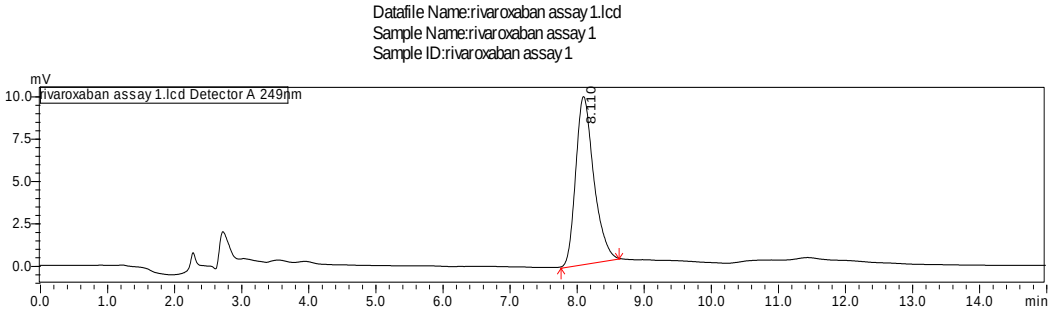


Table 50. Analysis of Rivaroxaban formulation

Formulation	Label claim	Amount found*	% Label Claim	SD	% RSD
Xarelto tablets	10 mg	10.06 mg	100.6	0.04	0.45

*Mean of six observations

Fig.71 HPLC Chromatogram of Rivaroxaban (Acid hydrolysis– 0hour)

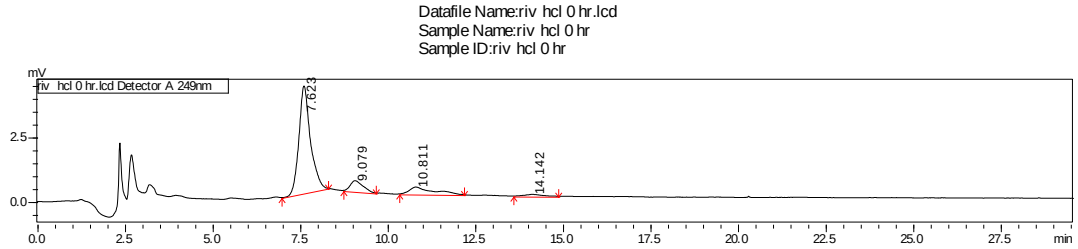


Fig.72 HPLC Chromatogram of Rivaroxaban (Acid hydrolysis– 4 hour)

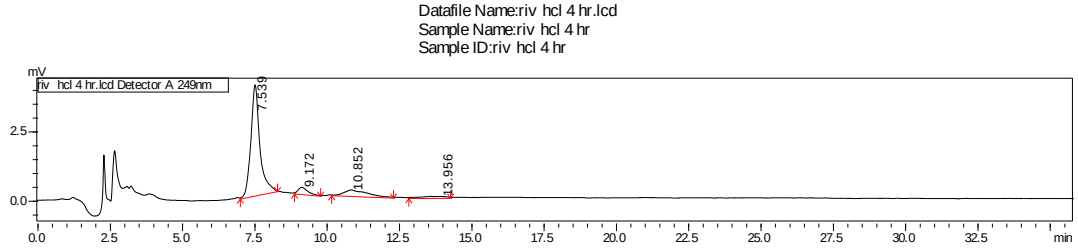


Table 51. Acid hydrolysis of Rivaroxaban

Time Hour	RT Min	Peak Area	% Drug Remaining	% degradation
0	7.623 9.079 10.811 14.142	94467 10180 12861 2018	43.42	56.58
4	7.539 9.172 10.852 13.956	85204 4835 9941 1759	39.16	60.84

Fig.73 HPLC Chromatogram of Rivaroxaban (Base hydrolysis– 0hour)

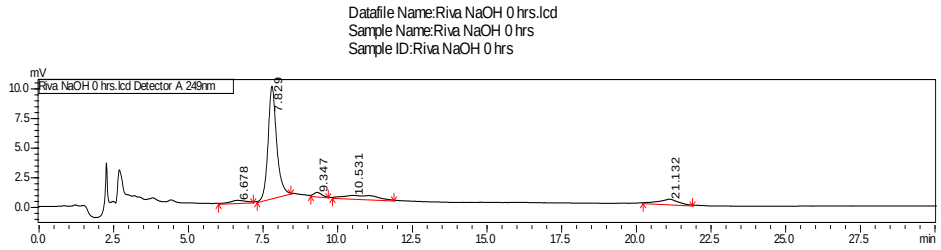


Fig.74 HPLC Chromatogram of Rivaroxaban (Base hydrolysis– 4 hour)

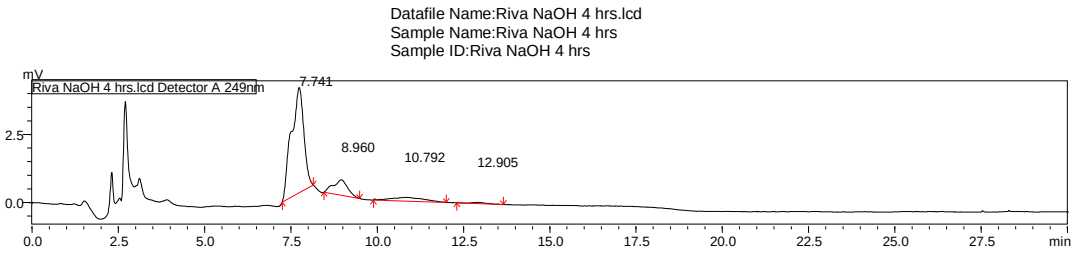


Table 52. Base hydrolysis of Rivaroxaban

Time Hour	RT Min	Peak Area	% Drug Remaining	% degradation
0	6.678 7.829 9.347 10.531 21.132	5067 185511 5142 19140 16257	85.27	14.73
4	7.741 8.960 10.792 12.905	79284 16620 9092 1374	36.44	63.56

Fig.75 HPLC Chromatogram of Rivaroxaban (Oxidation – 0hour)

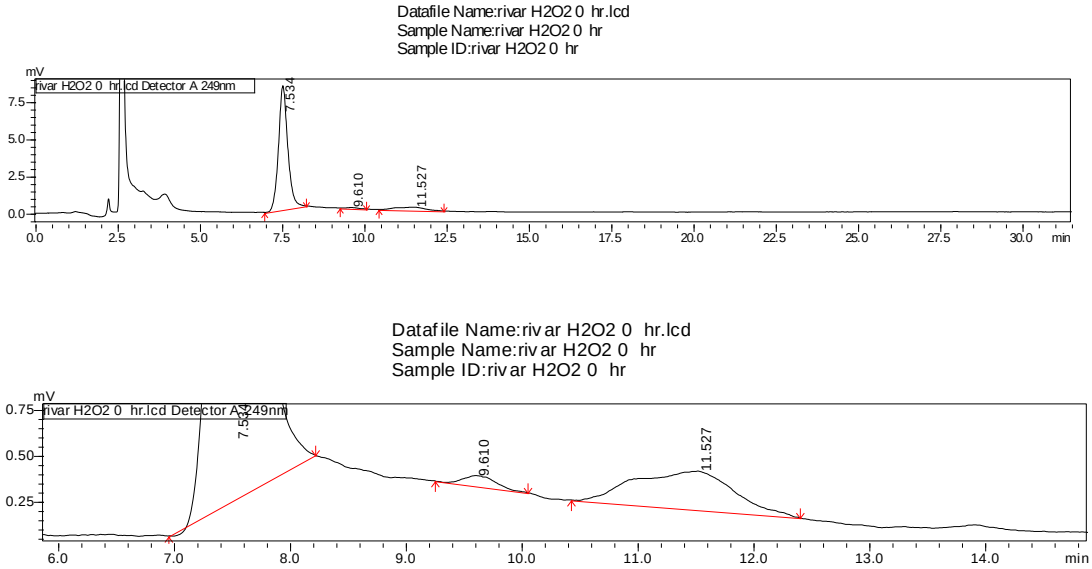


Fig.76 HPLC Chromatogram of Rivaroxaban (Oxidation – 4 hour)

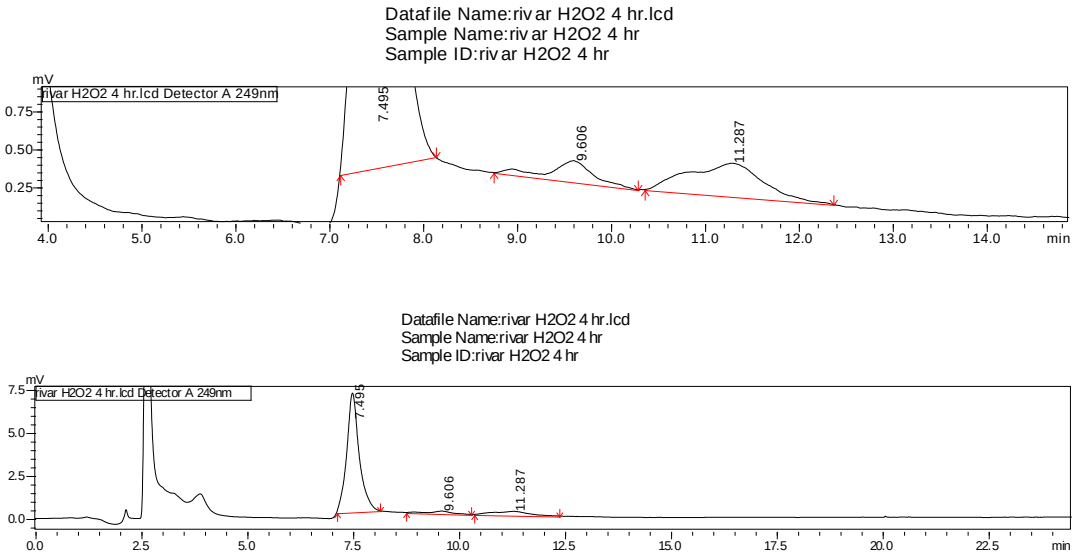


Table 53. Oxidation of Rivaroxaban

Time Hour	RT Min	Peak area	% Drug Remaining	% degradation
0	7.534 9.610 11.527	161177 1041 12156	74.02	25.98
4	7.495 9.606 11.287	140877 2534 11755	64.76	35.24

Fig.77 HPLC Chromatogram of Rivaroxaban (80ºC – 0hour)

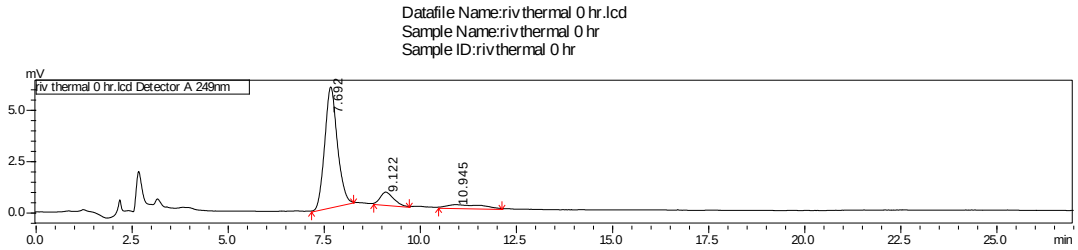


Fig.78 HPLC Chromatogram of Rivaroxaban (80ºC – 4 hour)

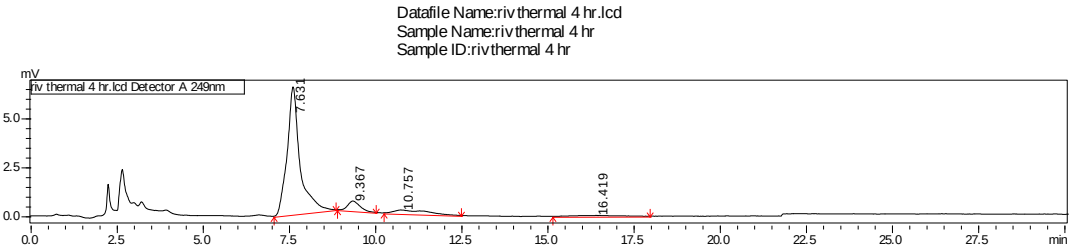


Table 54. Thermal degradation 80 °C

Time Hour	RT Min	Peak Area	% Drug Remaining	% degradation
0	7.692 9.122 10.945	132221 13952 8751	76.87	23.13
4	7.631 9.367 10.757 16.419	167239 11806 11785 3115	60.78	39.22

Fig.79 HPLC Chromatogram of Rivaroxaban (Photolysis- 0 hour)

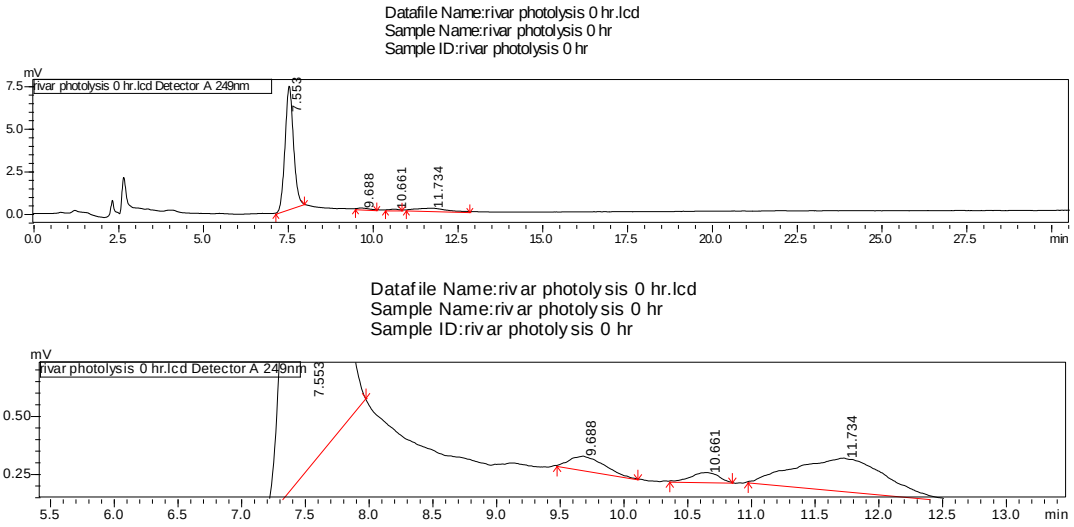


Fig.80 HPLC Chromatogram of Rivaroxaban (Photolysis- 4 hour)

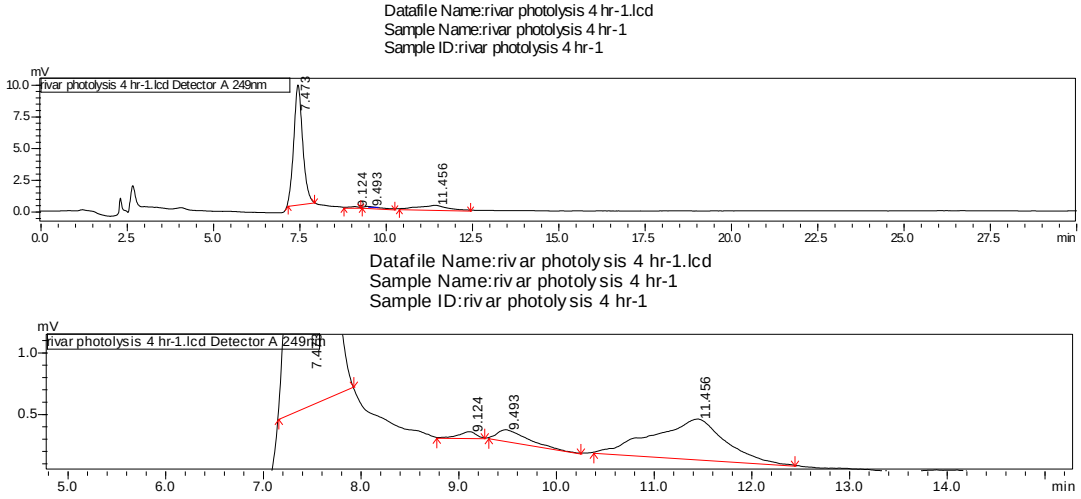


Table 55. Photolysis of Rivaroxaban

Time Hour	RT Min	Peak Area	% Drug Remaining	% degradation
0	7.473 9.124 9.493 11.456	162857 506 1750 16221	76.52	23.48
4	7.473 9.698 10.428 11.59	102076 1040 819 4707	46.61	53.39

Fig. 81HPLC chromatogram of Rivaroxaban API

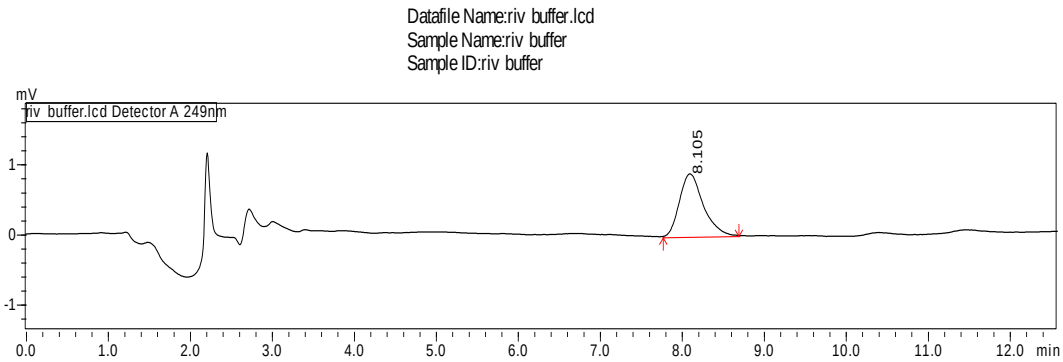


Fig. 82 HPLC chromatogram of Rivaroxaban Impurity

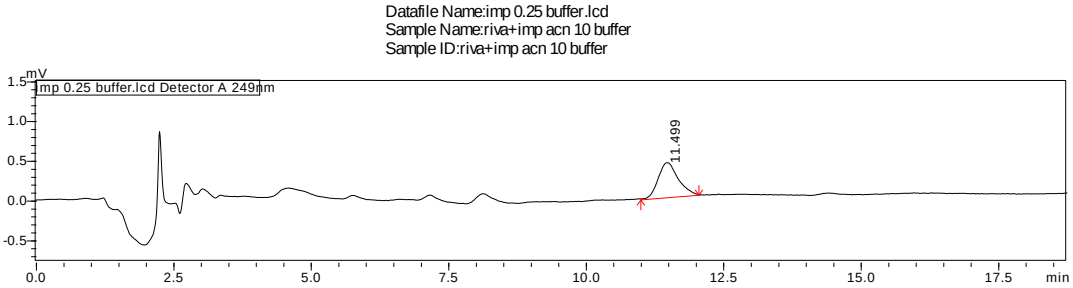


Fig.83 HPLC chromatogram of mixture (Rivaroxaban API+ impurity)

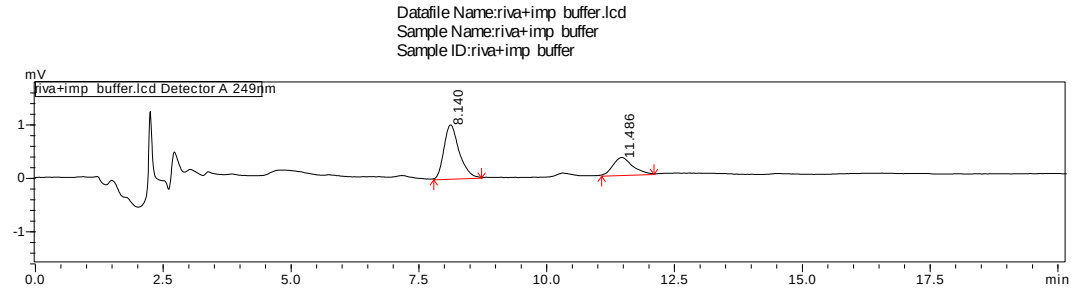


TABLE 56. The peak characters of rivaroxaban and its impurity

S.No	Sample name	Retention time	Peak area	Tailing factor	Peak resolution
1.	Rivaroxaban 1 mcg	8.105	17730	1.269	-
2.	Impurity 0.25 mcg	11.499	10591	1.188	-
3.	Riv+Imp 1+0.25 mcg	8.140 11.486	20309 8173	1.266 1.220	5.616

Fig. 84 Q3 Mass Spectra of Rivaroxaban API (m/z value 436)

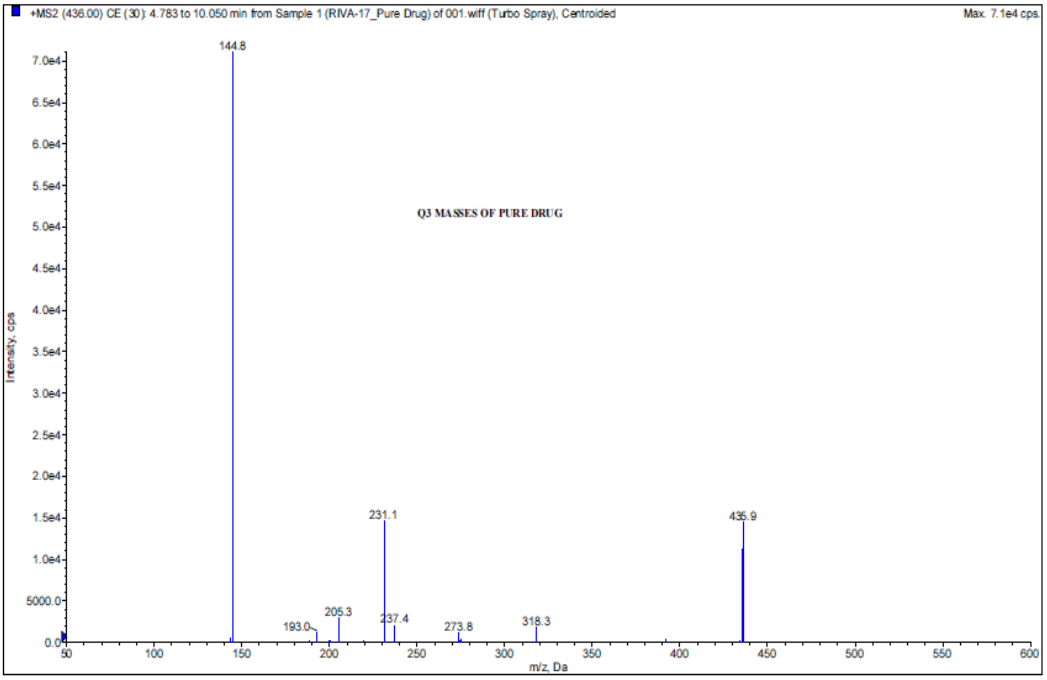


Fig.85Q1 Mass Spectra of Rivaroxaban impurity (m/z value 291)

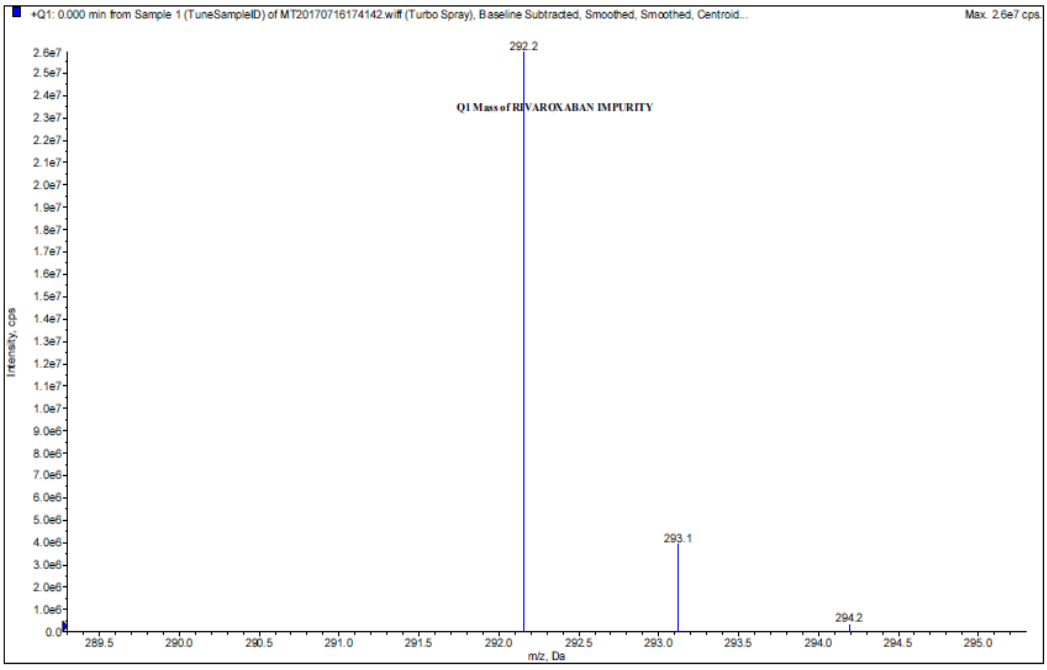


Fig.86 HPLC Chromatogram of rivaroxaban at 4 hours (Acid hydrolysis)

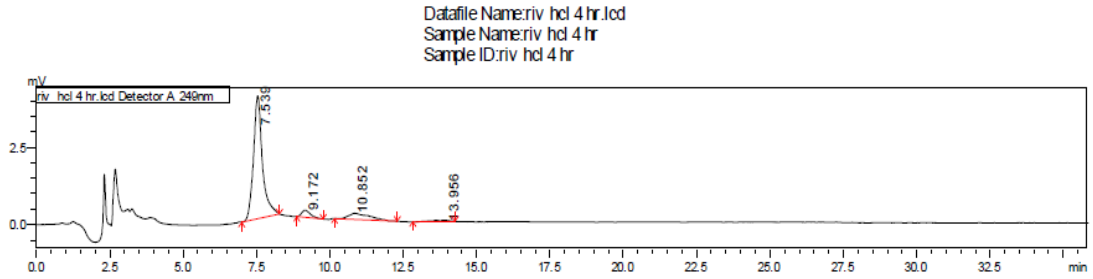


Fig.87 LC-MS/MS results of Rivaroxaban acid hydrolysis-7.5 Minute

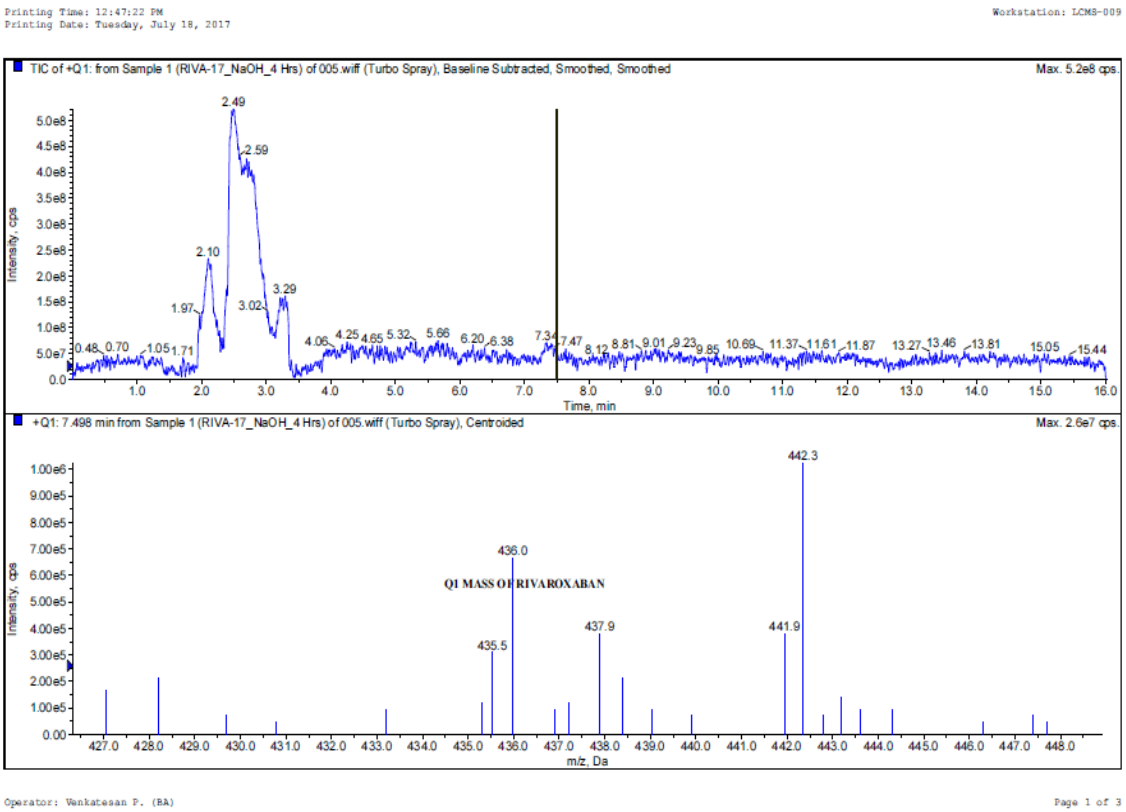


Fig. 88 Mass Spectra of Impurity at 10.8 Minute- m/z 292 (Acid hydrolysis)

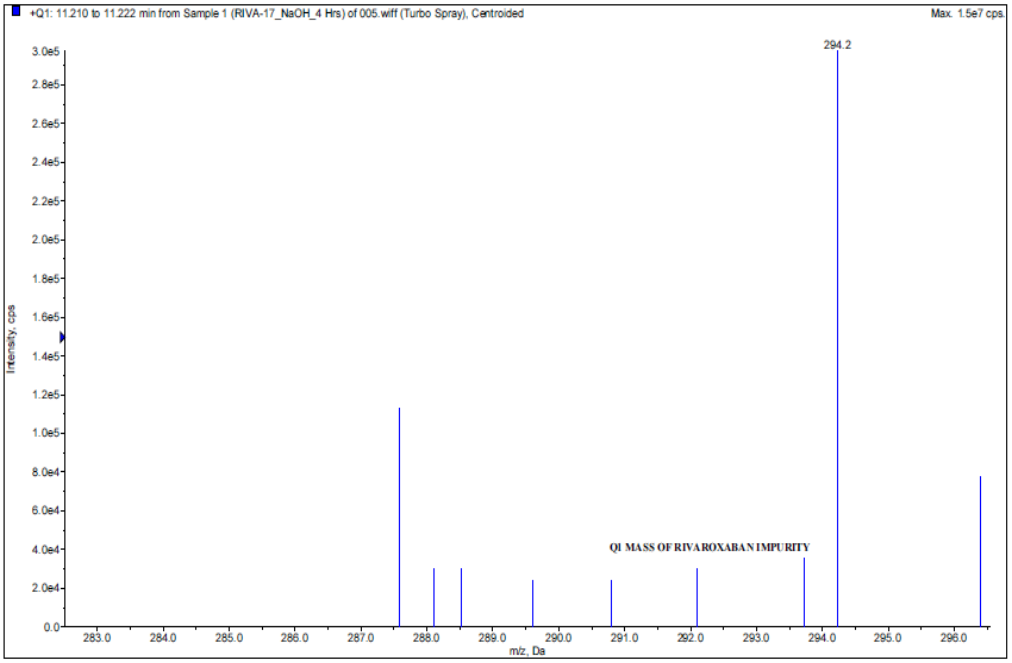


Fig.89 Mass spectra of Degradant at 13.9 Minute-m/z 274 (Acid hydrolysis)

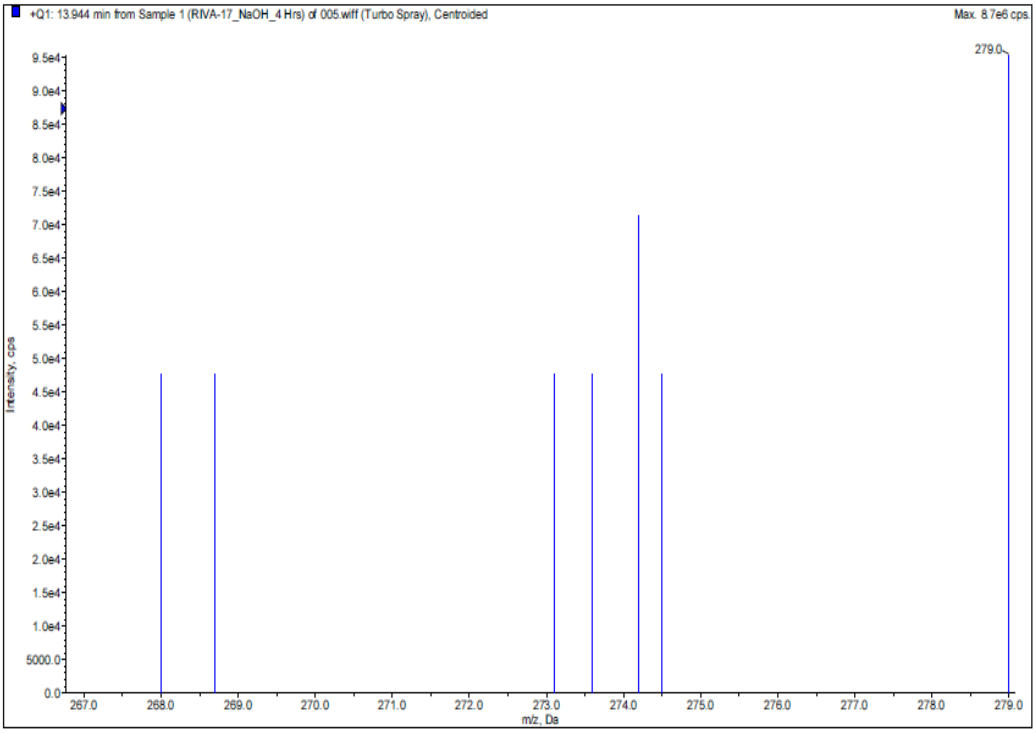


Table 57. Impurity profiling of acid hydrolyzed Rivaroxaban

RT Min.	Identified m/z value	Unidentified m/z value
7.53	436	427, 435.5, 437.9, 441.9, 442.3 (base peak)
10.85	292	287.5, 288.5, 289.6, 290.9, 293.8, 294.2 , 296.4
13.94	274	268, 268.6, 274.2, 274.5 and 279

Table 58. The proposed structure of identified impurities in Acid hydrolysis for Rivaroxaban

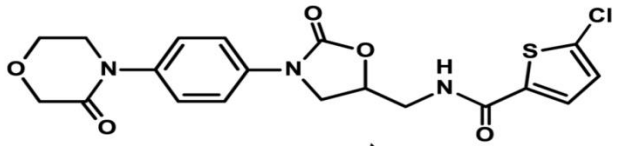
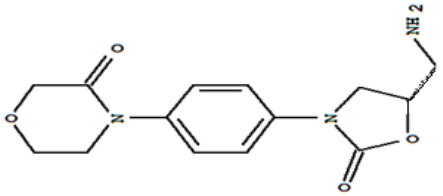
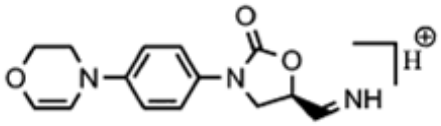
RT Min.	Identified m/z value	Structure
7.53	436	
10.85	292	
13.94	274	

Fig.90 HPLC Chromatogram of rivaroxaban at 4 hours (Base hydrolysis)

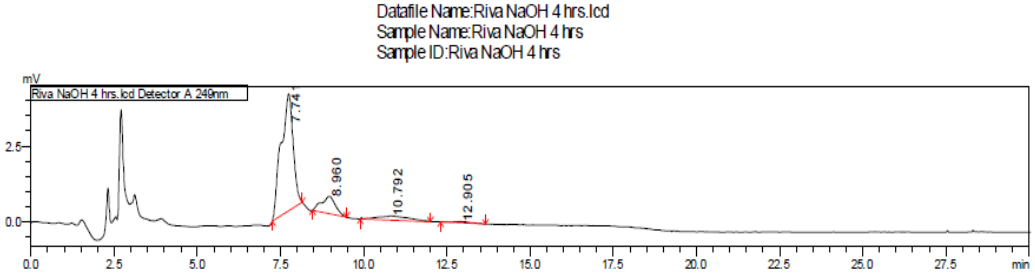


Fig. 91 LC-MS/MS Results for rivaroxaban at 7.4 Minute (Base hydrolysis)

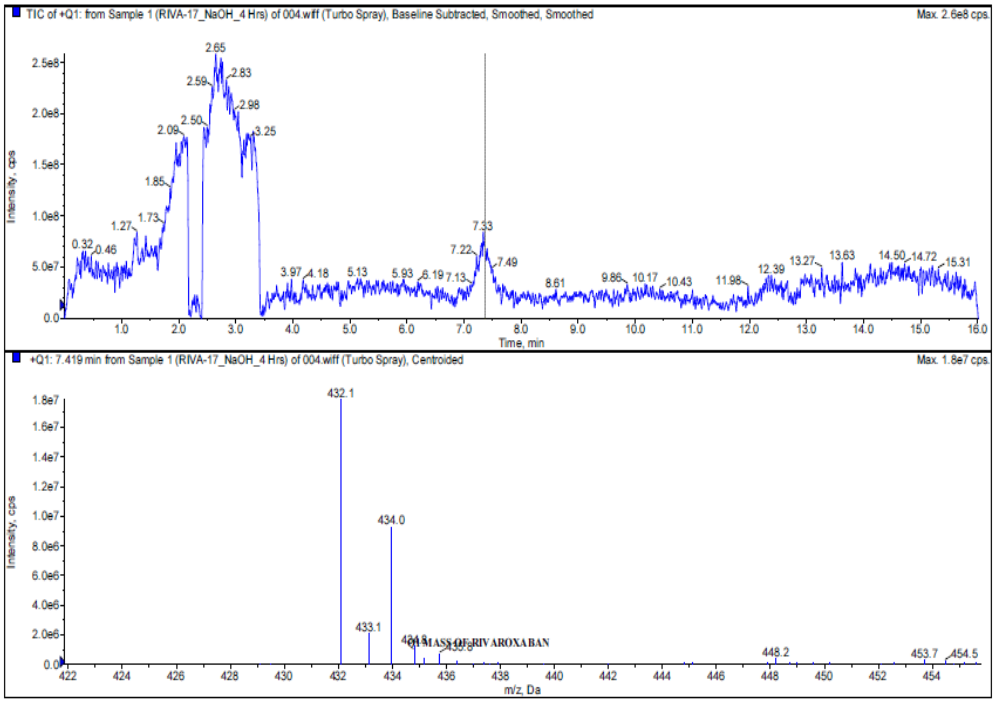


Fig.92Mass Spectra of Impurity at 10.7 minutes – m/z 292 (Base hydrolysis)

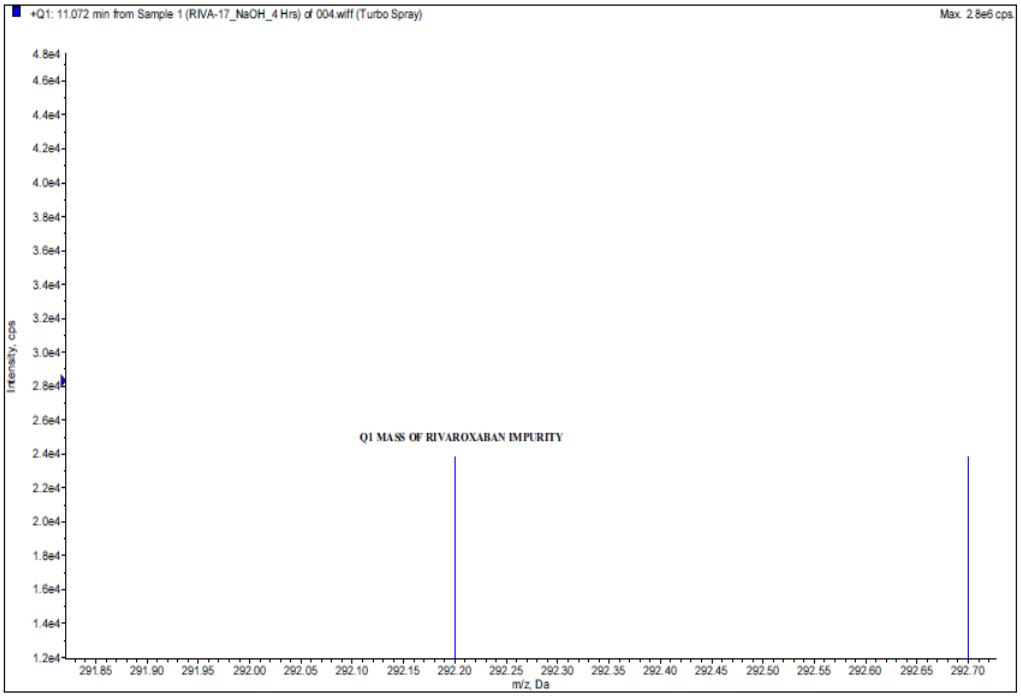


Fig. 93 Mass spectra of degradant at 8.9 minutes – m/z 205 (Base hydrolysis)

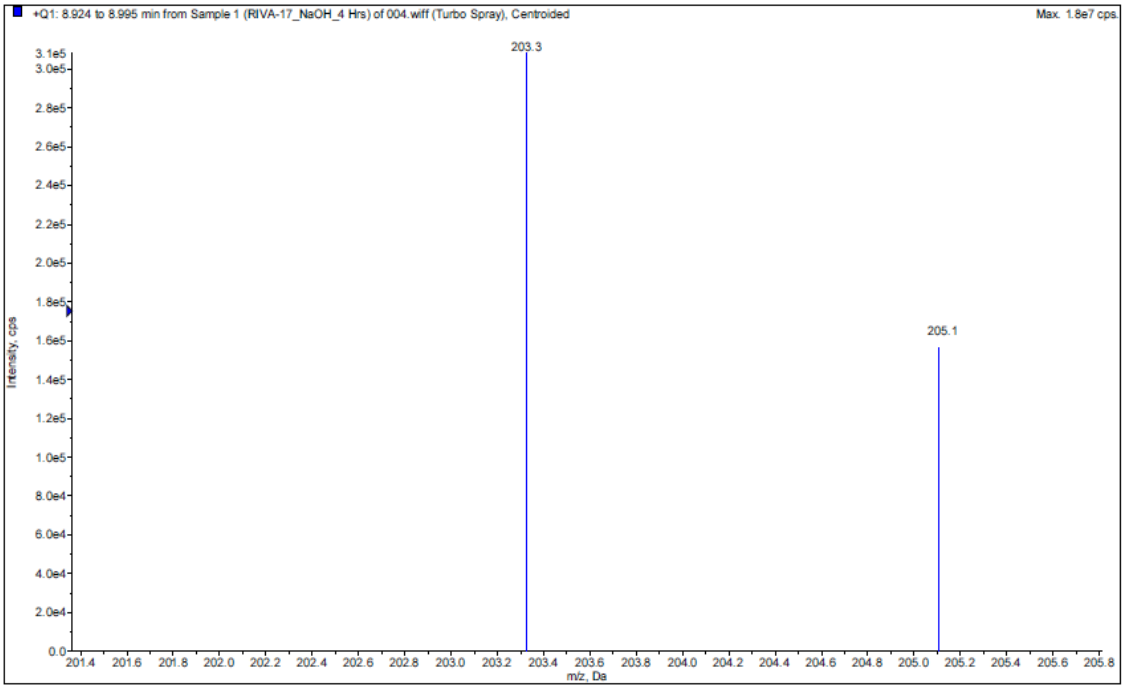


Fig. 94 Mass spectra of degradant at 12.9 minutes –m/z 193 (Base hydrolysis)

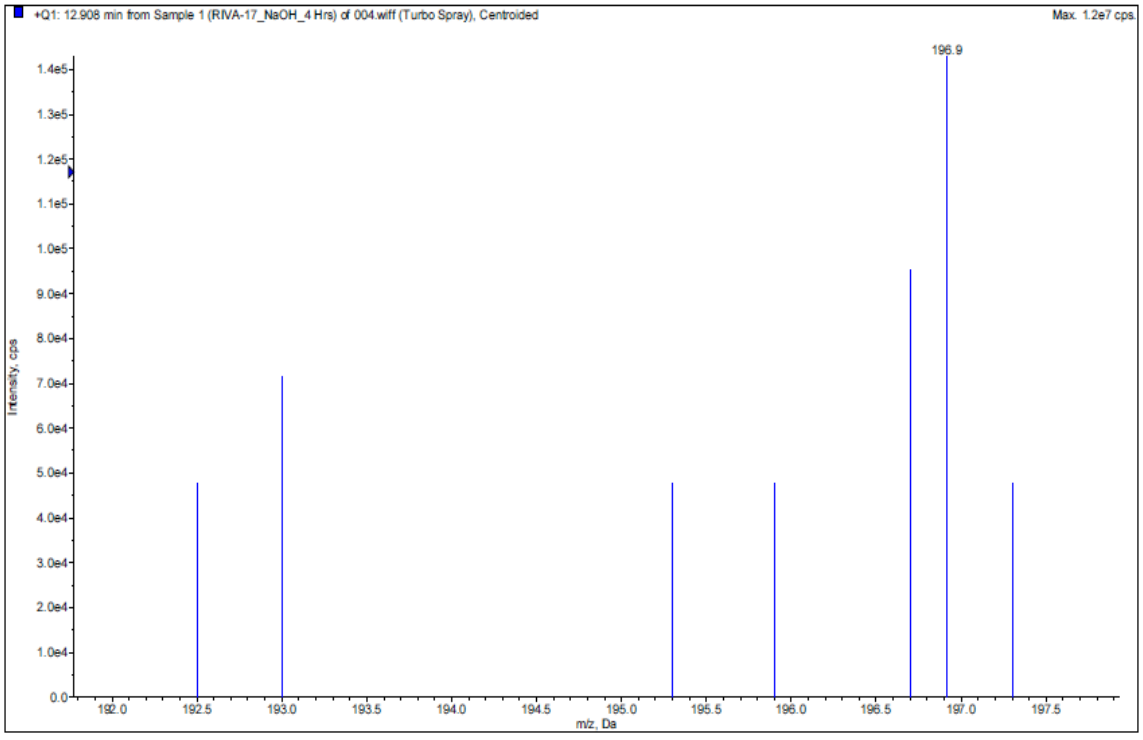


Table 59. Impurity profiling of base hydrolyzed Rivaroxaban

RT Min.	Identified m/z value	Unidentified m/z value
7.70	436	432.1 (base peak), 433.1, 434.6
8.98	205	203.3 (base peak)
10.70	292	Isotopic peaks 292.2 and 292.7
12.90	193	192.5, 195.3, 195.9, 196.6, 196.9 (base peak) and 197.4

Table 60. The proposed structure of identified impurities for rivaroxaban in Base hydrolysis

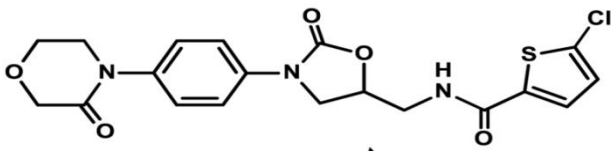
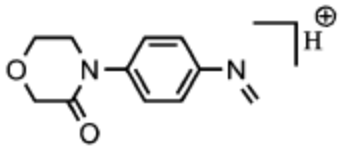
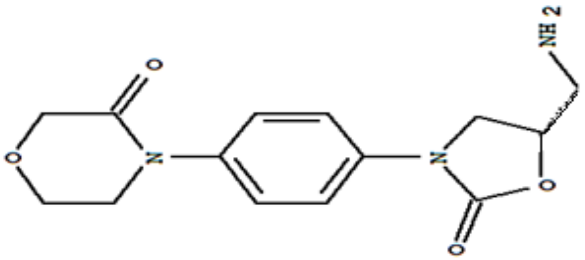
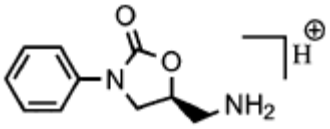
RT Min.	Identified m/z value	Structure
7.70	436	
8.98	205	
10.70	292	
12.90	193	

Fig. 95 HPLC Chromatogram of rivaroxaban at 4 hours (Oxidation)

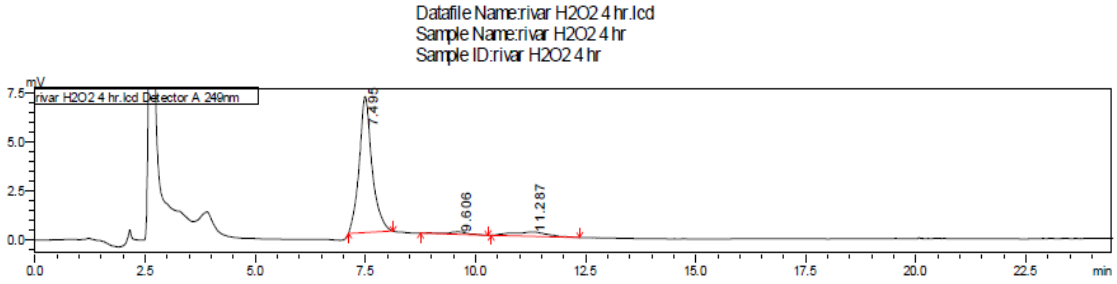


Fig. 96 LC-MS/MS Results for rivaroxaban at 7.4 Minute (Oxidation)

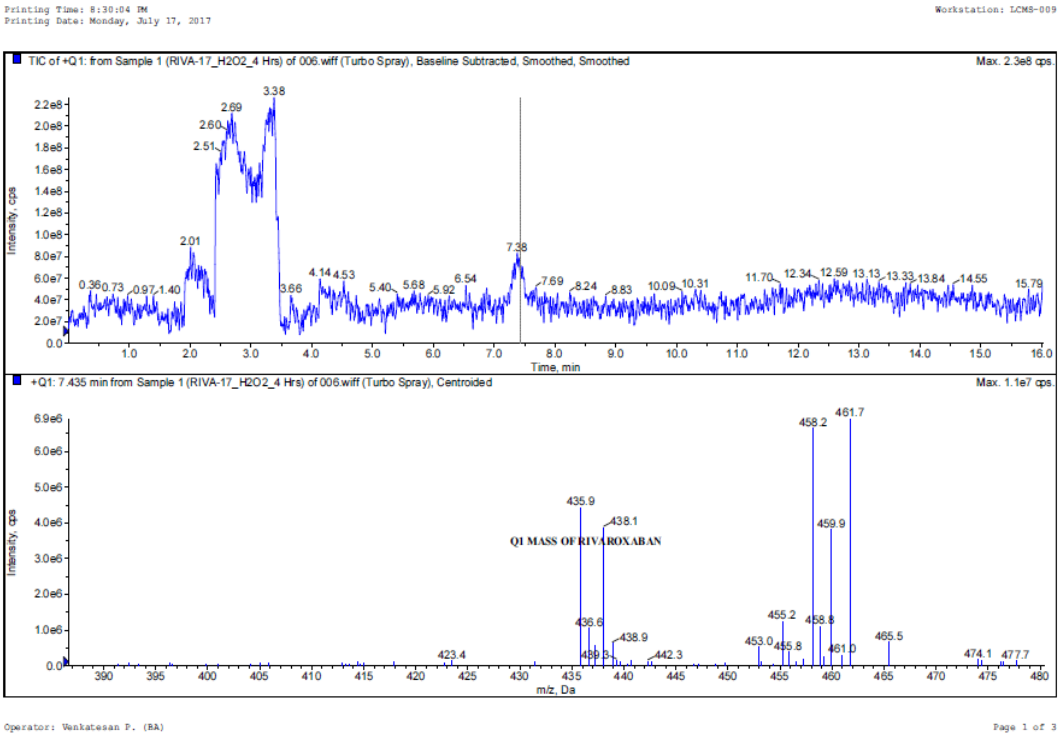


Fig. 97Mass spectra for degradant at 9.606minutes - m/z 231 (oxidation)

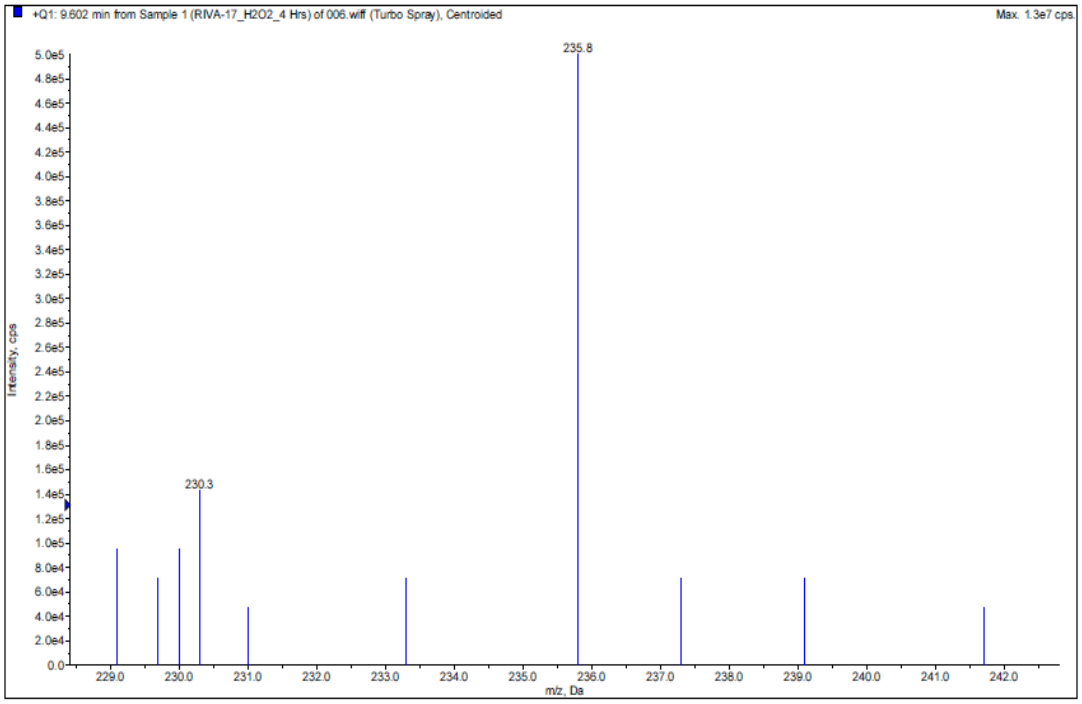


Fig.98 Mass spectra of Impurity at11.287minutes - m/z 292 (Oxidation)

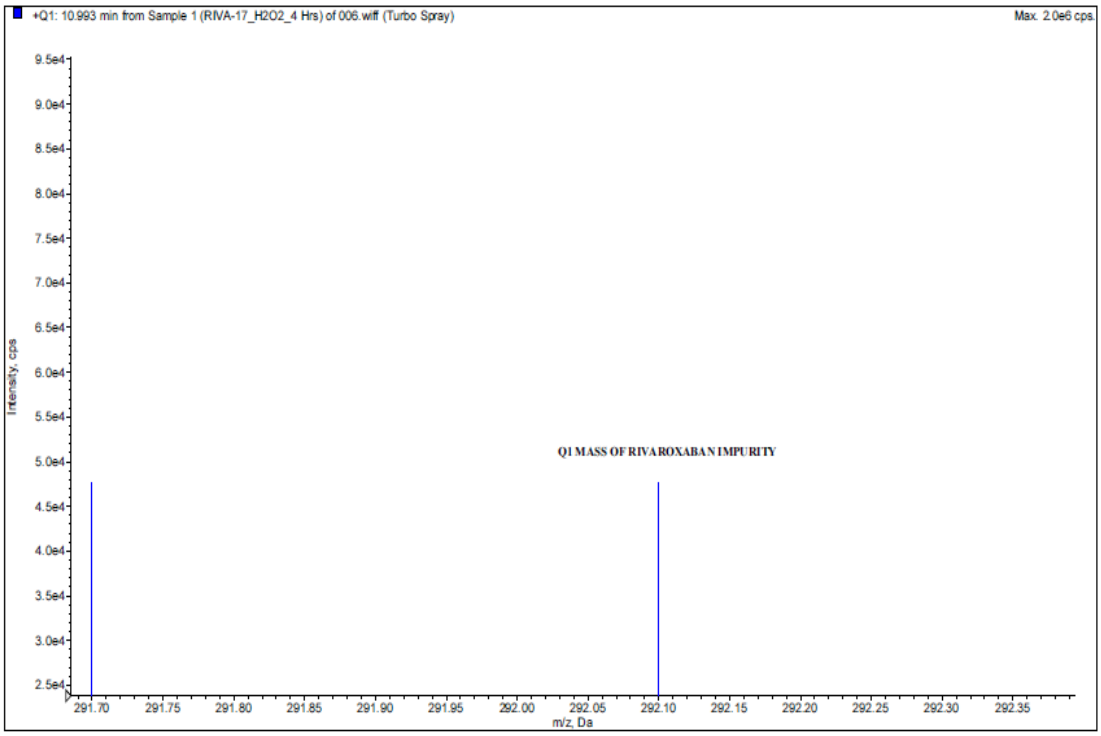


Table 61. Impurity profiling of Oxidized Rivaroxaban

RT Min.	Identified m/z value	Unidentified m/z value
7.49	436	435.9, 438.1, 438.9, 453, 455, 455.8, 458, 459.9, 461.7(base peak) and 465.5
9.60	237	229, 230.3, 233.5, 235.8 (base peak) 237.3, 239.1 and 241.
11.28	292	291.7(Isotopic peak)

Table 62. The proposed structure of identified impurities under Oxidationfor Rivaroxaban

RT Min.	Identified m/z value	Structure

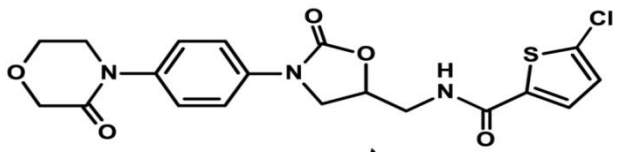
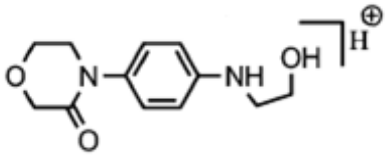
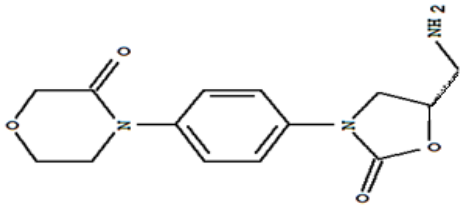
7.49	436	
9.60	237	
11.28	292	

Fig. 99 HPLC Chromatogram of rivaroxaban at 4 hours (Thermal degradation)

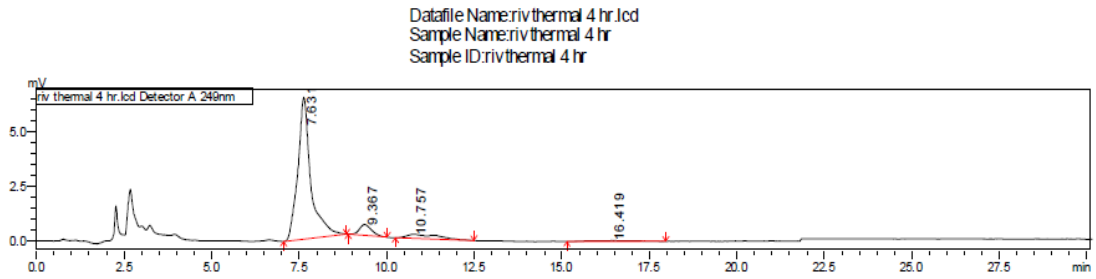


Fig. 100 LC-MS/MS Results for rivaroxaban at 7.4 Minute(Thermal degradation)

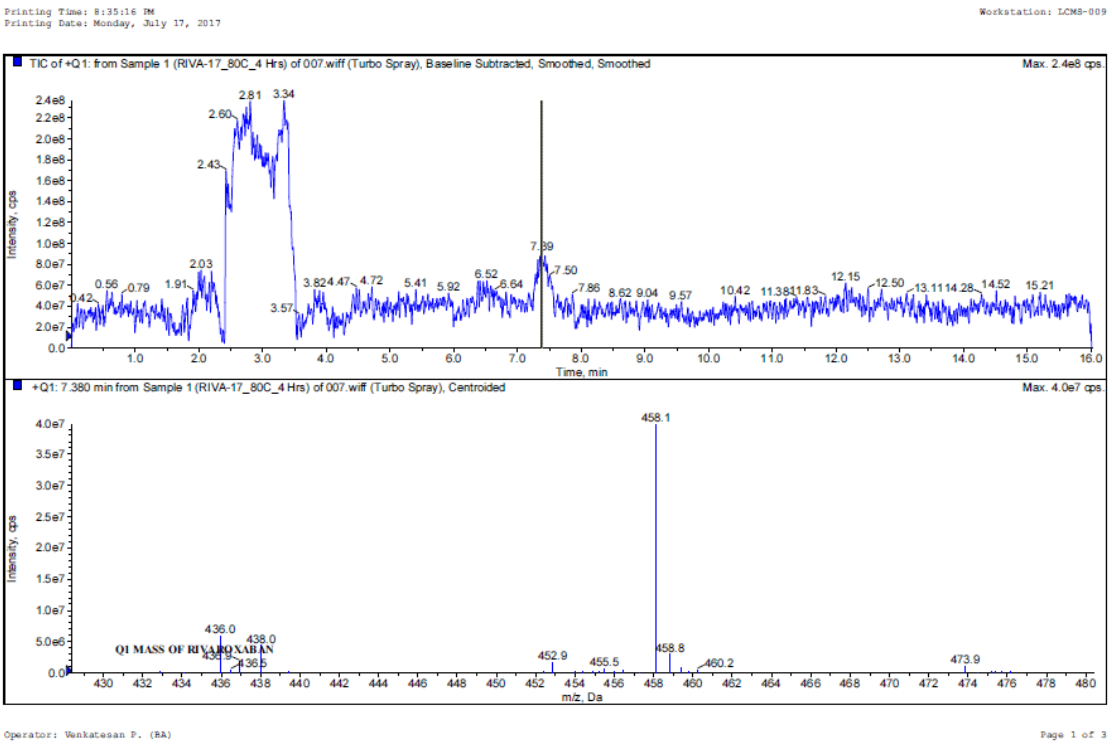


Fig. 101 Mass Spectra for impurity at 10.75 minutes – m/z 292 (Thermal degradation)

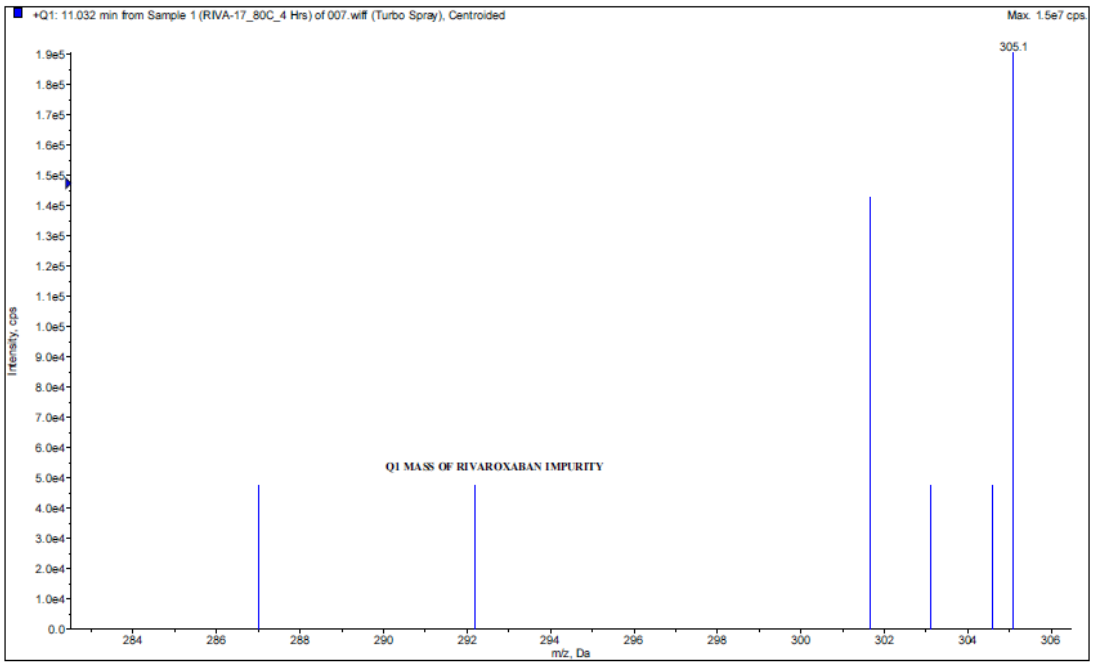


Table 63. Impurity profiling of Thermal degradation forRivaroxaban

RT Min.	Identified m/z value	Unidentified m/z value
7.62	436	436, 438, 452.9, 455.5, 458.1 (base peak) 458.8 and 460.
10.75	292	287, 292.1, 301.3, 303 and 305.1 (base peak)

Table 64. Proposed structure of the identified impurity of Rivaroxaban in Thermal degradation

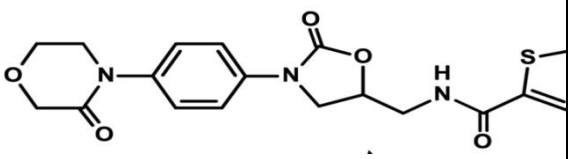
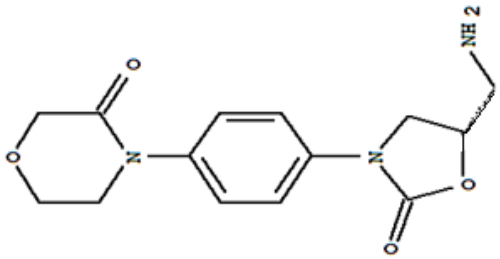
RT Min.	Identified m/z value	Structure
7.62	436	
10.75	292	

Fig. 102 HPLC Chromatogram of Rivaroxaban at 4 hours (Photolysis)

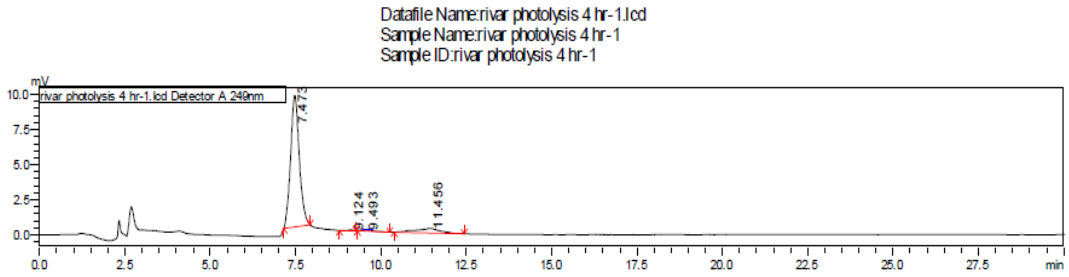


Fig. 103 LC-MS/MS Results for Rivaroxaban at 7.4 minutes (Photolysis)

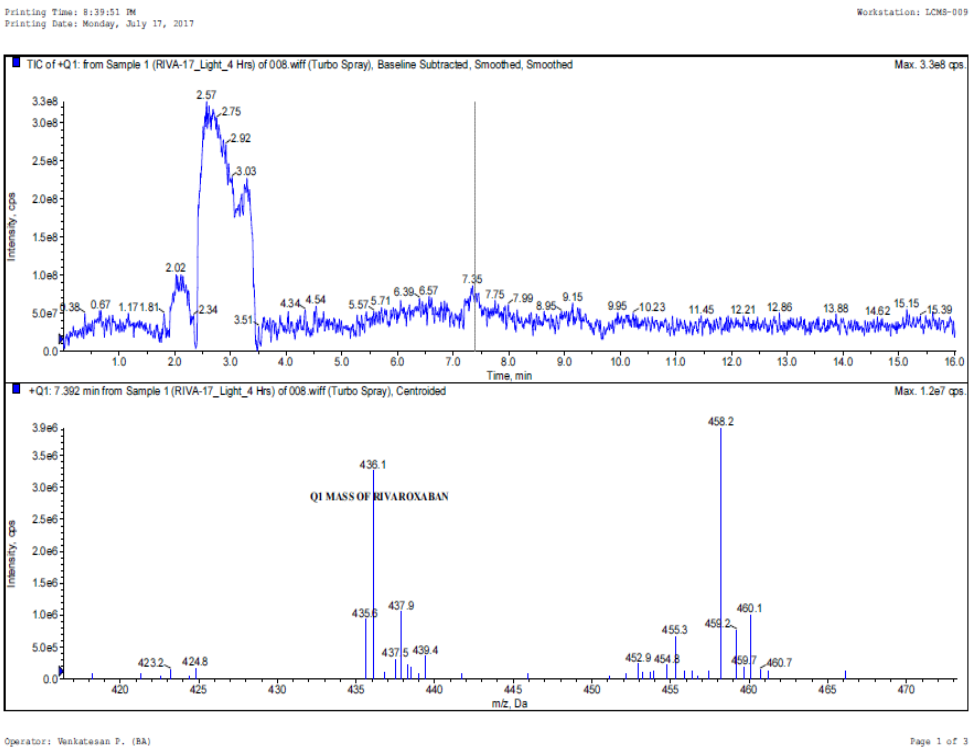


Fig. 104 Mass Spectra of degradant at 9.12 minutes – m/z 193(Photolysis)

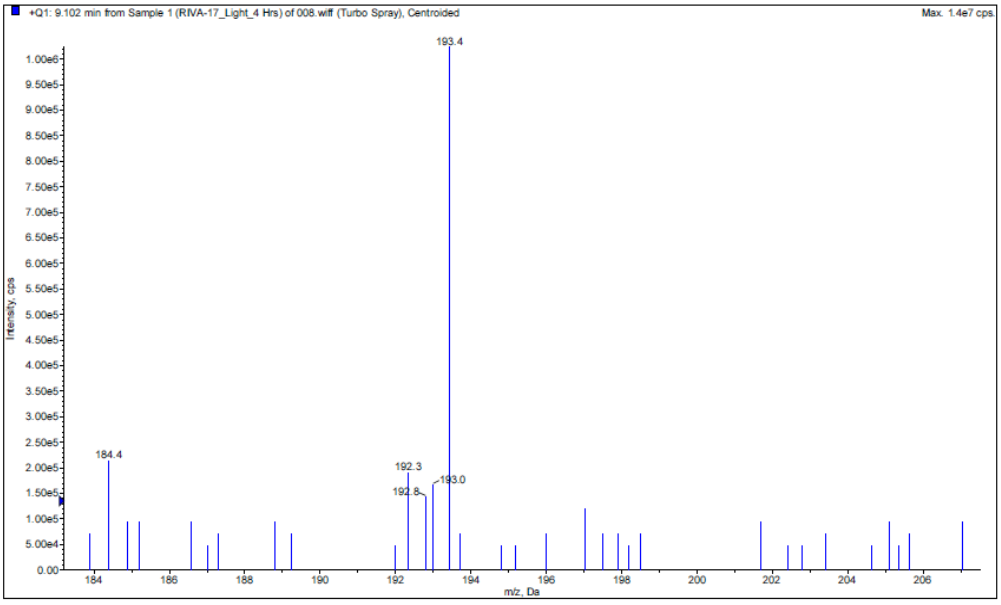


Fig. 105 Mass spectra of degradant at 9.49 minutes – m/z 273(Photolysis)

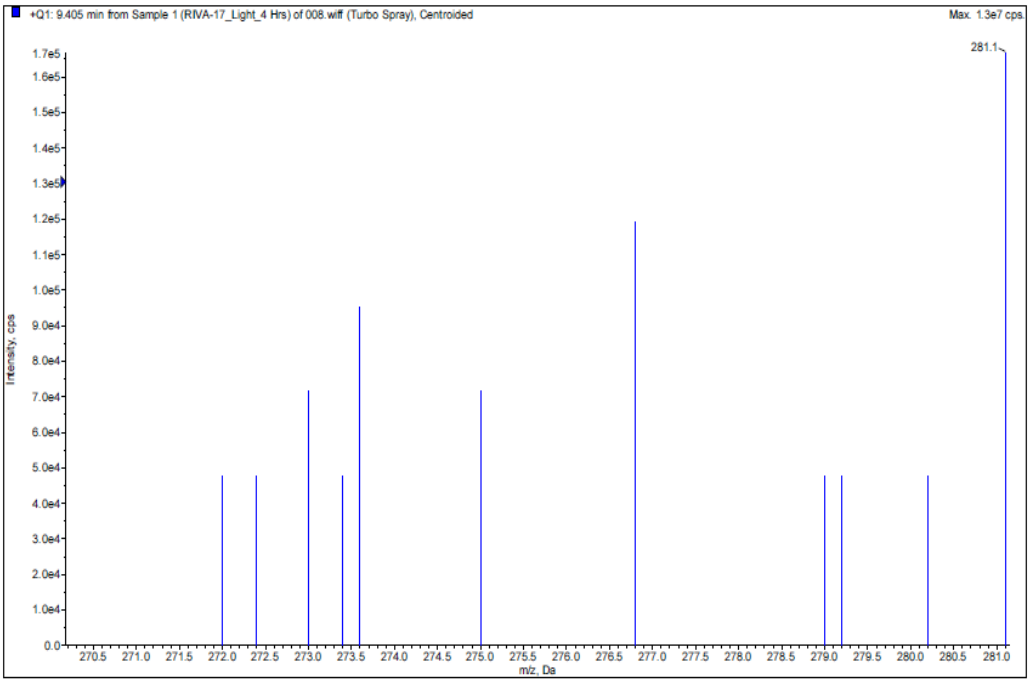


Fig. 106 Mass spectra for Impurity at 11.2 minutes – m/z 292(Photolysis)

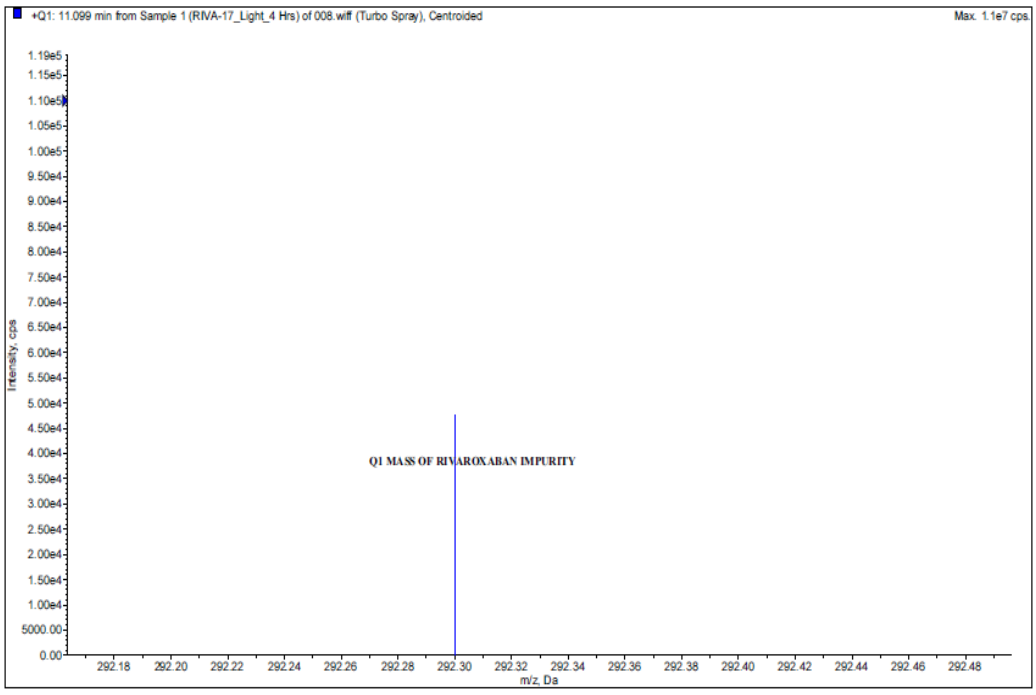
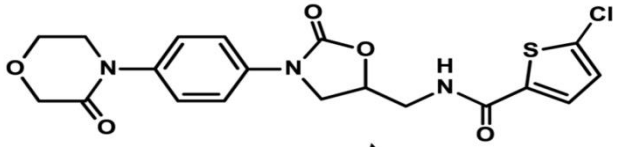
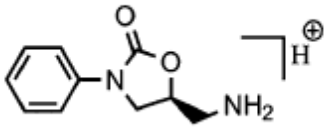
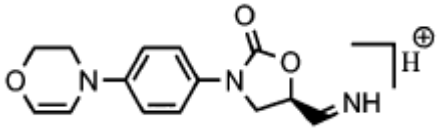
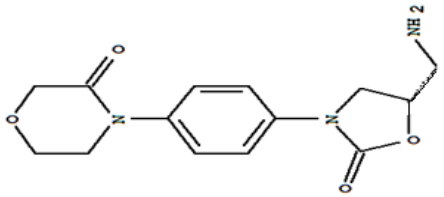


Table 65.Impurity profiling of photolysis for Rivaroxaban

RT Min.	Identified m/z value	Unidentified m/z value
7.47	436	435.1, 436.1, 437.5, 439.4, 452.9, 454.8, 456.3, 458.2 (base peak) 459.2, 459.7, 460.1 and 460.7
9.12	193(base peak)	184.4, 192.3, 197, 201, 205, 206.5
9.49	273	272, 273, 275, 276,8, 279, 280.2 and 281.1(base peak)
11.45	291	-

Table 66. Proposed structure in photolysisfor Rivaroxaban

RT Min.	Identified m/z value	Structure
7.47	436	
9.12	193	
9.49	273	
11.45	291	 -

7.3 IBUTILIDE FUMARATE

7.3.1 DEVELOPMENT OF VALIDATED UV SPECTROSCOPY METHOD FOR IBUTILIDEFUMARATE

7.3.1.1 Selection of the Experimental conditions

Ibutilide fumarate was found to be completely soluble in methanol. So the UV method for ibutilide was performed using methanol as solvent. A solution of 10 mcg/ml in methanol was scanned for the spectrum and found to give maximum absorbance at 228 nm (Figure 107). The method was carried out by fixing the slit width as 0.1mm, speed of spectral scan as 400 nm/min and a band width of 0.5 nm.

7.3.1.2 Validation of UV Spectroscopy Method

Linearity and range

Accurately measured volumes of the stock solution of ibutilide fumarate (100 mcg/ml)were transferred into the 10 ml volumetric flask to prepare concentration from 2 mcg/ml to 14 mcg/ml. The dilutions were done using methanol. The solutions were scanned between 200 – 400 nm and given as overlay spectrum in figure 108. The calibration data are shown in table 67 and the linear graph in figure 109.The slope, intercept, standard error and correlation coefficient was found to be 0.0385, 0.0143, 0.3014 and 0.997, respectively.

Precision studies

The precision of the established method was studied by determining the absorbance values of a fixed concentration of 10mcg/ml. The intraday precision was performed by six replicate measurements on the same day. The interday precision was performed on three different days. The values are presented in the table 68 for the intraday precision and in the table 69 for interday precision studies.

Robustness and Ruggedness

Robustness of the method was evaluated by measuring the absorbance at varied wavelength of ± 0.1 nm for the selected wavelength of 228 nm. The response obtained by two different analysts showed that the method was rugged. The operating conditions of the instrument and the experimental conditions were the same for both analysts.

Limit of detection and limit of quantification

Using the statistical formula for the detectable limit and quantifiable limit in consideration of the values of the slope of the graph and standard deviation of the measurements, the respective values were calculated. The amount detected was found to be 0.0624 mcg/ml and limit that can be quantified was found to be 0.1892 mcg/ml.

Stability of solution

In order to ascertain the stability of the solution prepared for the drug in methanol, the absorbance was checked for 4 hours at room temperature. The solution was found to be stable at room temperature for 4 hours as shown in table.The solution was stored in the fridge at 8^{o}C and absorbance measured at every 12 hours indicated that the solution was stable upto 72 hours after which there was decrease in the spectral response(Table 70).

Specificity of the method

The interference due to solvent is assayed by recording spectrum of methanol from 200-400nm. Figure 110 represents the overlay spectra of ibutilide fumarate and methanol. There was negligible absorbance of methanol at the selected wavelength.

Optical parameters for the method

The intrinsic property of ibutilide fumarate given by the molar absorptivity was calculated from the concentration range fitting the Beer's plot and pathlength. The E(1%,1cm), Sandell's sensitivity were derived and shown in table 71.

7.3.1.3 Application to injection drug solution

The ibutilide fumarate was prepared as per the product leaflet and analysed. After dilution, sample was analyzed by recording UV spectrum against a respective blank prepared omitting the sample.(Fig.111). The amount found was calculated and shown in table 72.

Fig. 107 UV absorption spectrum of Ibutilide fumarate 10mcg/ml

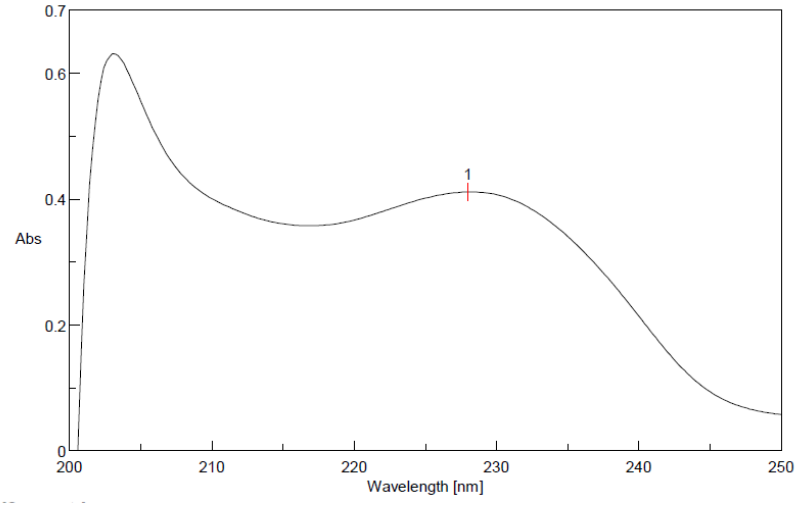


Fig. 108 Overlay Spectrum of Ibutilide fumarate

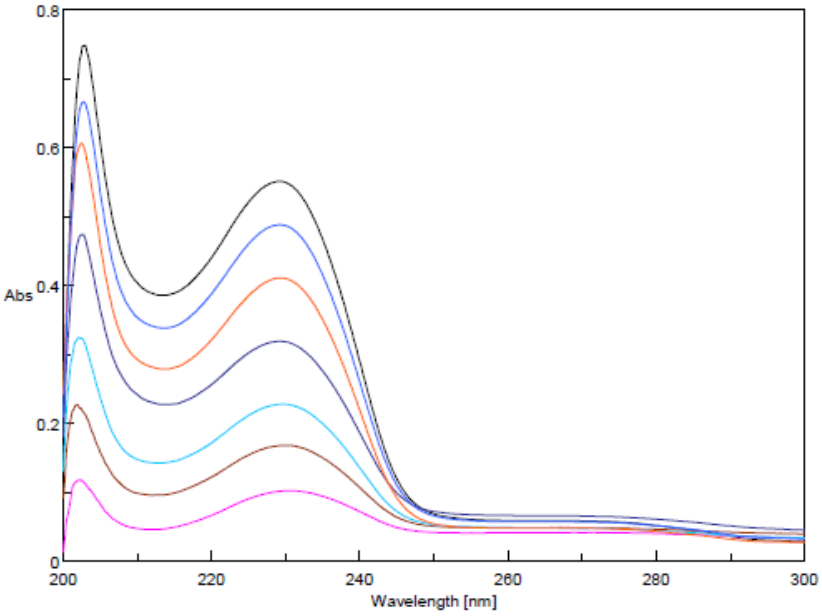


Table 67. Calibration data of Ibutilide fumarate

Concentration mcg/ml	Absorbance
2	0.1021
4	0.1680
6	0.2274
8	0.3181
10	0.4096
12	0.4864
14	0.5501

Fig. 109 Calibration graph for linearity of Ibutilide fumarate

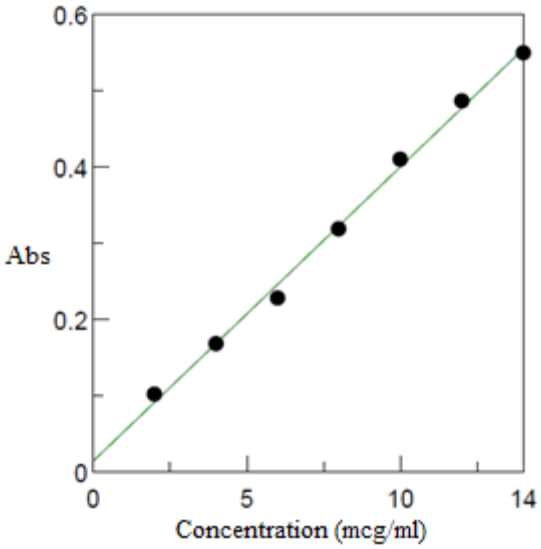


Table 68. Intraday Precision Studies of Ibutilide fumarate

Precision	Absorbance	SD*	%RSD*
Intraday 1	0.4089	0.003	0.29
Intraday 2	0.4093		
Intraday 3	0.4097		
Intraday 4	0.4088		
Intraday 5	0.4095		
Intraday 6	0.4096		

Table 69. Interday Precision Studies of Ibutilide fumarate

Precision	Absorbance*	SD	%RSD
Day 1	0.4099	0.013	0.020
Day 2	0.4093	0.019	0.007
Day 3	0.4094	0.012	0.033

*Mean of six determinations

Table 70. Stability of Ibutilide fumarate solution

Room Temperature		Refrigerator	
Time Hours	Absorbance	Time Hours	Absorbance
0	0.4099	6	0.4987
1	0.4082	12	0.4755
2	0.4069	24	0.4690
3	0.3922	48	0.4596
4	0.7880*	72	0.4503*
5	0.3644	84	0.3699

*Maximum time of stability

Fig. 110 Overlay spectrum of Ibutilide fumarate and methanol

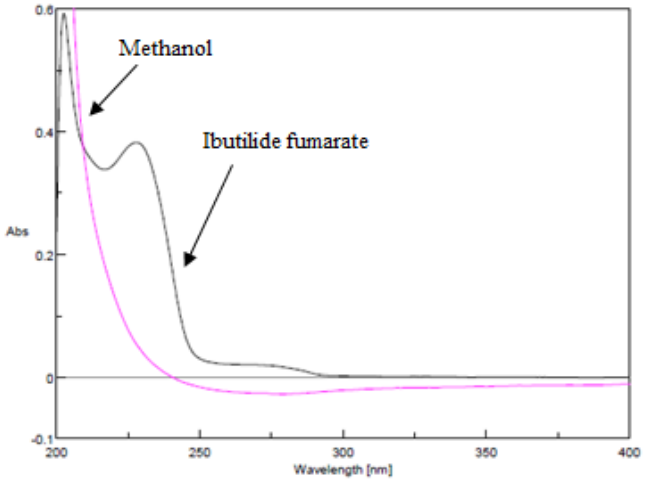


Table 71. Optical parameters of Ibutilide fumarate

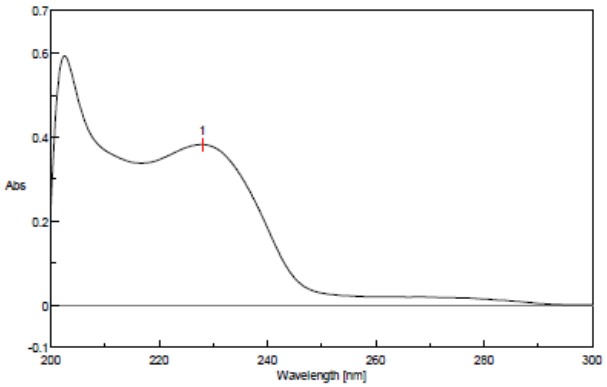
Parameter	Result
Molar absorptivity (L mol^{-1} cm^{-1})	1.687 x 10^5
Sandell's sensitivity (mcg/ml)	0.0261
E(1%,1cm)	381.8

Table 72. Analysis of Ibutilide fumarate drug solution

Amount of drug added	Amount of drug found	% drug found*	SD	% RSD
0.1 mg	0.0972 mg	97.2	0.29	1.52

*Mean of six observations

Fig.111 UV Spectrum for Ibutilide fumarate drug solution



7.3.2 DEVELOPMENT OF VALIDATED HPTLC METHOD FOR IBUTILIDE FUMARATE

7.3.2.1 Chromatographic conditions

Selection of stationary phase

HPTLC method for Ibutilide fumarate werecarried out using the precoated silica gel G_{60} F_{254} plates of 20 x 20 cm size. The plate was cut into appropriate size of 10 x 10 cm and 10 x 20 cm based on the necessity of the parameter involved.

Selection of mobile phase

Various composition of the mobile phase made of ethyl acetate, acetonitrile, methanol and toluene were used to retain Ibutilide on TLC plate(Table 73). The solvent system made of ACN:EA:MeOH was found to have Rf value less than 0.1, so isopropyl alcohol, ethyl acetate and toluene were put into trials. The results from both the composition were not satisfactory in the Rf value and the resolution between ibutilide peak and fumaric acid peak was found to be less than 2. Hence a system containing MeOH:EA:IPA was tried.

Optimization of variants in TLC

The impact of the solvents used in the mobile phase, chamber saturation time and distance travelled by the solvent were studied for the method development and then optimized.

Composition of mobile phase system

The solvent system made up of MeOH:EA:IPA was applied which resulted in acceptable Rf for ibutilide and resolution of more than 2. So the increase in volume of MeOH gave a acceptable Rf value of 0.31 for the drug and 0.73 for fumaric acid(Fig.114). Hence the composition MeOH:EA:IPA in ratio of 8:1:1 v/v/v was fixed as the mobile phase system. The effect of variation in the methanol ratio is given in the table 74.

Chamber saturation time

Different time for the saturation shows a effect on the peak shape and the nature of development on the plate. When the saturation time was modified from 5 to 30 minutes, the peak shape and symmetry was found to be varied. The selected chamber saturation time was 15 minutes which gave proper peak shape without fronting and tailing.

Distance of solvent front

Migration of the solvent from the base carrying the sample spots till the solvent front line has a marked shift in the Rf value. The mobile phase was allowed to run on the plate to various distances from 7 cm to 9 cm. The acceptable Rf value was attained with the distance of 8 cm of the solvent front and hence fixed for the studies.

Band width

The samples were spotted with the band width from 2 mm to 6 mm using the automatic sampler. The 2 mm band width produced a narrow peak with significant variation in the peak area. As the band width increases, there was exponential change in the peak area which were symmetric in shape. The band width of 5 mm was selected as the peak characteristics were good.

7.3.2.2 Validation of HPTLC method for Ibutilide fumatate

Linearity and range

Stock solution of ibutilide fumarate was prepared with methanol to give a concentration of 1000mcg/ml. From this volumes of 1 to 9 µl equivalent to 1000 to 9000 ng/band were spotted.The plates were developed in the chamberwith prerequisite

saturation time. They were scanned at the detection wavelength of 228 nm. The peak of the drug was studied for the R_f value and area. The regression equation was y = 1.3863*x +3257.17 with a correlation coefficient of r^2>0.99 and SE of 310.23. Overlay 3D densitogram is shown in figure 112.The values of linearity range and the calibration graph are given in table 76 and figure 113.

Precision studies

The interday and intraday precision studies were conducted by repeating the injections of a concentration 3000 ng/band for six times on the same day and on three consecutive days. The peak area were tabulated in table 77 and 78. The SD and % RSD values were shown in respective tables.

Robustness and Ruggedness

Intentional variations in the chamber saturation time by ± 2 min and mobile phase ratio ± 0.1 ml were carried out for Ibutilide fumarate. The peak area and the Rf value showed no significant changes. The results indicated the robustness of the method. Analysis of the drug by two different analyst on three days results in identical responses. Thus the method was found to be rugged.

LOD and LOQ

The sample spotted on the plate from 100 to 700 ng/band were scanned for the peak that can be detected and for the concentration that can be quantified. The limit of detection was found to be 100 ng/band giving a detectable peak area of 217.50. The limit quantifiable was 400 ng/band with peak area of 844.31.(Figure 115 and 116)

Stability of developed plate

The plate spotted with the standard solution of 3000ng/band were scanned for every one hour and checked for the responses. The study was executed periodically till 5 hours and the plate was found to be stable for 4 hours after which the responses had more than 10% change in the peak area. The values were depicted in the table 79.

System suitability studies

The method was checked for the suitability parameters for tailing factor, asymmetric factor and resolution of peaks of ibutilide and fumaric acid. The values examined were found to be within the acceptance criteria.(Table 80)

7.3.2.3 Application to drug solution

The ibutilide fumarate was prepared as per the product leaflet and analysed. After dilution, sample was spotted and the chromatogram was developed, peaks are noted and amount found was calculated (Table 81, Fig 117)

Table 73.Selection of Mobile Phase System for Ibutilide fumarate

Mobile phase	Ratio(v/v/v)	Observations
ACN:EA:MeOH	6:2:2	Peaks retain in the start line
	5:3:2	Peaks retain in the start line
	4:2:4	Rf = 0.1
	2:4:4	Resolution less than 1.0
IPA:EA:TLN	5:3:2	Rf = 0.20and resolution less than 1.0
	6:2:2	Rf = 0.41 and resolution less than 1.0
	7:2:1	Rf = 0.52 and resolution less than 2.0

Table 74. Composition of Mobile Phase System for Ibutilide fumarate

MeOH:EA:IPA (v/v/v)	R_f value		Resolution
	Ibutilide	Fumaric acid	
5:3:2	0.12	0.44	1.39
6:2:2	0.18	0.47	1.37
7:2:1	0.28	0.69	1.84
8:1:1	0.31	0.73	2.45

Table 75. Optimized Densitometric Conditions for Ibutilide fumarate

Parameter	Fixed Condition
Stationary phase	Precoated silica gel plate G_{60} F_{254}
Mobile phase	MeOH:EA:IPA
mobile phase ratio	8:1:1 v/v/v
Chamber saturation	15 minutes
Distance of solvent front	8 cm
Band width	5 mm
Slit dimensions	5 x 0.45 mm
Scan speed	20 mm/sec
Scan wavelength	228 nm

Fig. 112 Overlay 3D densitogram of Ibutilide fumarate

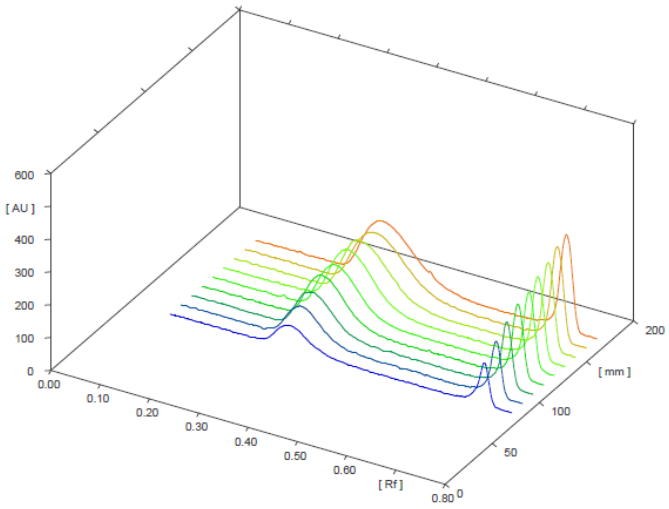


Table 76. Calibration data of ibutilide fumarate

Concentration ng/band	**R_f Value**	**Peak Area**
1000	0.31	4058
2000	0.33	6225
3000	0.33	7455
4000	0.33	9176
5000	0.33	10047
6000	0.33	12056
7000	0.34	13002
8000	0.33	14617
9000	0.34	15066

Fig. 113 Calibration graph for linearity of Ibutilide fumarate

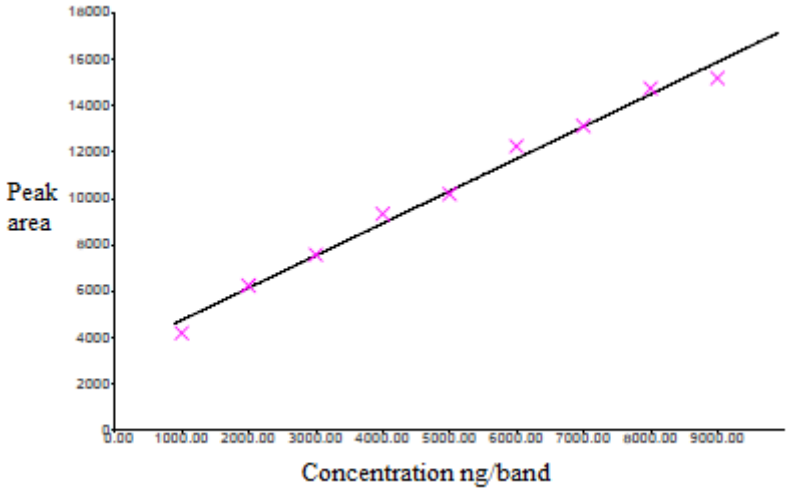


Fig. 114 Densitogram for fumaric acid

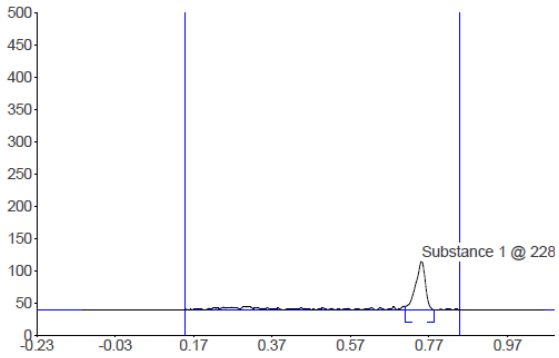


Table 77. IntradayPrecision studies of Ibutilide fumarate

Precision	Peak area	SD*	%RSD*
Intraday 1	7450	16.94	0.07
Intraday 2	7448		
Intraday 3	7452		
Intraday 4	7444		
Intraday 5	7450		
Intraday 6	7459		

*Average value

Table 78. InterdayPrecision studies of Ibutilide fumarate

Precision	Peak area*	SD	%RSD
Day 1	7456	18.99	0.54
Day 2	7449	14.42	0.06
Day 3	7459	16.07	0.41

*Mean of six observations

Fig. 115 LOD of ibutilide fumarate

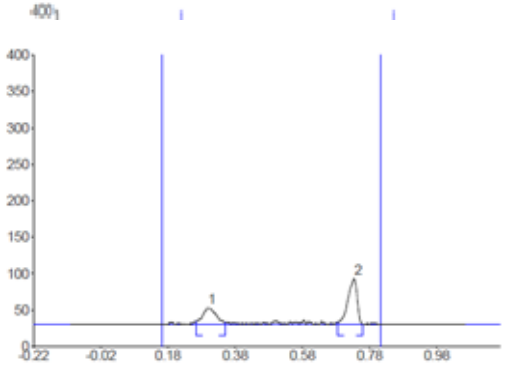
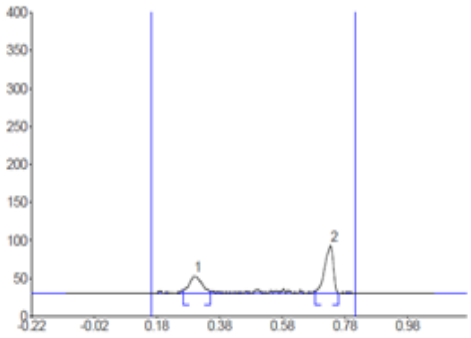


Fig. 116 LOQ of ibutilide fumarate



1	7399	34.10	0.52
2	7327		
3	7106		
4	6793*		
5	6621		

*Maximum time of stability

Table 80. System suitability parameters of Ibutilide fumarate

Tailing factor	Asymmetric factor	Efficiency
0.9346	1.0216	2659

Fig. 117 Analysis of Ibutilide fumarate drug solution

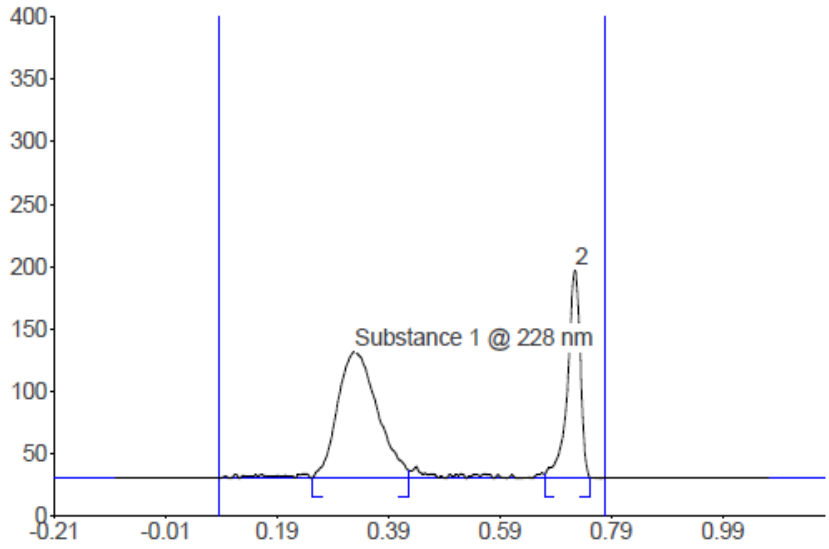


Table 81. Analysis of Ibutilide fumarate drug solution

Amount of drug added	Amount of drug found	% drug found*	SD	% RSD
0.1 mg	0.099 mg	99.0	0.68	0.572

*Mean of six observations

7.3.3 DEVELOPMENT OF VALIDATED RP-HPLC METHOD FOR IBUTILIDE FUMARATE

7.3.3.1 Chromatographic conditions

Selection of wavelength

Suitable wavelength for the analysis of ibutilide fumarate by RP-HPLC method was selected based on the maximum absorbance for the solution prepared in the solvent was methanol.A solution of 10mcg/ml was prepared using methanol and scanned

between 200-400 nm. The λ_{max} was found at 228 nm hence it was selected for the detection of ibutilide fumarate by RP-HPLC method(Figure 118).

Initial chromatographic conditions

RP-HPLC method development for ibutilide fumarate was carried out on a C18 Shim Pack column (250 mm x 4.6 µ id x 5 µm particle size). The mobile phase composition of methanol and water in a ratio of 50:50 (v/v) was initially carried out. The composition was varied for both solvents and the standard solution of ibutilide fumarate 10(mcg/ml) in methanol was run under each mobile phase composition.Two peaks obtained were found to be Ibutilide and fumaric acid. The peak obtained for the ibutilide and fumaric acid were evaluated for the peak shape, resolution between fumaric acid and the drug peak and tailing factor The results of the HPLC chromatogram of trials carried out were presented in the table 82 and figure 119 to 130.

Optimization of the chromatographic conditions

After fixation of the stationary phase and detection wavelength, the optimization of the mobile phase was carried out.The mobile phase system made up of water:methanol at fixed parameters of flow rate, injection volume and column temperature as mentioned in the table 83 were adopted for the studies. The figure 131 is a representation of the standard solution chromatogram.

7.3.3.2 Validation of RP-HPLC Method for Ibutilide fumarate

Linearity and range

The calibration curve was constructed by diluting the stock solution of Ibutilide fumarate with methanol to get a concentration range of 2-16 mcg/ml. The least square regression method of analysis was adopted with the peak area against concentration of drug. Figure 132 shows the overlay responses in the linearity range.The linearity graph was evaluated for the correlation coefficient, slope and intercept of the curve(Figure 133). The regression equation was found to be y=1109.42*x+33757.6 with a

correlation coefficient of 0.997 and standard error of 0.3454. The table 84 shows the peak area of ibutilide.

Specificity

To prove the specificity of methods fumaric acid solution and excipient solutions were injected individually to chromatographic conditions. It was observed that there is no interference from the above mentioned components. The chromatographs of fumaric acid are shown in figure 134.

Precision studies

Ibutilide fumarate was studied for the repeatability and reproducibility of the measurements by interday and intraday precision. Six replicate injection of the standard drug Ibutilide fumarate (10mcg/ml) at three times on the same day was carried out for the intraday precision and three consecutive days for the Interday precision studies. The relative standard deviation was found to be below 2 as given in table 85 for intraday precision and table 86 for interday precision.

Robustness and Ruggedness

Robustness of the method for Ibutilide fumarate was investigated by intentional changes in the mobile phase ratio ± 0.1ml/min, flow rate ± 0.1 ml and injection volume ± 0.1µl. The ruggedness was evaluated by two analysts recording the chromatogram under similar operating conditions with the same instrument. The method was found to be rugged and robust for the analysis of Ibutilide fumarate.The values are shown in table 87.

Limit of detection and limit of quantification

The limit detection was derived mathematically using the formula 3.3σ/S and the limit of quantification calculated from 10σ/S based on the signal to noise ratio. The LOD and LOQ were found to be 0.0051mcg/ml and 0.0084mcg/ml.

Stability of solution

Freshly prepared solution of ibutilide fumarate in methanol was diluted appropriately to obtain a concentration of 10mcg/ml. Zero hour sample was analyzed

immediately after dilution. The drug stability at room temperature was studied until the detector response recorded below 10% change in the peak area. Drug solution was stable upto 8 hours at room temperature. The drug solution stored in the refrigerator was found to be stable for 36 hours. The values are depicted in the table 88.

System suitability parameters

The asymmetric factor (A_s) of the peak, tailing factor of the peak and the number of theoretical plates were evaluated from the responses recorded for the standard drug. The values were found to be within the limits and are presented in the table 89.

7.3.3.3 Application to drug solution

The ibutilide fumarate was prepared as per the product leafletand analysed. After dilution, the solution was injected to get chromatogram and from peak area, the amount was calculated and tabulated (Figure 135 and Table 90)

7.3.3.4 Application of RP-HPLC method for the degradation studies

RP-HPLC method developed and validated according to guidelines was adopted for the degradation studies of ibutilide fumarate drug substance. Chromatograms recorded at 228nm were studied for the drug peak and degradant peak. The parameters observed were retention time, resolution and tailing factor. Ibutilide fumarate was subjected to the acid and base hydrolysis, oxidation, photolysis and thermolysis. The % degradation was calculated and tabulated for each stress condition.

Acid Hydrolysis (0.1 M HCl)

Ibutilide as a free base elutes at 5.5 min,fumarate is eluted at 2.27 and degradant at 4.9 min. The change in peak area was calculated for the amount of drug present at 4 hours represents the level of degradation. The percentage degradation is shown in table 91. The acid degraded sample of ibutilide fumarate was injected and the chromatogram is shown in figure 136 to 144.

Base hydrolysis (0.1 M NaOH)

The effect of 0.1 M NaOH on the drug solution was found to show the ibutilide peak at 5.4 minutes. There was a sequential reduction in the peak area of the free base ibutilide. The change in response was found to be more than 10% of the acceptable criteria. Ibutilide was found to be unstable in basic conditions of 0.1 M NaOH. The amount of degradation are shown in table 92. The results are depicted in figure 145 to 153.

Oxidation(3% v/v H_2O_2)

Ibutilide fumarate solution in 3% v/v hydrogen peroxide exhibits a constant change in the peak area at 7.2 minutes. Along the Ibutilide peak the degradant peak at 4.5 and 12.0 min was identified. The degree of degradation was found to be more than 10%.The results are depicted in the picture figure 154 to 162 and summarized in table 93.

Thermal degradation (40 ^oC)

Standard solution of Ibutilide fumarate prepared in methanol was subjected to a controlled temperature of 40 ^oC on a thermostat. The peak at the retention time 2.8, 4.5 and 10.1 minutes signifies no appreciable change as shown in the chromatograms in figure 163 to 170. Ibutilide fumarate found to be stable at 40 ^oC till four hours. The results for the oxidation are summarized in the table 94.

Thermal degradation (60 ^oC)

The 100 mcg/ml Ibutilide fumarate solution prepared in methanol was maintained at80^oC till 4 hours. Ibutilide peak at 5.5 minutes had a change in response below 10% till 90 minutes. The additional peak at 4.4 minutes had a parallel increase in the response. Ibutilide was found to be stable at 60^oC upto 90 minutes and more than 10% after 90 mins.The outcomes are illustrated in the figure 171 to 178 and degradation for every 30 minutes are given in table 95.

Photolysis – 220 V light

Stability of Ibutilide fumarate was demonstrated under the effect of light till 8 hours for 100 mcg/ml standard solution. The chromatogram was examined for change in peak area at 2.3 minutes. There was negligible change in the peak area for the drug. The outcome of the studies for the amount of degradation are shown in table 96 and the results are depicted in figure 179 to 187.

7.3.3.5 Application of RP-HPLC for *in-vitro* drug-drug interaction studies of ibutilide fumarate with selected cardiovascular drug

Selection of wavelength

The UV spectrum of ibutilide fumarate in methanol shows the maximum absorbance at 228 nm and verapamil was found to have λ_{max} of 230 nm. The wavelength for the study was selected as 228 nm as both the drugs show appreciable absorbance. The overlay UV spectrum of ibutilide fumarate and verapamil is shown in the figure 195.

Chromatographic conditions

The mobile phase system was varied with an increase in the composition of acetonitrile to get a good resolution between peaks. The solvent system made up of methanol, acetonitrile and water were used for trials and observed for the peak characters. Finally the solvent system fixed was methanol, water and acetonitrile in the ratio of 50:25:25v/v/v, in which the retention time of ibutilide was 4.071 and verapamil was 11.414minutes. The validation and system suitability parameters for ibutilide fumarate and verapamil are shown in table 97.

***In-vitro* drug-drug interaction studies of ibutilide fumarate**

Interactions in simulated Conditions

Before conducting the interaction in the enzymes, the study was performed in simulated conditions. The procedure is described in earlier section. The drug ibutilide fumarate and verapamil under simulated conditions were found to get eluted at 3.6 and 14.0 minutes (Figure 196). The study was conducted for 4hours and the chromatograms are shown in Figure 197. Summary of the peak area changes are shown in table 98.

***In vitro* interaction study with rat liver microsomes**

The invitro interaction study was carried out individually for ibutilide fumarate and verapamil in separate interaction flask and treated with enzyme in a specified condition as described earlier.

The interaction study was conducted for ibutilide in presence of verapamil with liver microsomal enzymes as per stipulated conditions.

The samples were withdrawn at regular intervals from 0 hour to 4 hours, after appropriate dilutions, they were injected and chromatograms were recorded.

The chromatograms of enzymatic interaction study for ibutilide (Figure 198&199), verapamil (Figure 200&201) and mixture (Figure 202&203) are presented. The amount of drugs remaining after 4 hours of the study is shown in table 98.

In vitro interaction of ibutilide fumarate with enzymes have shown that the amount remained was very high (91%) while present with verapamil than in individual (8%). This shows a significant role of verapamil in metabolic pathway of ibutilide and clinical effect.

Fig. 118 UV Spectrum of Ibutilide fumarate

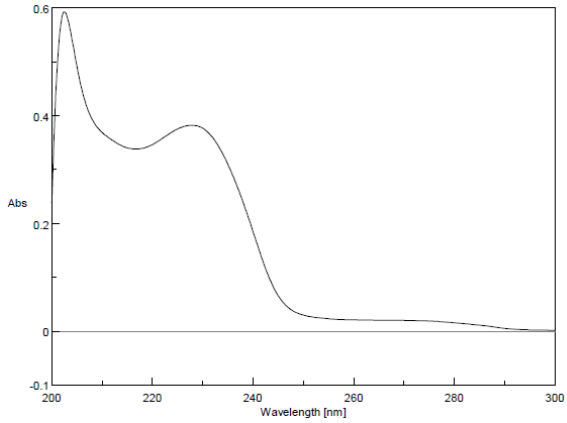


Table 82. Initial Chromatographic Conditions for Ibutilide fumarate

Trial	mobile phase ratio v/v	Fumaric acid		Ibutilide	
		RT Min.	Tailing factor	RT Min.	Tailing factor
1	50:50	2. 635	1.319	6.502	2.015
2	50:50	2.614	1.311	6.529	1.551
3	50:50	2.360	1.300	5.939	1.771
4	50:50	2.383	1.226	5.975	1.935
5	50:50	2.151	1.187	5.393	1.750
6	60:40	2.137	1.161	4.688	1.947
7	60:40	2.126	1.091	4.688	1.806
8	60:40	2.110	1.193	4.692	2.121
9	60:40	2.093	1.223	4.689	1.533
10	55:45	2.596	1.238	6.285	1.564
11	55:45	2.310	1.276	5.550	1.586
12	55:45	2.085	1.420	5.013	1.997

Fig.119 HPLC chromatogram of trial 1

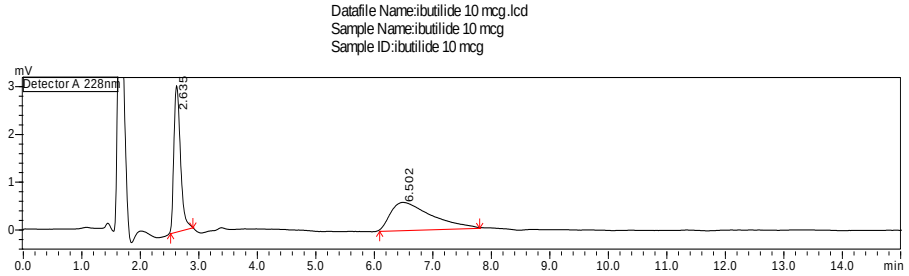


Fig.120 HPLC chromatogram of trial 2

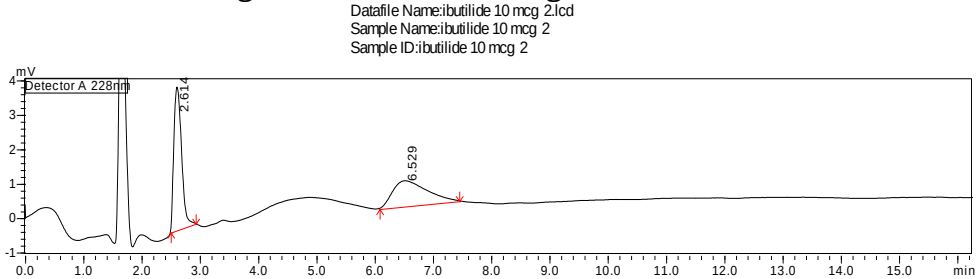


Fig. 121 HPLC chromatogram of trial 3

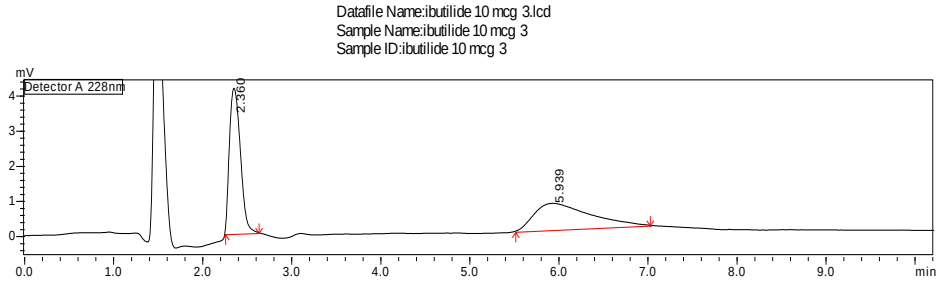


Fig. 122 HPLC chromatogram of trial 4

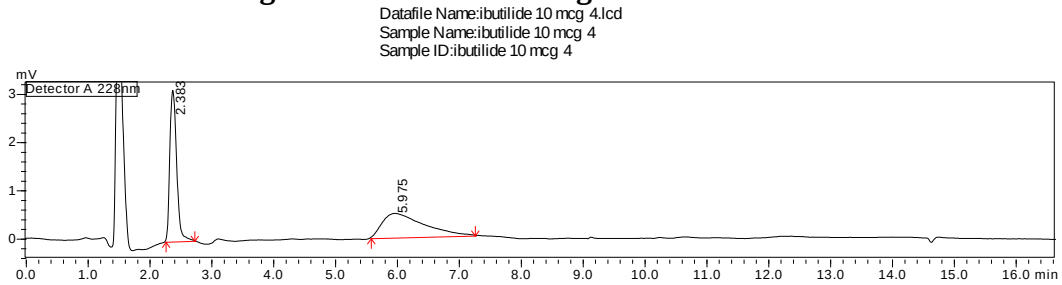


Fig. 123HPLC chromatogram of trial 5

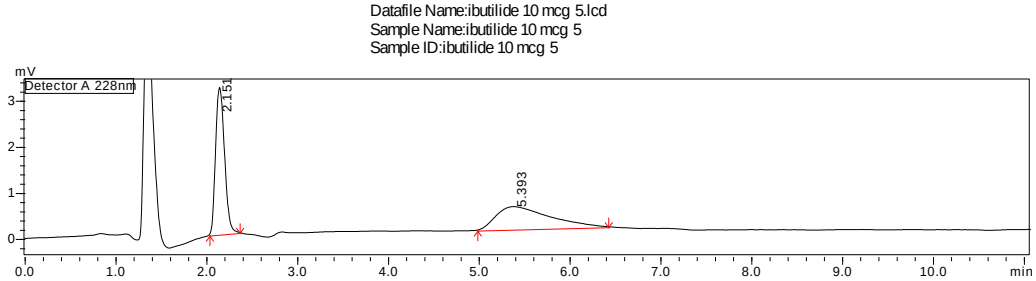


Fig. 124 HPLC chromatogram of trial 6

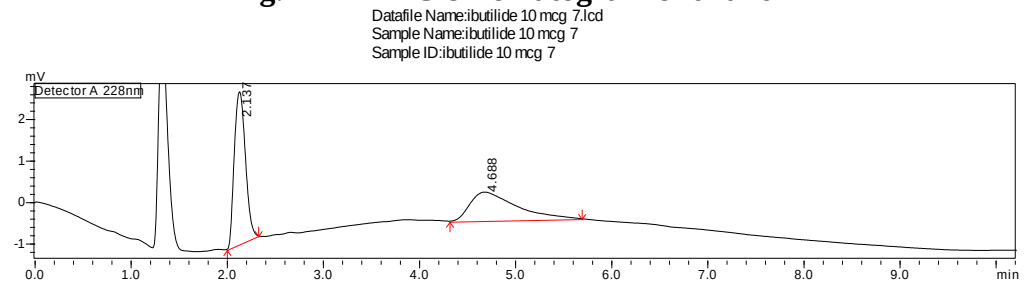


Fig. 125 HPLC chromatogram of trial 7

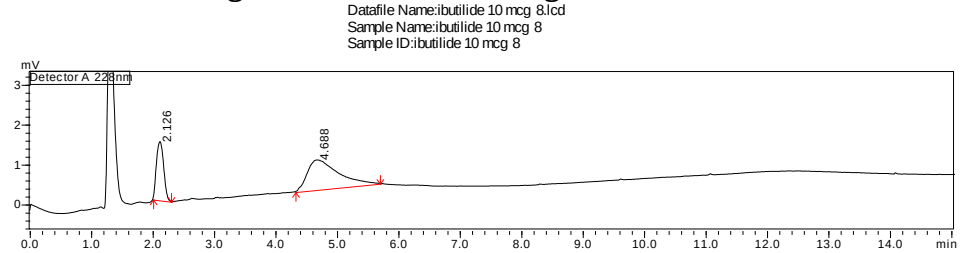


Fig. 126 HPLC chromatogram of trial 8

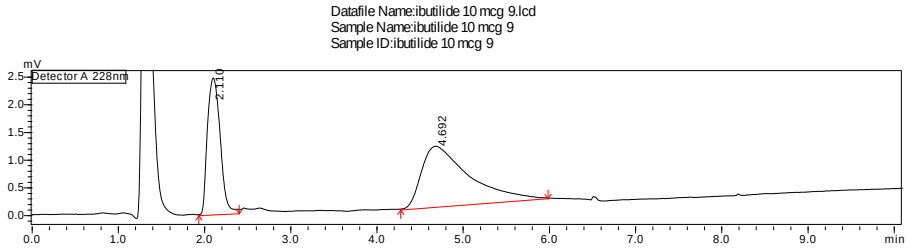


Fig. 127 HPLC chromatogram of trial 9

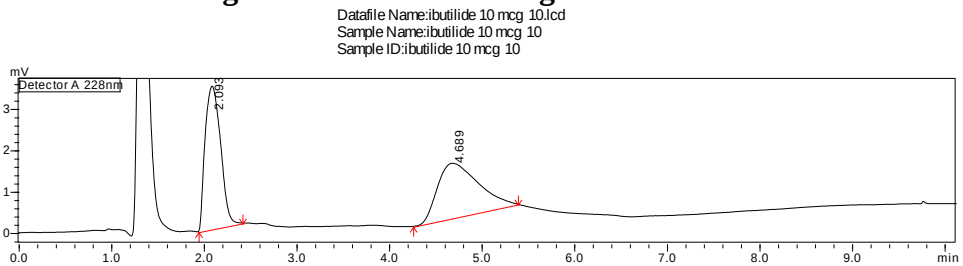


Fig. 128 HPLC chromatogram of trial 10

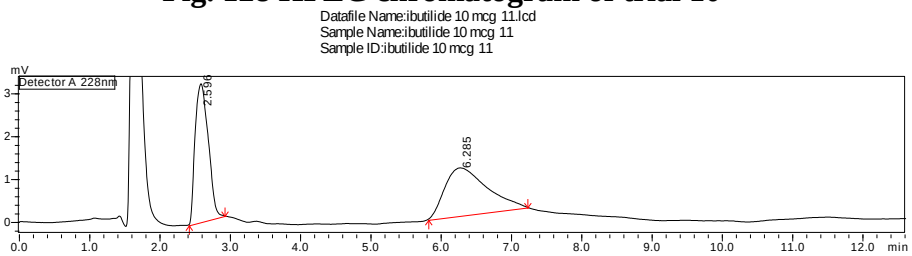


Fig. 129 HPLC chromatogram of trial 11

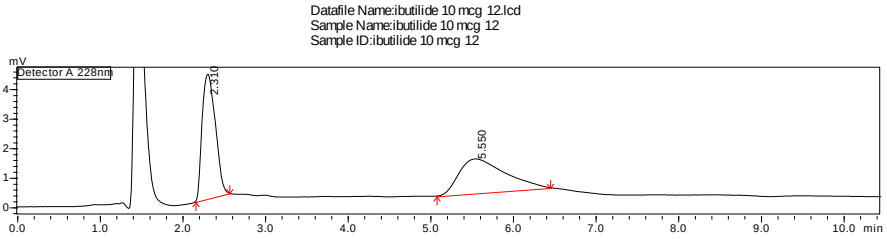


Fig. 130 HPLC chromatogram of trial 12

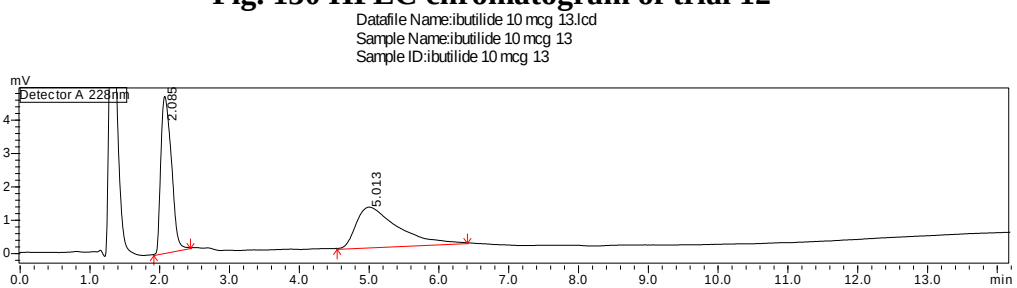


Fig. 131 Representative Chromatogram of ibutilide fumarate

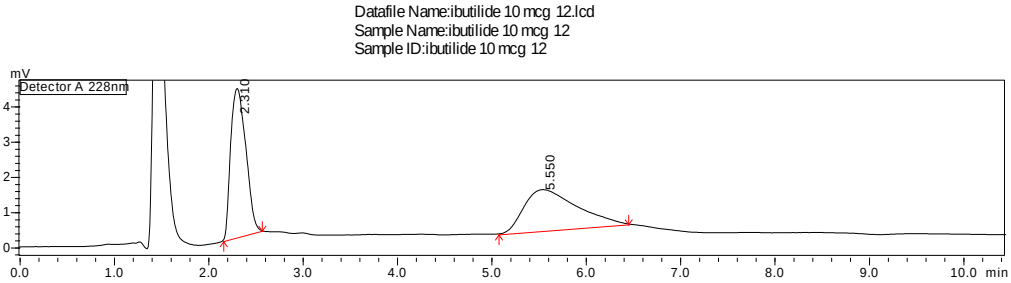


Table 83 Optimized chromatographic conditions for Ibutilide fumarate

Parameters	Fixed experimental conditions
Column	250 mm x 4.6 id x 5 µ particle size
Wavelength(nm)	228 nm
Mobile phase	H_2O:MeOH
Ratio of mobile phase(v/v)	55:45
Flow rate(ml/min)	0.9
Injection volume(µl)	20
Temperature of column(^oC)	Ambient

Fig. 132 Overlay chromatogram of Ibutilide

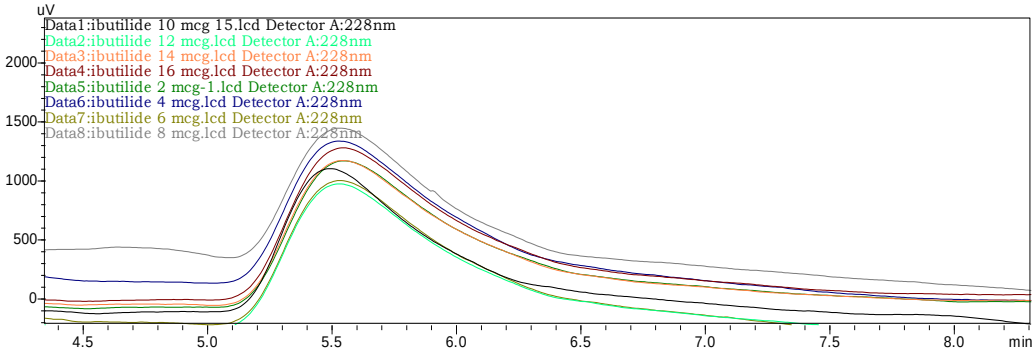


Table 84.Calibration dataof Ibutilide

Concentration mcg/ml	Ibutilide	
	RT Min	Peak area
2	5.551	36372
4	5.532	38310
6	5.536	40446
8	5.533	42069
10	5.011	44951
12	5.531	46947
14	5.547	49381
16	5.546	51964

Fig. 133 Calibration graph for linearity

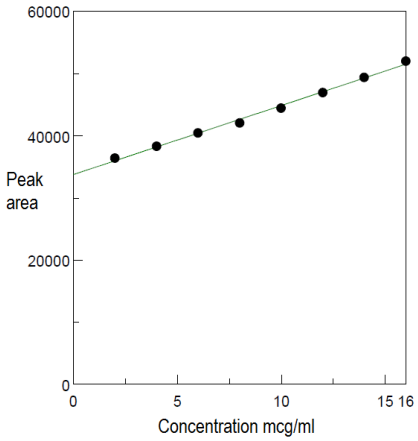


Fig. 134 Representative chromatogram of Fumaric acid

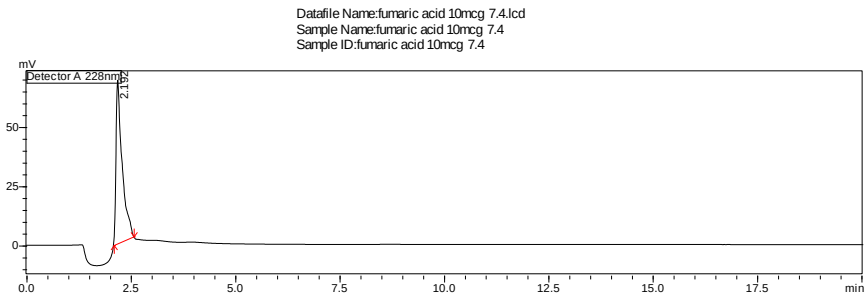


Table 85 Intraday precision studies for Ibutilide

Precision	Peak Area	SD*	%RSD*
Intraday 1	44943	20.40	0.63
Intraday 2	44958		
Intraday 3	44944		
Intraday 4	44899		
Intraday 5	44943		
Intraday 6	44947		

*****Average value

Table 86. Interday precision studies for Ibutilide

Interday Precision	Peak Area*	SD	%RSD
Day 1	44955	13.59	0.04
Day 2	44939	22.18	0.17
Day 3	44966	17.65	0.33

*Mean of six determinations

Table 87.Robustness and Ruggedness

Parameter	Variation	Peak area	RT Min.
Flow rate	0.95 ml	33698 45885	2.214 3.846
	0.85ml	37715 48339	2.452 4.276
Injection volume	19.9 µl	38454 45221	2.231 5.448
	20.1 µl	39567 45973	2.257 5.517
Mobile phase ratio(v/v)	+0.1ml	37203	2.006

		44845	5.011
	-0.1ml	38992 45773	2.596 5.285
Ruggedness	Analyst 1	38554 46565	2.151 5.393
	Analyst 2	36409 45994	2.257 5.497

Table 88. Stability studies of Ibutilide

Room Temperature		**Refrigerator**	
Time Hours	**Peak area**	**Time Hours**	**Peak area**
0	44949	0	44938
2	44018	4	44231
4	43856	8	43984
6	42664	12	42885
8	41674*	24	42643
12	40381	36	41675*
24	39588	48	40022

*Maximum time of stability

Table 89. System suitability studies of Ibutilide

Tailing factor	**Efficiency (N)**	**Asymmetric factor**
1.501	45083	0.994

Fig. 135 HPLC chromatogram of drug solution for Ibutilide fumarate

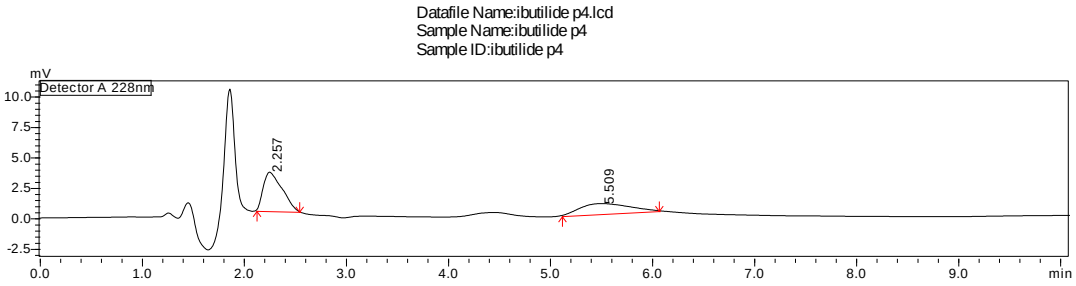


Table 90. Analysis of Ibutilide fumarate drug solution

Amount of drug added (mg)	Amount found (mg)*	SD	% RSD
0.1	0.105	6.78	0.825

* Mean of six observations

Fig.136 Chromatogram of Ibutilide fumarate (Acid hydrolysis – 0 Hr)

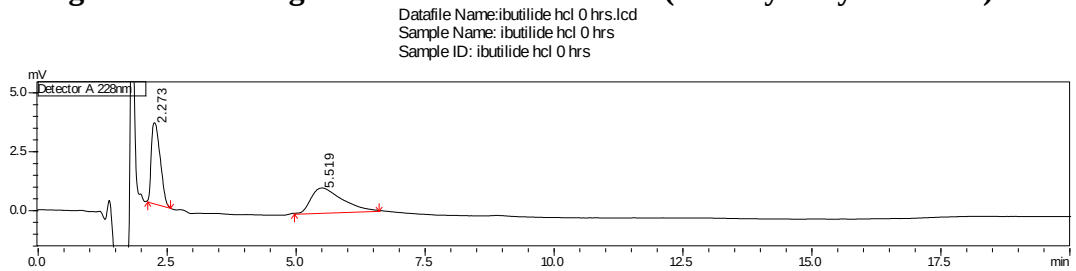


Fig.137 Chromatogram of Ibutilide fumarate(Acid hydrolysis -0.5 Hr)

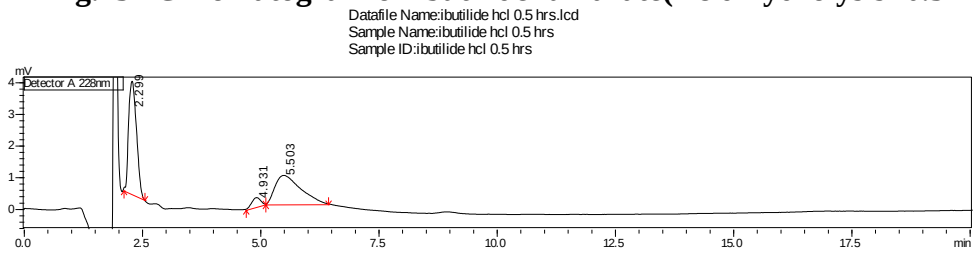


Fig.138 Chromatogram of Ibutilide fumarate (Acid hydrolysis-1 Hr)

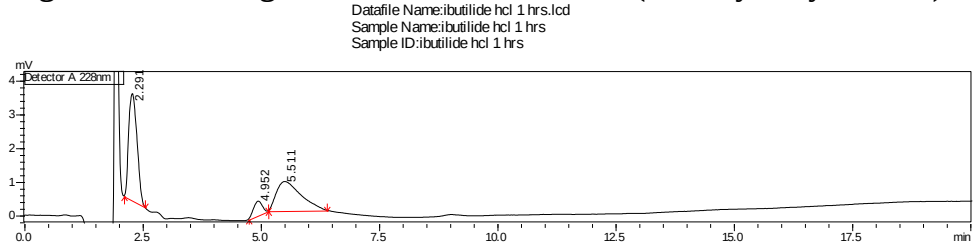


Fig.139Chromatogram of Ibutilide fumarate (Acid hydrolysis- 1.5 Hr)

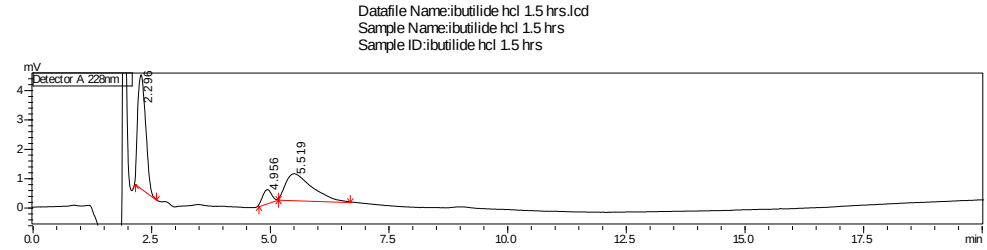


Fig.140 Chromatogram of Ibutilide fumarate (Acid hydrolysis -2 Hr)

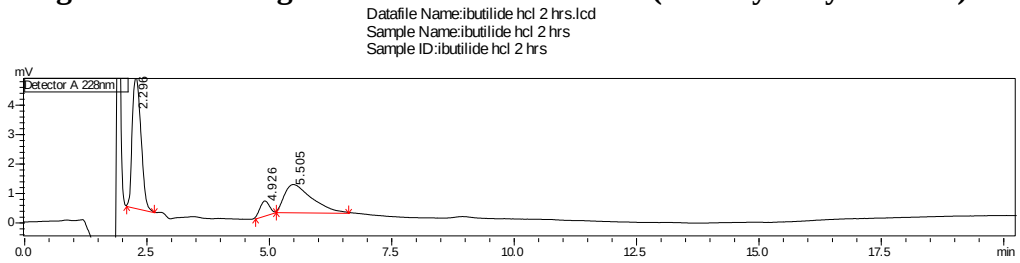


Fig.141– Chromatogram of Ibutilide fumarate (Acid hydrolysis -2.5 Hr)

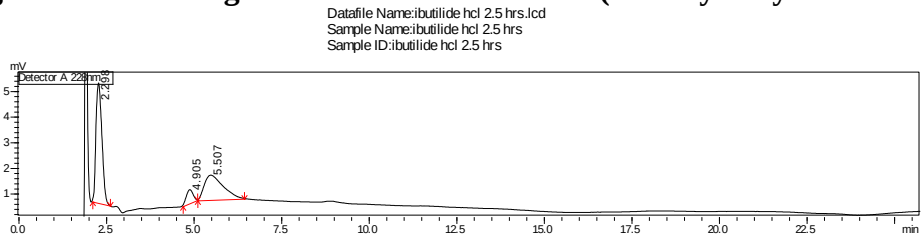


Fig.142. Chromatogram of Ibutilide fumarate (Acid hydrolysis 3 Hr)

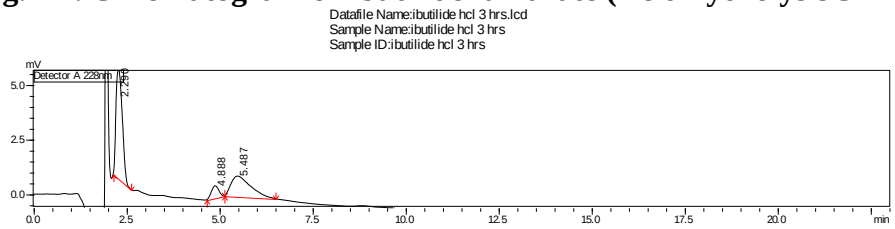


Fig.143 Chromatogram of Ibutilide fumarate (Acid hydrolysis -3.5 Hr)

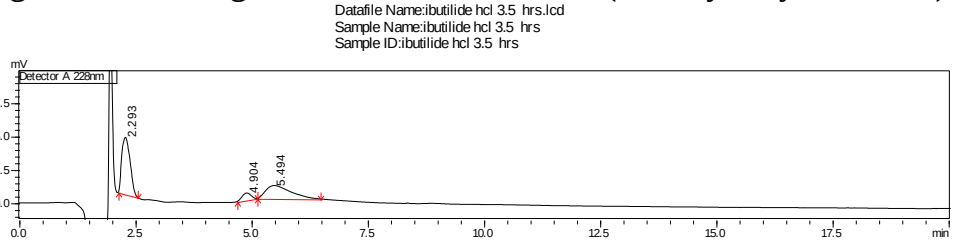


Fig.144 Chromatogram of Ibutilide fumarate (Acid hydrolysis- 4 Hr)

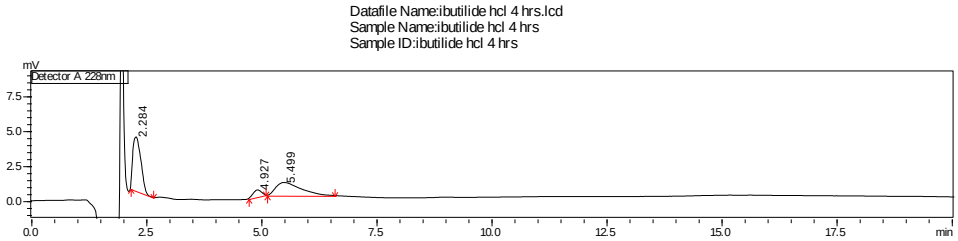


Table 91. Acid hydrolysis of Ibutilide fumarate

Time Hours	RT Min.	% Degradation	Additional peaks at RT Min.
0	5.519	6.30	-
30	5.503	24.39	4.93
60	5.511	24.16	4.95
90	5.519	25.74	4.95
120	5.505	25.75	4.92
150	5.507	26.44	4.90
180	5.487	27.05	4.88
210	5.494	28.07	4.90
240	5.499	29.82	4.92

Fig.145–Chromatogram of Ibutilide fumarate (Base hydrolysis- 0 Hr)

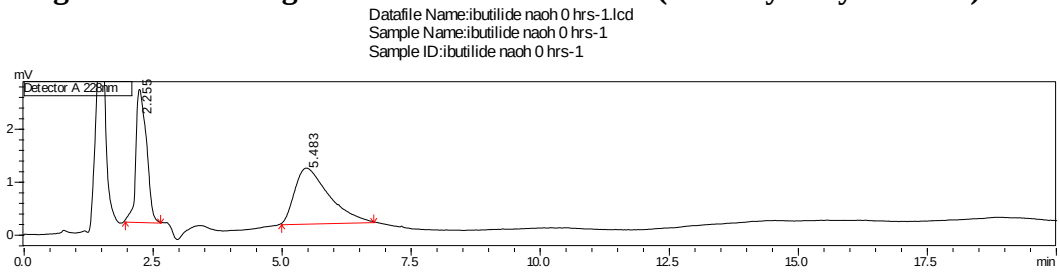


Fig.146 Chromatogram of Ibutilide fumarate (Base hydrolysis 0.5 Hr)

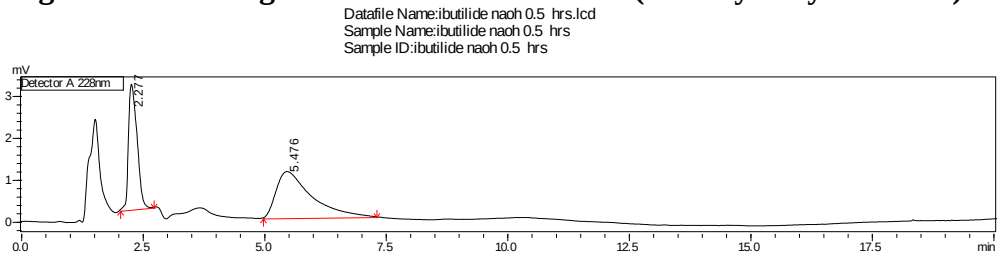


Fig.147Chromatogram of Ibutilide fumarate (Base hydrolysis 1 Hr)

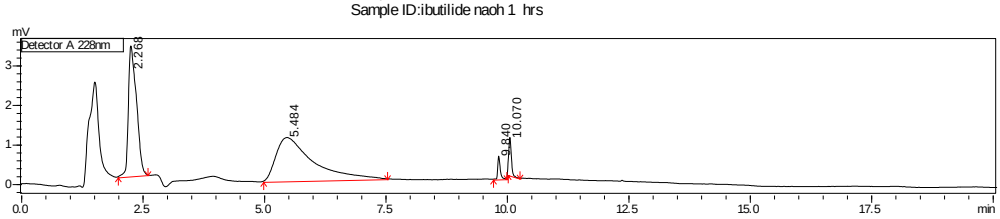


Fig.148 Chromatogram of Ibutilide fumarate (Base hydrolysis 1.5 Hr)

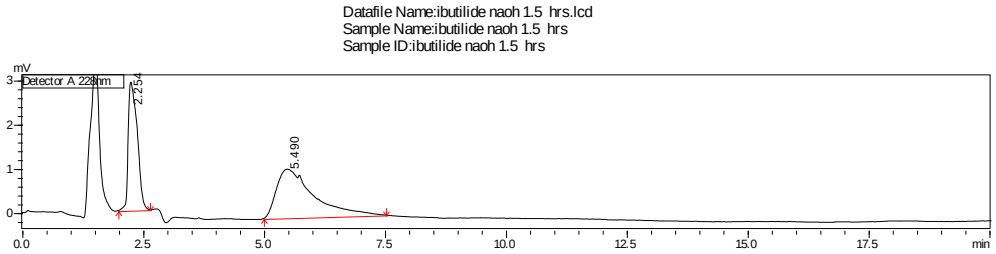


Fig.149 Chromatogram of Ibutilide fumarate (Base hydrolysis 2Hr)

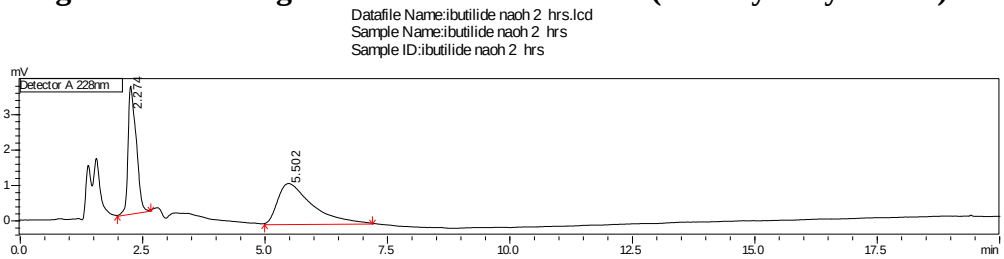


Fig.150 Chromatogram of Ibutilide fumarate (Base hydrolysis 2.5 Hr)

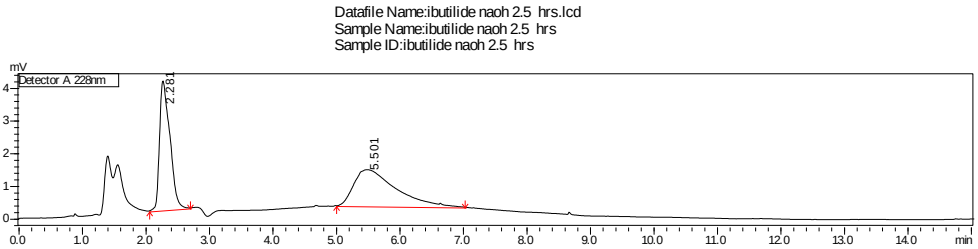


Fig.151 Chromatogram of Ibutilide fumarate (Base hydrolysis 3Hr)

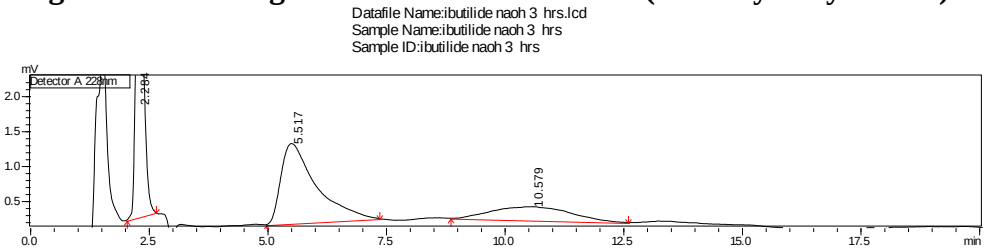


Fig.152 Chromatogram of Ibutilide fumarate (Base hydrolysis 3.5Hr)

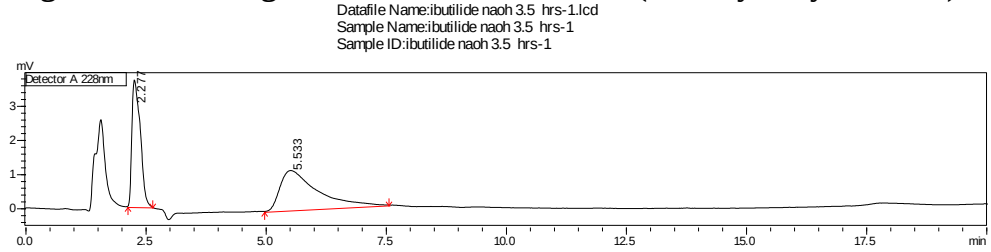


Fig.153 Chromatogram of Ibutilide fumarate (Base hydrolysis 4Hr)

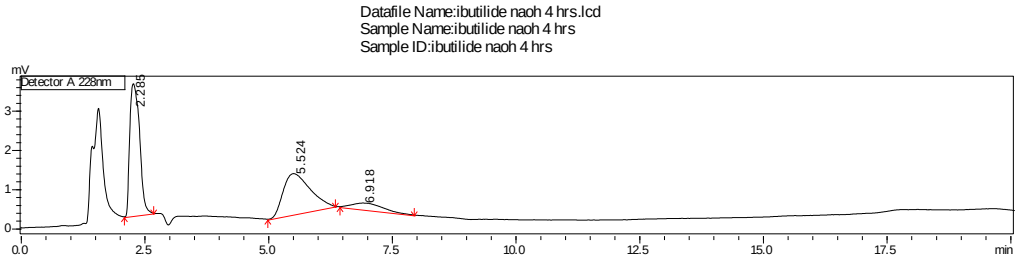


Table 92. Base hydrolysis of Ibutilide fumarate

Time Hours	RT Min.	% Degradation	Additional Peaks at RT Min.
0	5.483	5.97	
30	5.476	17.00	
60	5.484	22.03	
90	5.490	24.42	
120	5.502	26.59	
150	5.501	28.74	
180	5.517	30.11	10.579
210	5.533	32.87	
240	5.524	34.04	6.978

Fig.154 Chromatogram of Ibutilide fumarate (Oxidation – 0 Hr)

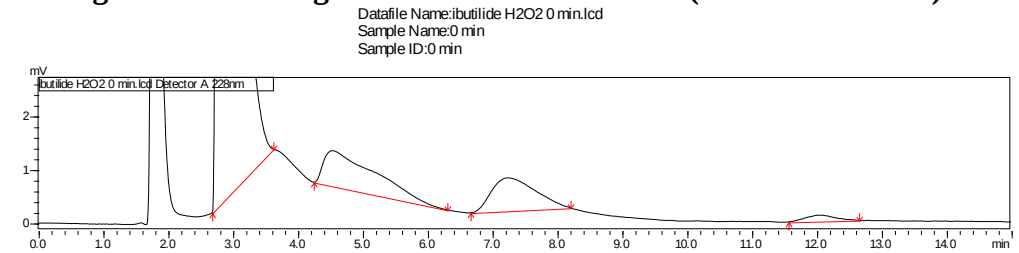


Fig.155 Chromatogram of Ibutilide fumarate (Oxidation – 0.5 Hr)

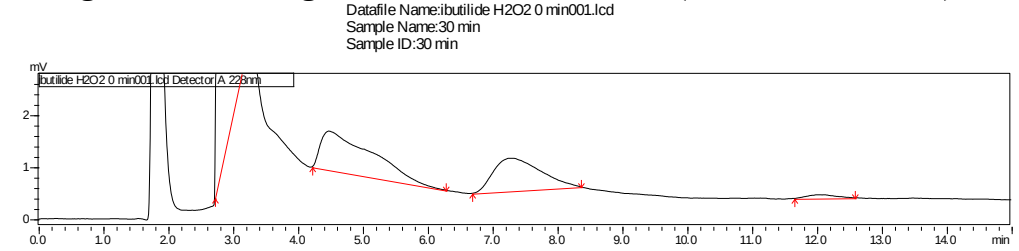


Fig.156 Chromatogram of Ibutilide fumarate (Oxidation – 1 Hr)

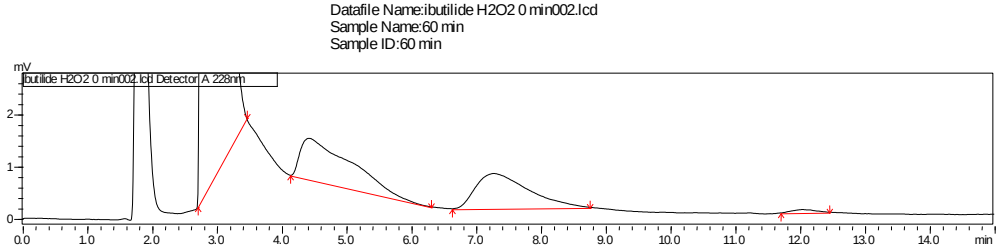


Fig.157 Chromatogram of Ibutilide fumarate (Oxidation – 1.5 Hr)

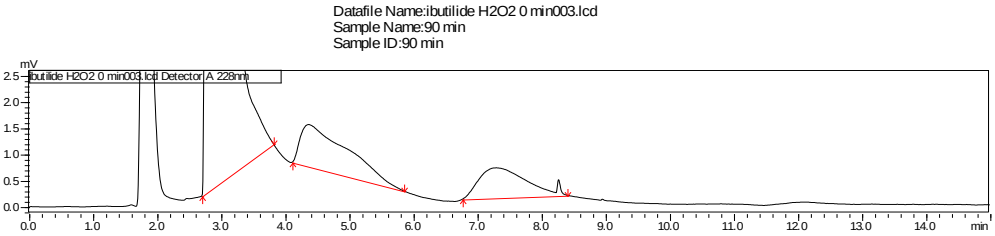


Fig.158 Chromatogram of Ibutilide fumarate (Oxidation – 2 Hr)

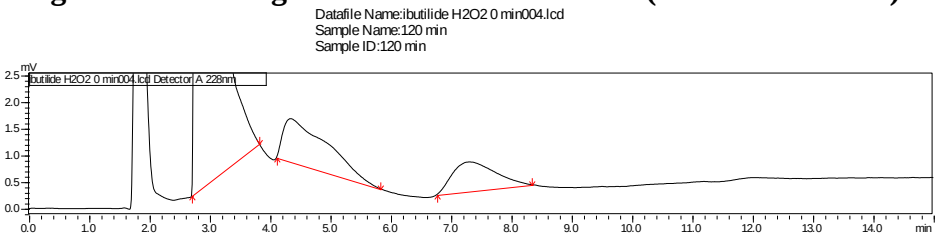


Fig.159 Chromatogram of Ibutilide fumarate (Oxidation – 2.5 Hr)

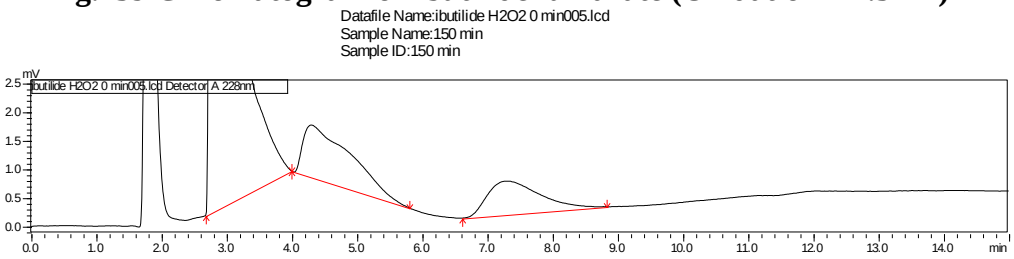


Fig.160 Chromatogram of Ibutilide fumarate (Oxidation – 3 Hr)

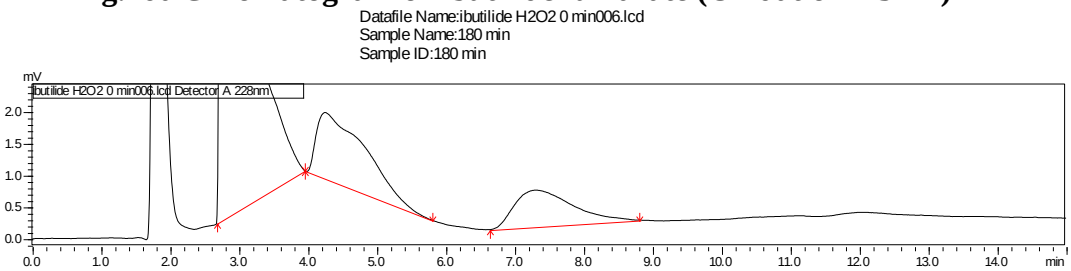


Fig.161 Chromatogram of Ibutilide fumarate (Oxidation – 3.5 Hr)

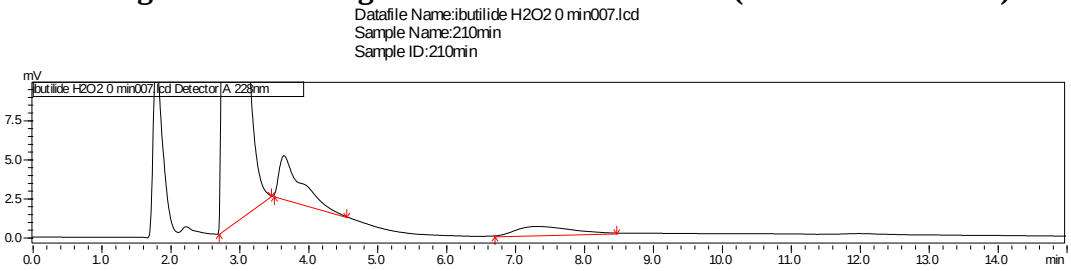


Fig.162 Chromatogram of Ibutilide fumarate (Oxidation – 4 Hr)

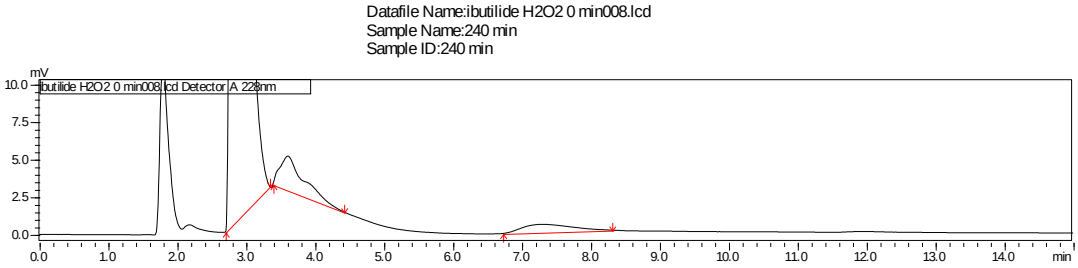


Table 93. Oxidation of Ibutilide fumarate

Time Hours	RT Min.	% Degradation	Additional peaks at RT Min.
0	7.230	14.54	4.534 12.045
30	7.315	11.16	4.481 12.018
60	7.276	11.62	4.427 12.037
90	7.298	16.29	4.369
120	7.313	19.01	4.341
150	7.321	22.28	4.300
180	7.316	22.66	4.250
210	7.348	25.85	3.653
240	7.295	37.60	3.608

Fig.163 Chromatogram of Ibutilide fumarate (Thermal 40ºC – 0.5 Hr)

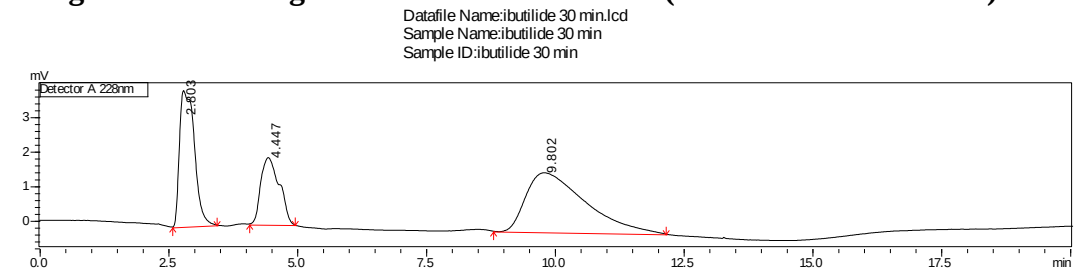


Fig.164 Chromatogram of Ibutilide fumarate (Thermal 40ºC – 1Hr)

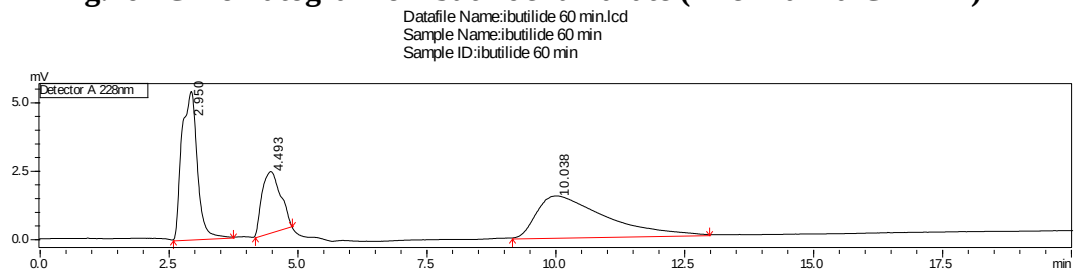


Fig.165 Chromatogram of Ibutilide fumarate (Thermal 40ºC – 1.5Hr)

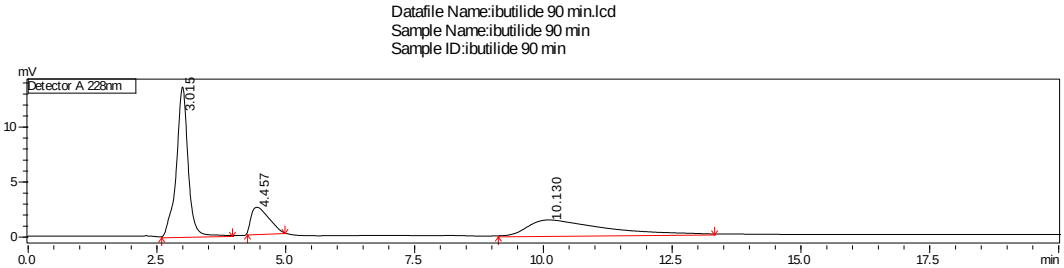


Fig.166 Chromatogram of Ibutilide fumarate (Thermal 40ºC – 2 Hr)

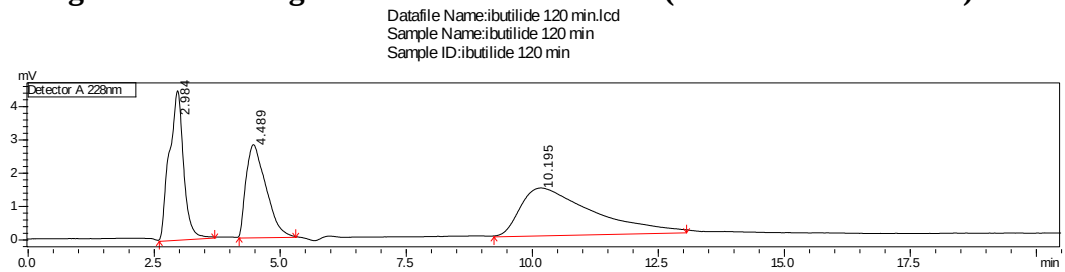


Fig.167 Chromatogram of Ibutilide fumarate (Thermal 40ºC – 2.5 Hr)

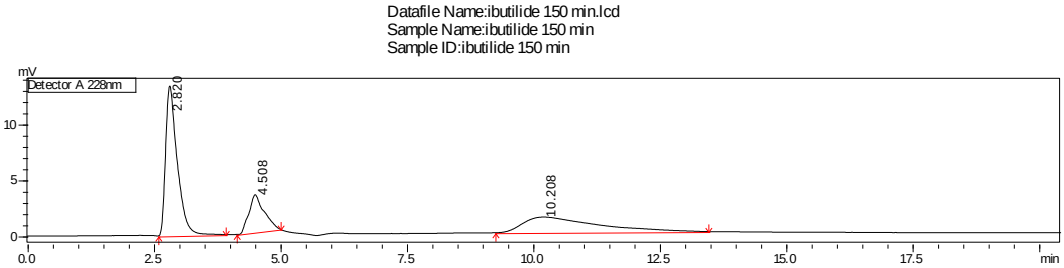


Fig.168 Chromatogram of Ibutilide fumarate (Thermal 40ºC – 3Hr)

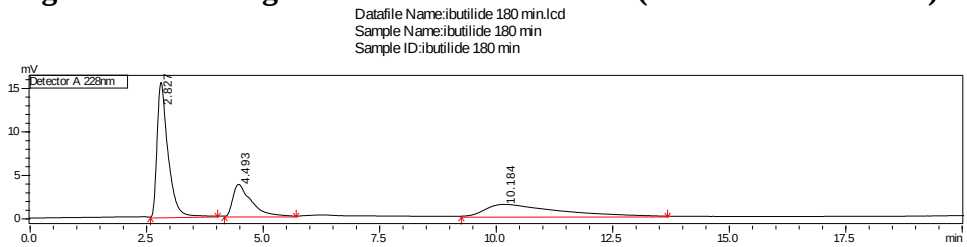


Fig.169 Chromatogram of Ibutilide fumarate (Thermal 40ºC – 3.5Hr)

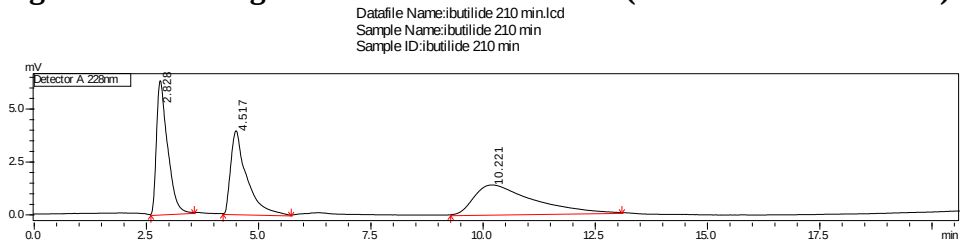


Fig.170 Chromatogram of Ibutilide fumarate (Thermal 40ºC – 4Hr)

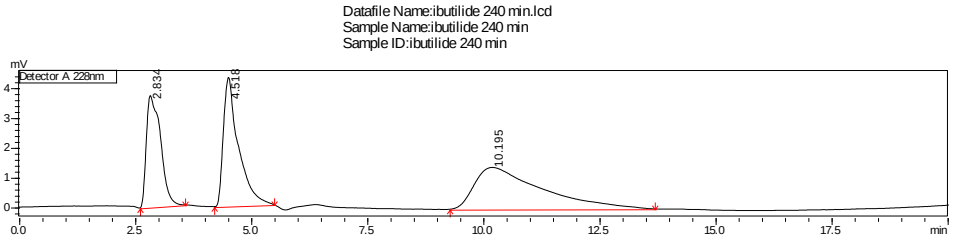


Table 94. Thermal degradation of Ibutilide fumarate at 40 ºC

Time Hours	RT Min.	% Degradation	Additional peak at RT Min.
0	6.285		
30	4.447	-	9.802
60	4.493	0.18	10.038
90	4.457	0.579	10.130
120	4.489	1.11	10.195
150	4.508	1.46	10.208
180	4.493	2.56	10.184
210	4.517	2.57	10.221
240	4.518	2.614	10.195

Fig.171 Chromatogram of Ibutilide fumarate (Thermal 60ºC – 0.5 Hr)

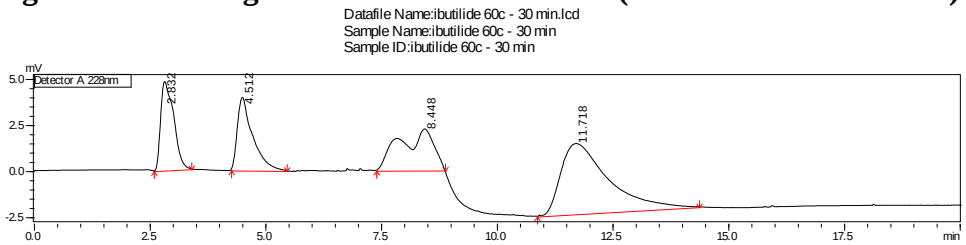


Fig.172 Chromatogram of Ibutilide fumarate (Thermal 60ºC – 1Hr)

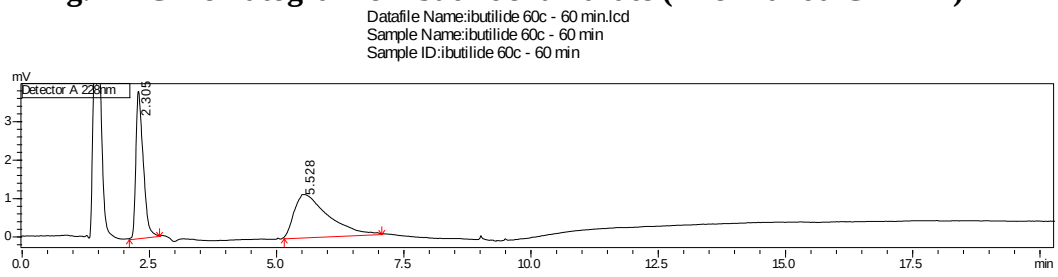


Fig.173 Chromatogram of Ibutilide fumarate (Thermal 60ºC – 1.5Hr)

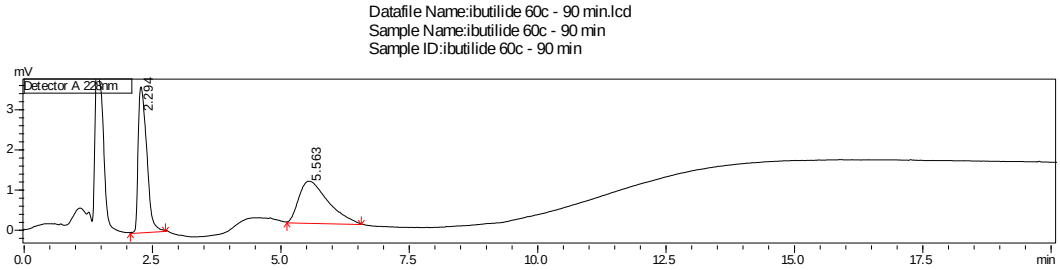


Fig.174Chromatogram of Ibutilide fumarate (Thermal 60ºC – 2Hr)

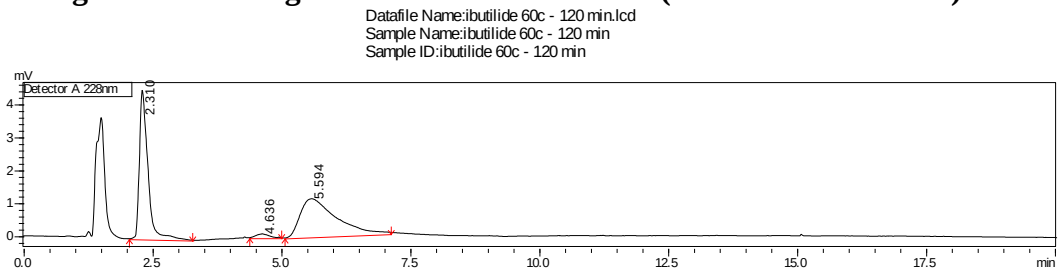


Fig.175 Chromatogram of Ibutilide fumarate(Thermal 60ºC – 2.5Hr)

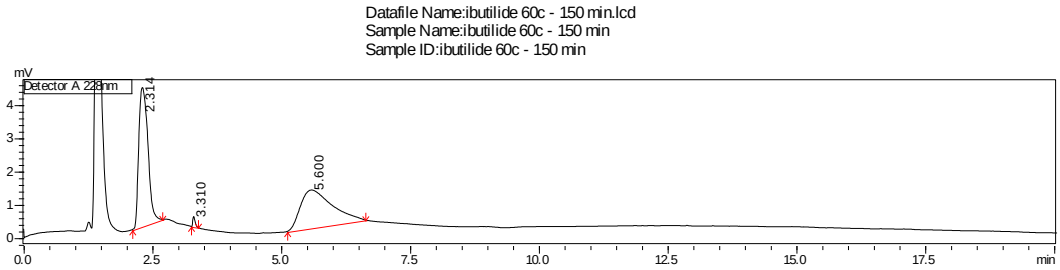


Fig.176 Chromatogram of Ibutilide fumarate (Thermal 60ºC – 3Hr)

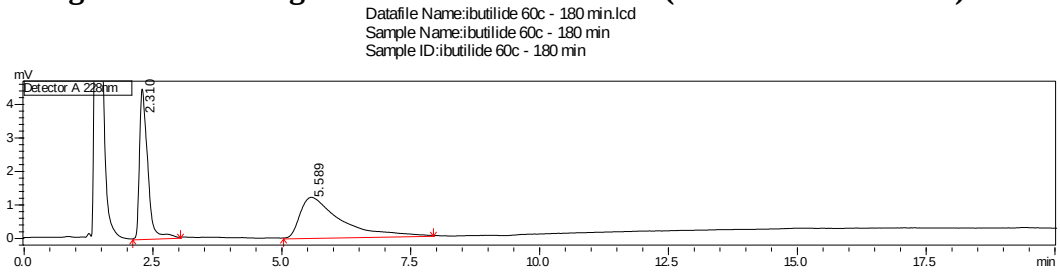


Fig.177 Chromatogram of Ibutilide fumarate (Thermal 60ºC – 3.5Hr)

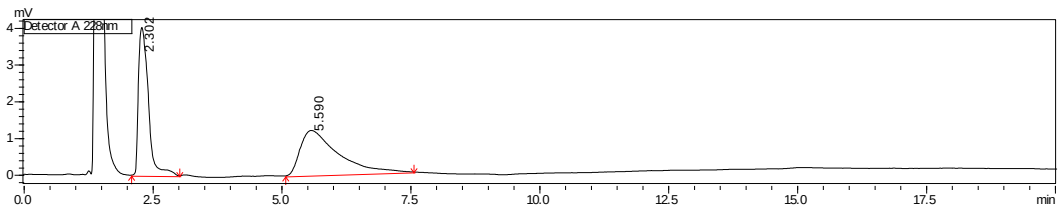


Fig.178 Chromatogram of Ibutilide fumarate (Thermal 60ºC – 4Hr)

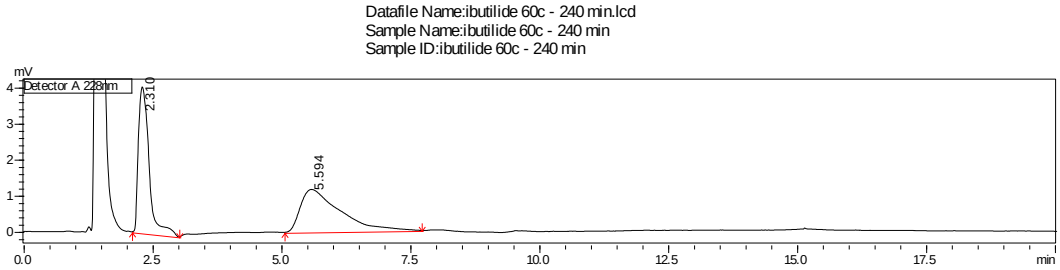


Table 95. Thermal degradation of Ibutilide fumarate at 60 ºC

Time Hours	RT Min.	% Degradation	Additional peak at RT Min.
0	6.285	-	
30	4.512	-	8.448 11.718
60	5.528	8.91	-
90	5.563	9.73	-
120	5.594	13.13	4.636
150	5.600	25.65	3.310
180	5.589	38.22	-
210	5.590	40.24	-
240	5.594	43.71	-

Fig.179 Chromatogram of Ibutilide fumarate (Photolysis – 0 Hr)

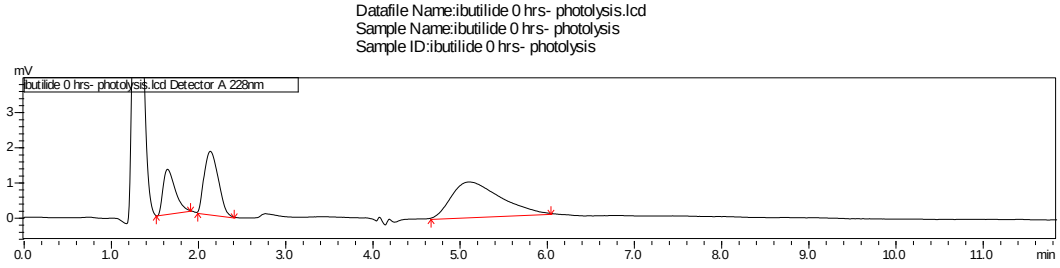


Fig.180 Chromatogram of Ibutilide fumarate (Photolysis – 0.5Hr)

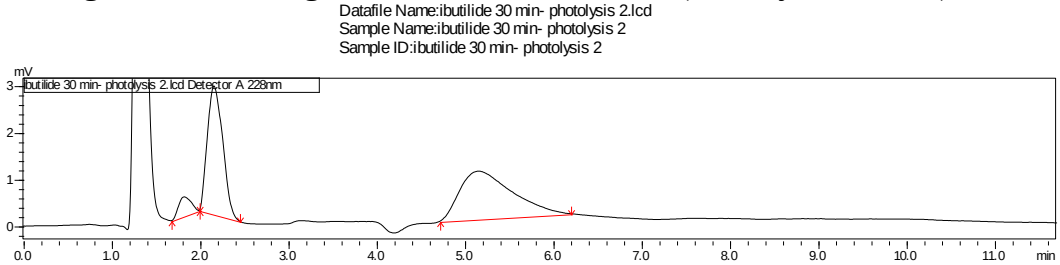


Fig.181 Chromatogram of Ibutilide fumarate (Photolysis – 1Hr)

Datafile Name:ibutilide 60 min- photolysis.lcd
Sample Name:ibutilide 60 min- photolysis
Sample ID:ibutilide 60 min- photolysis

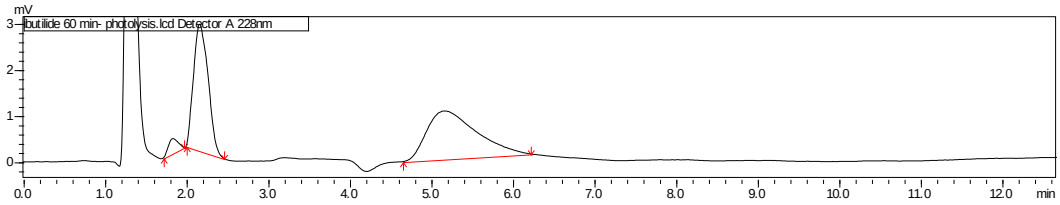


Fig.182 Chromatogram of Ibutilide fumarate (Photolysis – 1.5Hr)

Datafile Name:ibutilide 90 min- photolysis.lcd
Sample Name:ibutilide 90 min- photolysis
Sample ID:ibutilide 90 min- photolysis

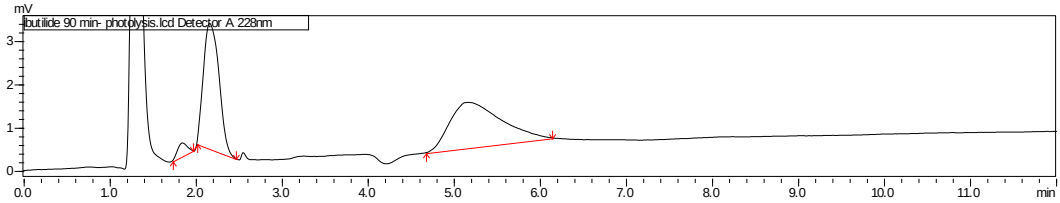


Fig.183 Chromatogram of Ibutilide fumarate (Photolysis – 2Hr)

Datafile Name:ibutilide 120 min- photolysis.lcd
Sample Name:ibutilide 120 min- photolysis
Sample ID:ibutilide 120 min- photolysis

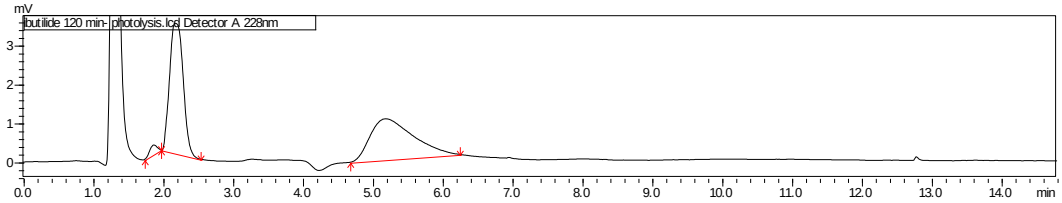


Fig.184 Chromatogram of Ibutilide fumarate (Photolysis – 2.5Hr)

Datafile Name:ibutilide 150 min- photolysis.lcd
Sample Name:ibutilide 150 min- photolysis
Sample ID:ibutilide 150 min- photolysis

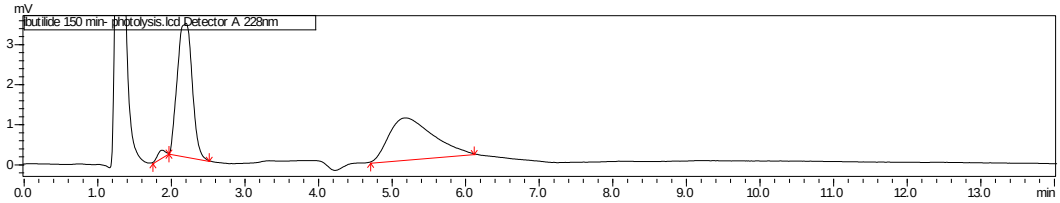


Fig.185 Chromatogram of Ibutilide fumarate (Photolysis – 3Hr)

Datafile Name:ibutilide 180 min- photolysis.lcd
Sample Name:ibutilide 180 min- photolysis
Sample ID:ibutilide 180 min- photolysis

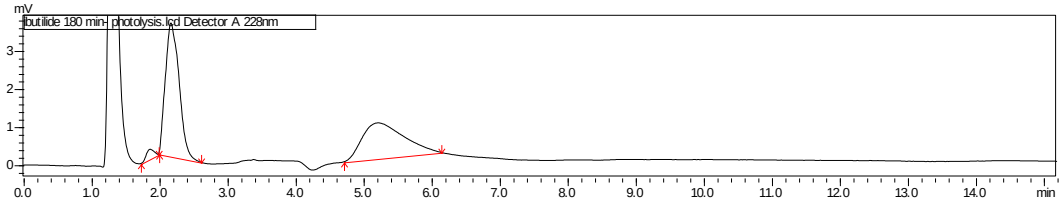


Fig.186 Chromatogram of Ibutilide fumarate (Photolysis – 3.5Hr)

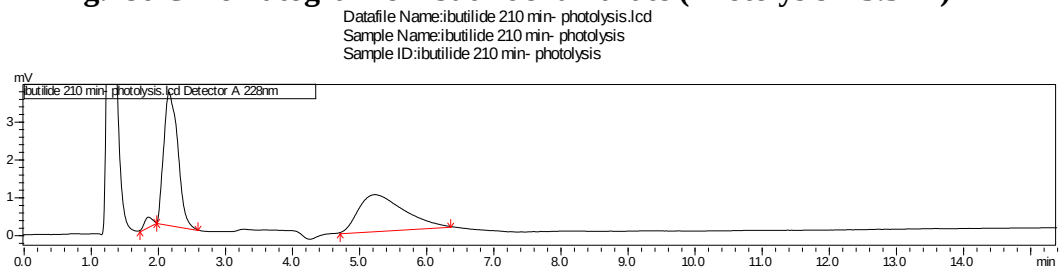


Fig.187 Chromatogram of Ibutilide fumarate (Photolysis – 4Hr)

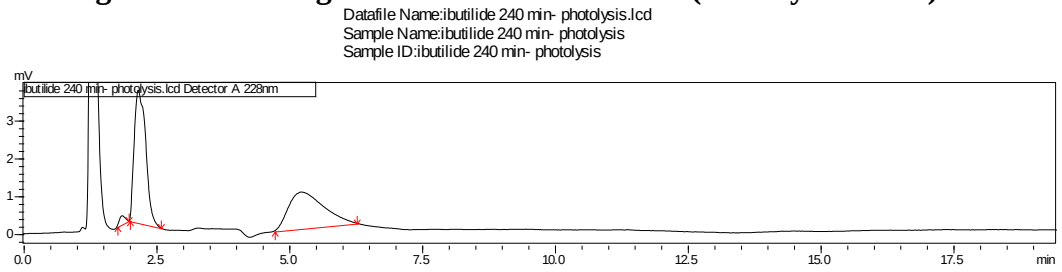


Table 96. Photolysis of Ibutilide fumarate

Time Hours	RT Min.	% Degradation
0	5.112	8.91
30	5.159	10.1
60	5.167	12.01
90	5.181	13.59
120	5.189	15.52
150	5.189	18.16
180	5.217	19.24
210	5.244	19.81
240	5.234	21.04

Fig. 188 Overlay spectrum of Ibutilide fumarate and verapamil

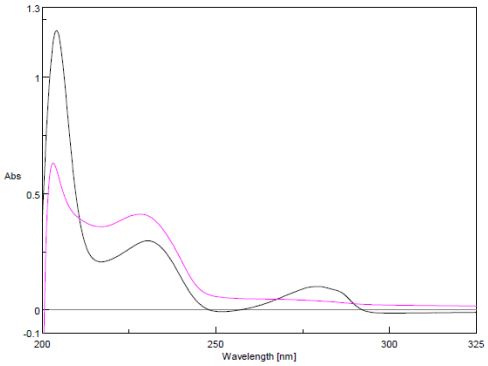


Table 97. Validation and system suitability parameter of Ibutilide fumarate and verapamil

Parameter	Ibutilide fumarate	Verapamil
RT Min.	4.07	11.4
R_s	-	12.8
No. of theoretical plates	9436	8864
A_s	1.0	1.1
T_f	1.54	1.23
Linearity (µg/ml)	0.5-16	0.2-3.2
Correlation coefficient	0.9999	0.9999
LOD (µg/ml)	0.003	0.001
LOQ(µg/ml)	0.09	0.05
Precision(%RSD)	0.439	0.992

Fig. 189 Chromatogram of ibutilide fumarate and verapamil (Simulated condition 0Hr)

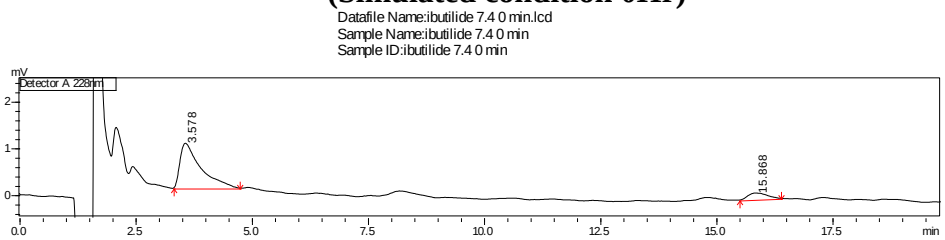


Fig. 190 Chromatogram of ibutilide fumarate and verapamil(Simulated condition 4Hr)

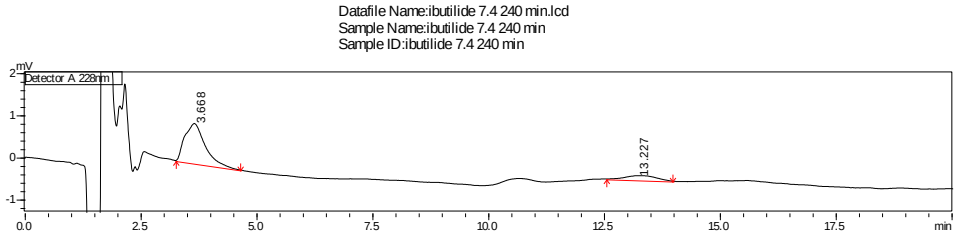


Fig. 191 Chromatogram of Ibutilide fumarate(With enzymes 0Hr)

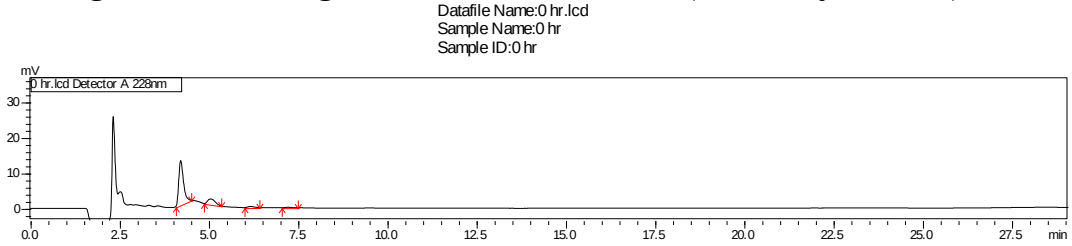
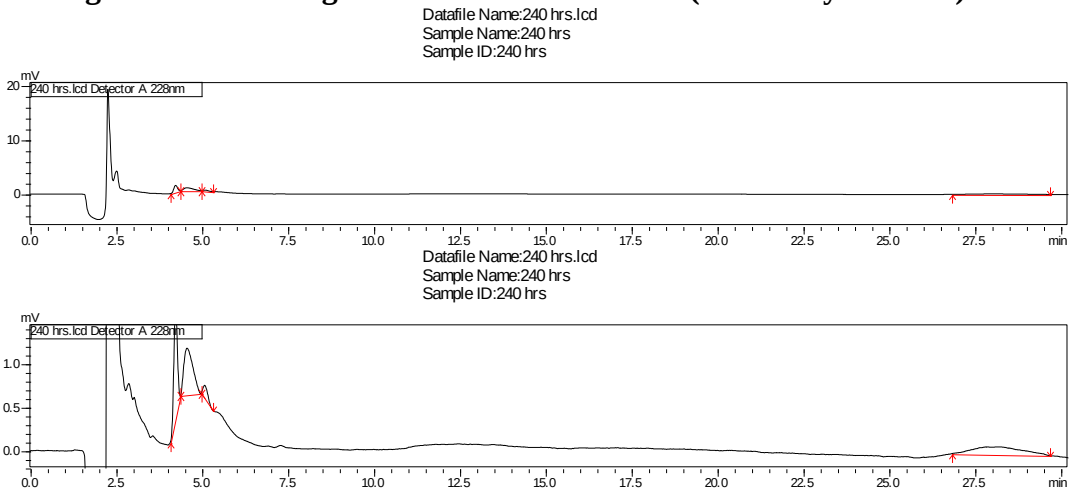


Fig. 192 Chromatogram of Ibutilide fumarate(With enzymes 4Hr)



. **Fig.193 Chromatogram of verapamil (With enzymes 0Hr)**

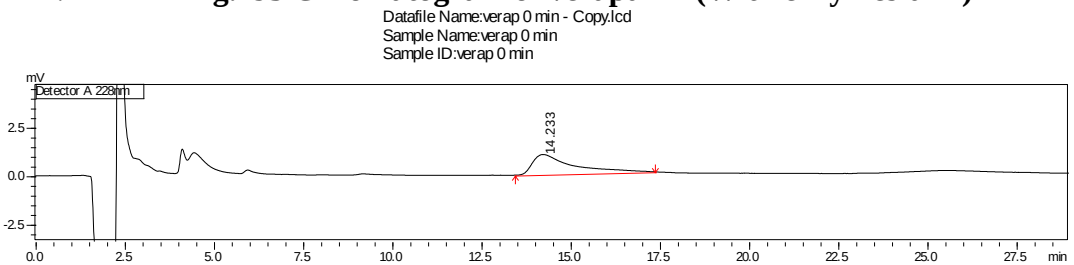


Fig.194 Chromatogram of verapamil (With enzymes 4Hr)

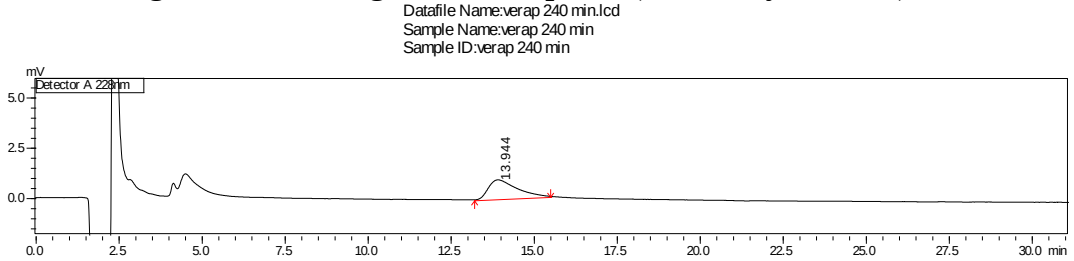


Fig.195 Chromatogram of ibutilide fumarate and verapamil(With enzymes 0Hr)

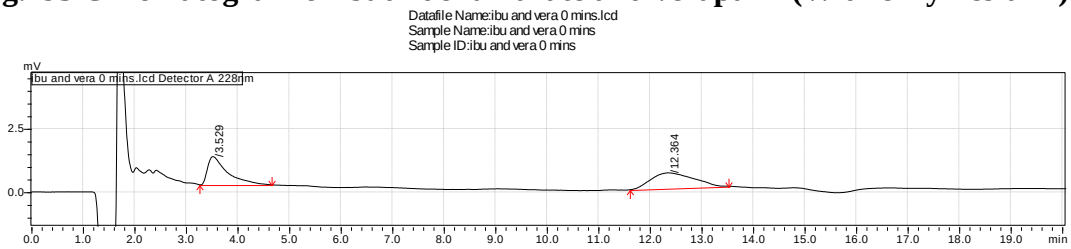


Fig.196 Chromatogram of ibutilide fumarate and verapamil(With enzymes 4Hr)

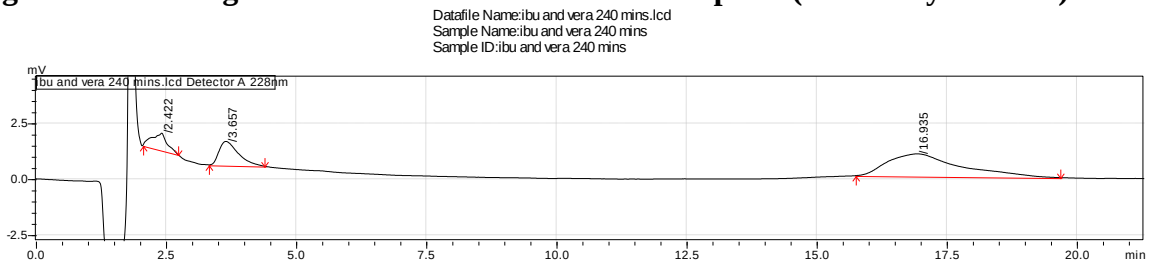


Table 98. Result of *Invitro* drug-drug interaction study of Ibutilide fumarate and verapamil

Drug	% Remaining					
	Simulated condition		With enzyme		With enzymes	
	Mixture of drugs		Individual drug		Mixture of drugs	
	0hour	4thhour	0hour	4thhour	0hour	4thhour
Ibutilide fumarate	100%	96%	100%	8%	100%	91%
Verapamil	100%	37%	100%	19%	100%	56%

7.4 DOFETILIDE

7.4.1 DEVELOPMENT OF VALIDATED UV SPECTROSCOPY METHOD FOR DOFETILIDE

7.4.1.1Selection of experimental conditions

Pure drug dofetilide was found to be completely soluble in methanol. The UV spectrum was recorded for the drug in methanol. A 10 mcg/ml solution was scanned between 200 to 400 nm which gave a Gaussian curve and the λ_{max} was found at 231 nm. TheOptimized instrumental parameters include scan speed of 400 nm/min, slit width of 0.1 mm and a band width of 0.5 mm. In these conditions dofetilide gave the smooth spectrum with highest absorbance (Figure 197).

7.4.1.2 Validation of UV Spectroscopy Method

Linearity and range

The calibration curve was constructed by preparing methanolic solution of the drug dofetilide with the concentration range between 2 – 18 mcg/ml. Linearity was evaluated by linear regression analysis, which was calculated by least square regression method with the concentration versus absorbance for plotting the linearity graph. The Y=A*X+B equation was derived with slope (A) = 0.0786, intercept (B) = 0.0191, SE = 0.2929 and correlation coefficient of 0.9987. The linear absorbance measured are shown in table 99. The figure 198&199 represents the overlay spectra of dofetilide and the calibration graph in linear concentrations.

Precision studies

Repeatability of the detector response for the standard solution was checked for six times on same day was performed for the intraday precision studies. Each time the solution was scanned six times. The interday precision was observed by measuring the absorbance on three different days with six replicate measurements. The data of absorbance for the intraday and interday precision studies are presented in table 100 and 101 .

Accuracy

Accuracy of the method was determined by analysing three solution prepared from admixture. The two different level of addition (50% and 100%) of the standard drug was added to the admixture. The observations and % recovery are given in the table 102.

Robustness and Ruggedness

The absorbance observed at a wavelength difference of 231±0.1nm, werenot to have any significant change. The response obtained by two analyst shows that the method was rugged. The operating conditions of the instrument and the experimental conditions were the same for both the analyst.

Limit of detection and Limit of quantification

LOQ and LOD were calculated by the use of the equation 3.3 σ/S and 10 σ/S based on the signal to noise parameter of the instrument. The detectable limit was found to be 0.0591mcg/ml and amount that can be quantified was found to be 0.1791mcg/ml.

Stability of solution

The stability of a methanolic solution of dofetilide (10mcg/ml) was studied shows that while stored at room temperature it was stable till 44 hours and refrigeration for 8days.The values are presented in table 103.

Specificity of the method

To prove the specificity of methanol, spectrum of methanol was recorded using water as blank, placed in the reference cell. The absorbance of methanol at the λ_{max} of the drug i.e., 231 nm was noted. Methanol was found to have negligible absorbance at the selected wavelength. The spectrum of the admixture solution prepared in methanol was found to be having no interference at the selected wavelength. The figure 200 is for the overlay spectra of drug dofetilide and methanol. The overlay spectra of drug and excipients is illustrated in figure 201.

Optical parameters of the method

The experimental data were used for calculating the molar extinction coefficient for the drug. The Sandell's sensitivity value for the concentration with 0.001 absorbance and E(1%,1cm) were calculated are presented in table 104.

7.4.1.3 Application of UV spectroscopy method for the analysis of admixture

Six replicate measurements of absorbance were performed for admixture 0.500 mg and the average value was calculated. The amount of drug present was found and the % RSD was evaluated. The SD and % RSD were calculated and shown in the table 105. The representative spectrum of the admixture is shown in figure 202.

Fig. 197 UV Absorption spectrum of Dofetilide (10 mcg/ml)

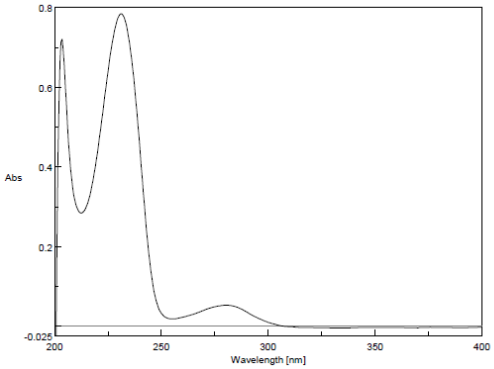


Fig.198 Overlay Spectrum of dofetilide

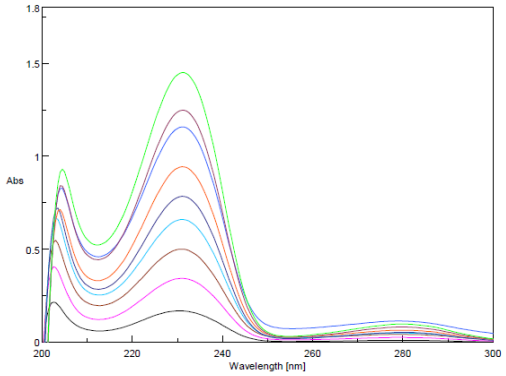


Table 99. Calibration data of Dofetilide

Concentration mcg/ml	Absorbance
2	0.1679
4	0.3427
6	0.4997
8	0.6591
10	0.7875
12	0.9427
14	1.1567
16	1.2469
18	1.4491

Fig.199 Calibration graph of Dofetilide

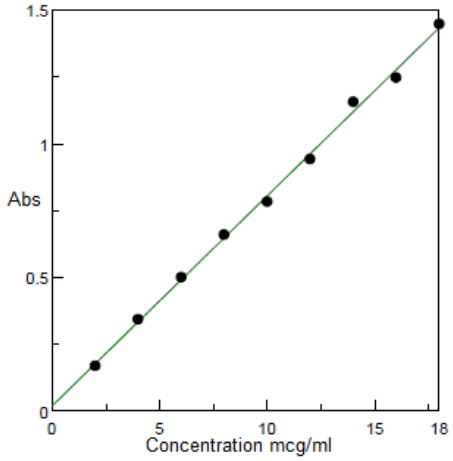


Table 100. Intraday Precision Studies of Dofetilide

Precision	Absorbance	SD*	% RSD*
Intraday 1	0.7896	0.006	0.014
Intraday 2	0.7877		
Intraday 3	0.7889		
Intraday 4	0.7864		
Intraday 5	0.7888		
Intraday 6	0.7895		

*Average value

Table 101. Interday Precision Studies of Dofetilide

Precision	Absorbance*	SD	%RSD
Day 1	0.7902	0.18	0.36
Day 2	0.7895	0.07	0.04
Day 3	0.7879	0.22	0.40

*****Mean of six observations

Table 102. Accuracy studies of Dofetilide

Level	Amount of Std added (mg)	Amount of Std* recovered(mg)	% Recovery of the Std*	SD	% RSD
50%	0.250	0.255	102.0	0.54	0.20
100%	0.500	0.504	100.8	0.71	0.31

*Mean of three determinations

Table 103.Stability of Dofetilide solution

Room Temperature		Refrigerator	
Time Hours	Absorbance	Time Hours	Absorbance
0	0.7877	0	0.7869
4	0.7865	12	0.7843
8	0.7858	24	0.7801
12	0.7803	36	0.7732
16	0.7779	48	0.7711
24	0.7732	60	0.7690
36	0.7688	84	0.7655
44	0.7637*	96	0.7640*
48	0.7016	108	0.7144

*Maximum time of stability

Fig.200 Overlay spectrum of Dofetilide and Methanol

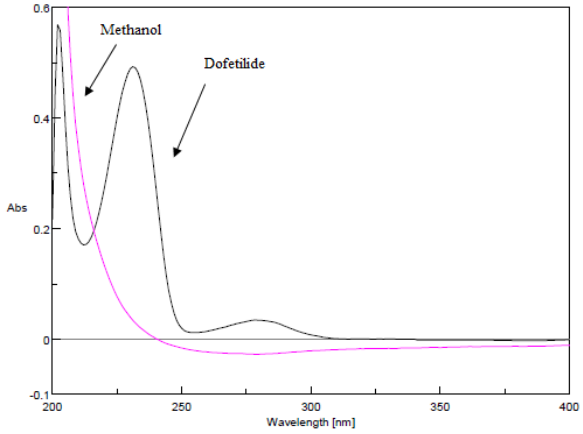


Fig.201 Overlay spectrum of Dofetilide and Excipients

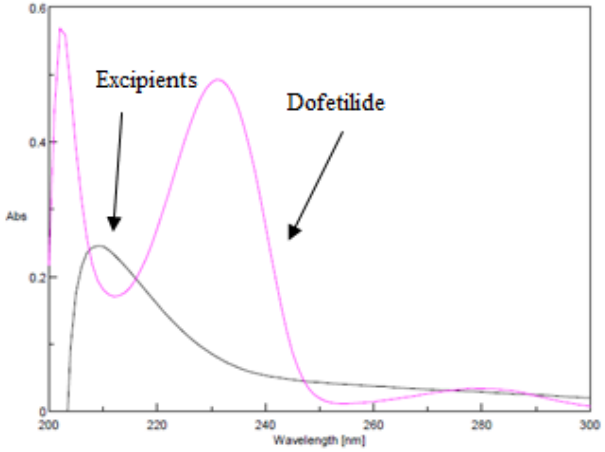


Table 104. Optical parameters of Dofetilide

Parameter	Result
Molar absorptivity (L mol^{-1} cm^{-1})	3.5 x 10^5
Sandell's sensitivity (mcg/ml)	0.0123
E (1%,1cm)	810.3

Fig.202 Representative spectrum of dofetilide admixture

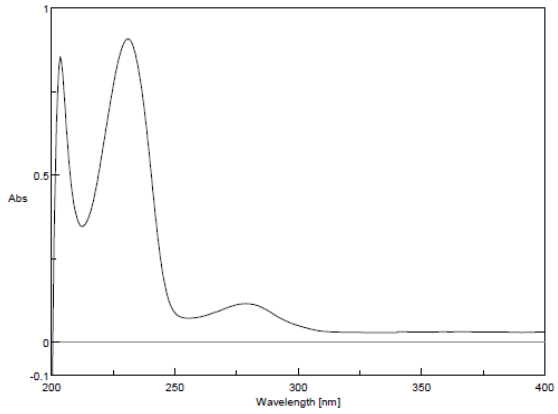


Table 105. Analysis of Dofetilide Admixture

Amount in admixture	Amount found*	% label claim*	SD*	% RSD*
0.500 mg	0.502 mg	100.4	0.67	0.030

*Mean of six observations

7.4.2 DEVELOPMENT OF VALIDATED HPTLC METHOD FOR DOFETILIDE

7.4.2.1 Chromatographic conditions

Selection of stationary phase

Precoated silica gel G_{60} F_{254} plates were used which were of 20 x 20 cm size. As per the requirement for the method the plate was concisely cut into appropriate size of 10 x 10 cm and 10 x 20 cm. The detection wavelength used was 233nm.

Selection of mobile phase

The mobile phase system tried in combination of petroleum ether, tetrahydrofuran, acetonitrile, methanol, dichloromethane, water, DMSO and toluene were found to carry the drug till the solvent front with Rf more than 0.87. The composition of THF:MeOH: Water have retained dofetilide. The interferences of trials are shown in table 106.

Optimization of variants in TLC

The impact of solvent ratio of the mobile phase, chamber saturation time and distance travelled by the solvent were studied for the method development and then optimized.

Composition of mobile phase system

The solvent system made up of MeOH:TLN:ACN was applied which resulted in acceptable R_f value. The increase in volume of MeOH gave a acceptable Rf value of 0.55 (Table 107). Hence the composition MeOH:TLN:ACN in ratio of 4:2:4 v/v/v was fixed as the mobile phase system.

Chamber saturation time

Different time for the saturation shows an effect on the peak shape and the nature of development on the plate. When the saturation time was modified from 5 to 30 minutes, the peak shape and symmetry of the peak was found varying. The selected chamber saturation time was 20 minutes which gave proper peak shape without fronting or tailing.

Distance of solvent front

Migration of the solvent from the base carrying the sample spots till the solvent front shifts in the the Rf value. The mobile phase was allowed to run on the plate to various distances from 7 cm to 9 cm. The acceptable Rf value was attained with the distance of 8.5 cm of the solvent front and hence fixed for the studies.

Band width

The samples were spotted with the bandwidth from 2 mm to 6 mm using the semiautomatic sampler. The 2 mm band width produced a narrow peak with peak area variation. As the band width increases, the peak area was found to show exponential change,which were symmetric in shape. The band width of 5 mm was selected as the peak characteristics were good.The optimized densitometric conditions are shown in table 108.

7.4.2.2 Validation of HPTLC method for Dofetilide

Linearity and range

A stock solution of 100µg/ml was used from which 0.5µl to 5µl were spotted. The sample spots developed on the plate were scanned at 233 nm. The linearity was found between 50-500 ng/band after which the non-linearity in peak area was observed. The overlay 3D chromatogram of standard doefitilide is shown in figure 203 and the peak area of standard presented in table109. The calibration graph plotted is shown in figure 204.

Precision studies

The degree of agreement between the results for the six determinations of the standard sample was assessed by the %RSD value for the intraday precision. The interday precision was carried out by repeating the six replicate injections of the standard and %RSD are given in the table and found to be less than 2. The figure represents the precision studies. The table 110 and 111 is the summary of the intraday and interday precision studies with the calculated value of SD and % RSD.

System precision

A standard solution of Dofetilide was injected forsix times to determine the system precision of the method and %RSD was calculated for Dofetilide and the six replicate scanning of the plate were run and the peak area of dofetilide were noted. The SD and %RSD of system precision was found and stated in the table 112.

Accuracy

The accuracy was determined by spiking the dofetilide admixture with the standard drug at 50% and 100% level. The % recovery was calculated for the standard added. The %RSD calculated for the recovered sample were presented in the table 113.

Robustness and ruggedness

The effect of variations in the saturation time by ± 2 min and small changes in the mobile phase ratio by ± 0.1 ml were studied. A change less than 10% was observed in the peak area and the Rf value. The results of analyzing dofetilide by two different analysts with the similar operating conditions had no significant variation. The %RSD was found to be below 2.

LOD and LOQ

The LOD and LOQ of the method by spotting the stock solution in the concentration below the linearity range and detected for the peak area. The concentration of 20 ng/spot with a peak area of 189.26 was observed as the limit of detection and the concentration of 40 ng/spot had a quantifiable peak area of 471.05. The chromatogram of LOD (Figure 205) & LOQ (Figure 206) are presented.

Stability of the developed plate

The volume spotted for the stability studies was 3µl (100 mcg/ml) of the working standard. The stability of plate was checked periodically till 18 hours and the plate was found to be stable for 14 hours after which the responses had notable change of peak area of above 10% (Table 114).

System suitability parameters

The HPTLC method was studied for the suitability of various parameters in order to analyze the admixture. The tailing factor, asymmetric factor and efficiency were calculated and shown in the table 115.

7.4.2.3 Applications to admixture

An admixture of dofetilide capsule contains 0.5mg of drug was prepared. It was further dissolved and diluted, using methanol. After filtration a 100µg/ml was prepared and spotted. From peak area observed, the amount present was calculated. The chromatogram of dofetilide admixture and amount present are shown in figure 207 and table 116 respectively.

Table106. Selection of mobile phase system for Dofetilide

Mobile phase	mobile phase ratio v/v/v	Observations
MeOH:PET:TLN	4:3:3	0.95
	5:2:3	Spot moved with solvent front
	6:2:2	
	3:4:3	0.88
	3:3:4	Spot moved with solvent front
	2:4:4	
THF:MeOH: H_2O	5:0.5:4.5	
	4:1.5:3.5	
	3:3:4	
	2:4:4	0.91
	2.5:4.5:3	0.87
	1:5:4	Spot moved with solvent front
MeOH: H_2O:DMSO	1:3:6	
	2:2:6	
	3:1:6	
	2:6:2	
	1:3:6	

Table 107.Composition of Mobile Phase System for Dofetilide

MeOH:TLN:ACN v/v/v	R_f value
1:2:7	0.89
2:3:5	0.77
3:3:4	0.74
4:2:4	**0.54±0.02**

Table 108. Optimized densitometric conditions for Dofetilide

Parameter	Fixed Condition
Stationary phase	Precoated silica gel plate G_{60} F_{254}
Mobile phase	MeOH: TLN:CAN
mobile phase ratio	4:2:4 v/v/v
Chamber saturation	20 minutes
Distance of solvent front	8.5 cm
Band width	5 mm
Slit dimensions	5 x 0.45 mm
Scan speed	20 mm/sec
Scan wavelength	233 nm

Fig 203. Overlay 3D densitogram of Standard Dofetilide

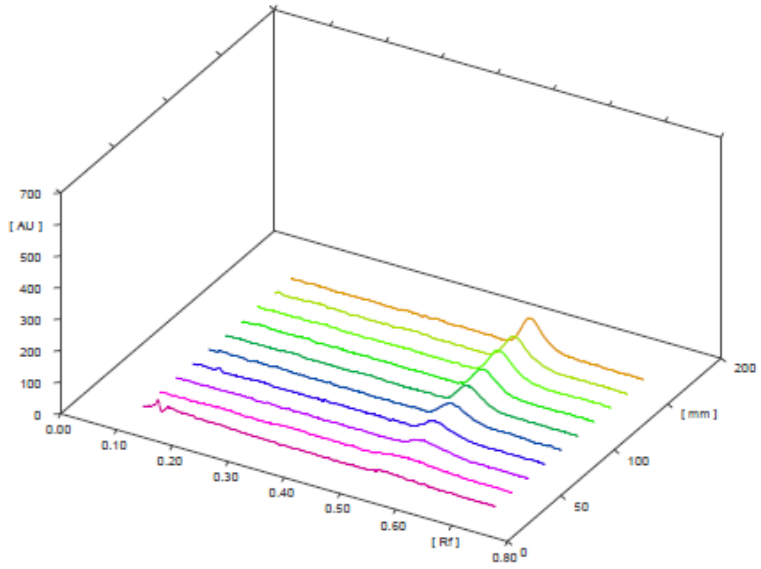


Table 109. Calibration data of Dofetilide

Conc (ng/band)	R_f value	Peak area
50	0.54	572
100	0.54	947
150	0.53	1513
200	0.53	2036
250	0.53	2593
300	0.53	2790
350	0.53	3195
400	0.53	3546
450	0.53	3696
500	0.53	3868

Fig. 204 Calibration graph of Dofetilide

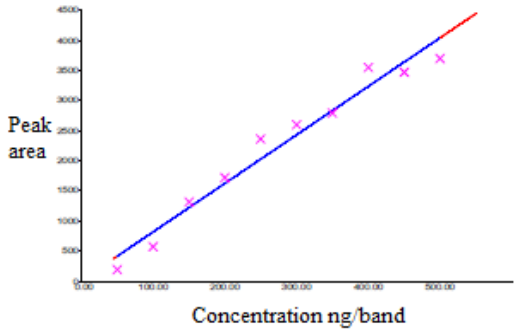


Table 110. Intraday Precision Studies for Dofetilide

Precision	Peak area	SD*	%RSD*
Intraday 1	2785	4.11	0.001
Intraday 2	2789		
Intraday 3	2779		
Intraday 4	2786		
Intraday 5	2785		
Intraday 6	2791		

*Average value

Table 111. Interday Precision Studies for Dofetilide

Precision	Peak area*	SD	%RSD
Day 1	2793	7.22	0.02
Day 2	2788	11.32	0.12
Day 3	2790	8.48	0.03

***Mean of six observations

Table 112. System precision for Dofetilide

Parameter	Peak area*	SD	%RSD
Repeatability of injection	2794	17.45	0.06
Repeatability of scanning	2783	18.38	0.10

***Mean of six observations

Table 113. Accuracy studies of Dofetilide

Level	Amount of Std added (mg)	Amount of Std recovered (mg)	% recovery of Std.*	SD	% RSD
50%	0.250	0.256	102.4	77.9	0.98
100%	0.500	0.503	100.6	44.2	0.27

*Mean of six observations

Fig.205 LOD of dofetilide

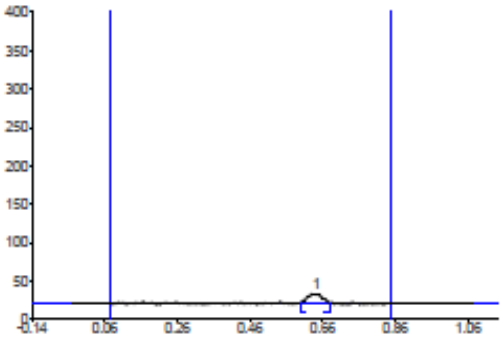


Fig.206 LOQ of dofetilide

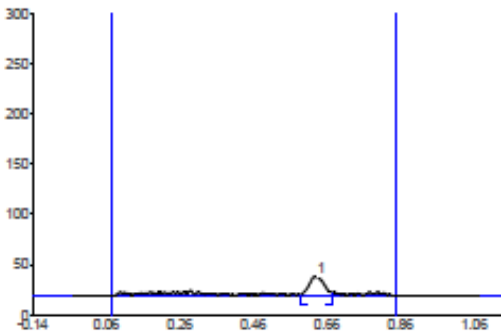


Table 114. Stability of plate for Dofetilide

Time in Hours	Peak area	SD	% RSD
0	2786	73.43	0.91
3	2720		
6	2609		
9	2555		
14	2531*		
18	2495		

*****Maximum time of stability

Table 115. System suitability parameters of Dofetilide

Tailing factor	Asymmetric factor	Efficiency
0.978	1.1178	1844

Fig.207. Densitogram of dofetilide admixture

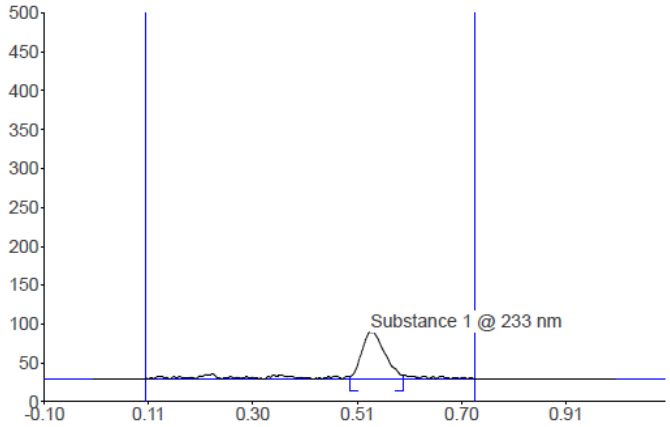


Table 116. Analysis of dofetilide admixture

Amount of drug added	Amount of drug found*	% of drug found*	SD*	% RSD*
0.5mg	0.493	98.6	9.92	0.74

*Mean of six observations

7.4.3 DEVELOPMENT OF VALIDATED RP-HPLC METHOD FOR DOFETILIDE

7.4.3.1 Chromatographic conditions

Selection of wavelength

Wavelength selection for the RP-HPLC analysis of the drug dofetilide was fixed from the UV spectrum recorded. The drug solution in 10mM Orthophosphoric acid was scanned and the λ_{max} was selected (figure 208). The selected wavelength of 233nm was applied for the studies.

Initialchromatographic conditions

TheShimadzu Shim pack GWS (250 mm x 4.6 µ id x 5 µm particle size) was used as the stationary phase for the chromatographic analysis of dofetilide. The sample solution of the drug was run with different proportions of mobile phase consisting of two phase system of acetonitrile and water and then with methanol and water. The chromatographic parameters were verified and as the responses were not according to the required criteria. Hence, the mobile phase of methanol, acetonitrile and water were chosen in various ratio and the chromatogram were evaluated. The interference of trials are shown in table 117 & figure 209 to 212.

Optimization of the chromatographic conditions

The mobile phase system made up of methanol, acetonitrile and water with a flow rate of 1 ml/min was used for the analysis of dofetilide detected at a wavelength of 233 nm. The trialand were performed with the solution prepared in acetonitrile. After which the solution was prepared in 10 mM Orthophosphoric acid was investigated for the responses. The ratio of 10:35:55(v/v/v) and injection volume of 20 µl were fixed for the method of analysis. The sample were analysed at ambient temperature. The chromatogram are shown in figure 213 to 215. The optimization of trials and optimized condition are presented in the table 118&119 respectively.

7.4.3.2 Validation of RP-HPLC Method for dofetilide

Linearity and range

The calibration curve was constructed by diluting the working standard solution of dofetilide with 10 mM Orthophosphoric acid to give a range of 10-50mcg/ml solution. The calibration plot was subjected to line of best fit and calculated for the slope (4.99941E-05), intercept(-7.5828) and correlation coefficient(0.9931) . The standard chromatogram are shown in the figure 216 to 220 and the calibration graph in figure 221. The linear data values are presented in the table 120.

Precision studies

The intraday precision was studied by six replicate injection of the standard drug dofetilide at three times on the same day and studied on three consecutive days for the Interday precision studies. The standard deviation and relative standard deviation were calculated and found to be below 1. The results are shown in table 121&122.

Accuracy

Accuracy of the method was determined by analysing three solution prepared from admixture. The two different level of addition (50% and 100%) of the standard drug was added to the admixture. The observations and % recovery are given in the table 123.

Specificity

The specificity studies using blank (OPA) and matrix (Excipients) proven there is no interference at the elution time of dofetilide.

Robustness and Ruggedness

The robustness of the method was evaluated by minor changes in the parameters fixed. The flow rate, injection volume, column temperature, mobile phase ratio were modified and the responses obtained had no significant difference from the standard conditions adopted.

Limit of detection (LOD) and limit of quantification(LOQ)

Representative concentration of the drug that can be detected from the fixed chromatographic conditions was derived from the calibration data and found to be 0.0042mcg/ml. The mathematically derived value of the amount of drug which can be quantified was 0.0130mcg/ml.

Stability of solution

A solution of 10mcg/ml in 10mM Orthophosphoric acid was used to study the stability of solution. The solution was examined at regular intervals and found to be stable till 44hours while stored at room temperature and for 7 days in refrigerator. The responses are noted and presented in table 124.

System suitability parameters

The suitability of the method for the analysis of dofetilide in admixture was computed from the instrument by the values observed for the tailing factor, asymmetric factor and theoretical plates in the column (Table 123)

7.4.3.3 Application of RP-HPLC method for theanalysis of Dofetilide admixture

An admixture of dofetilide capsule was analysed in the strength of 0.5mg. The assay sample of 100mcg/ml was prepared in methanol by dissolving the drug, filtering and making up the volume. Further dilution with 10mM OPA was made to produce a solution of 10mcg/ml.The chromatogram of six replicate injections were observed for the retention time and peak area. The amount of drug dofetilide present was calculated by comparing with the standard drug response. The values are presented in table 124 and the chromatogram of formulation shown in figure 222.

7.4.3.4 Application of RP-HPLC method for the degradation studies

Degradation studies of dofetilide was performed using ICH guidelines. The stress condition employed were 0.1 M HCl, 0.1M NaOH, 3 % v/v H_2O_2, Thermal at 80^oC and light. The study was carried out under each condition up to 8 hours. The level of degradation was studied and values were calculated in reference to the acceptable criteria of 10 % degradation.

Acid Hydrolysis(0.1 M HCl)

The acid hydrolysed dofetilide sample was injected into a chromatographic conditions and they are presented in the figure 223 and 224. The drug dofetilide was found to elute at 9.7 in zero hour sample. By the 8 hour of the stress study, the degradants were found to elute separately at 12.0, 12.6, 13.2, 14.9min where the drug peak was found at the retention time of 16.01min. The drug was stable in acid condition till 4 hours after which the level of degradation was more than 10%. The peak areas at each time interval are presented in the table 127.

Base hydrolysis (0.1 M NaOH)

When dofetilide was treated under base condition the peak was identified at 9.7 minutes at the 0 and 4 hours. At 8 hours of hydrolysis, the drug was found at 8.9 and 10.1 minutes. The additional peaks were formed at different retention times of 8.9, 10.1, 10.7, 11.4 and 13.0 minutes. The figure 225 and 226 represents the degradation of dofetilide from 0 to 8 hours. The table 127 shows the values of the response at the selective time.

Oxidation (3% v/v H_2O_2)

Dofetilide was found to be stable in the oxidation condition till 4 hours which is evident from the peak area change identified to be below 10%. The area decreases gradually by 8 hours with additional peak formed at 5.0 and 8.9 minutes. Based on the m/z value the drug peak was found as the base peak at 7.3 minutes. The level of degradation was calculated tabulated. The peaks eluted are shown in figure 227 to 228.

Thermal degradation 80 ^oC

Standard solution of dofetilide prepared in methanol was studied at 80^oC on a thermostat. The retention of the drug at 8.4 minutes was found to have variation in the response of more than 10% by 8 hours. Hence dofetilide was susceptible to degradation at 80^oC. Chromatograms representing the degradation at 0,4,8 hours are shown in figure 229 to 230.

Photolysis – 220 V light

Stability of dofetilide was studied using a lamp of constant source of light till 8 hours. The chromatogram was examined for change in peak area at a retention time of 8.9 min till 4 hour after which the peaks were observed at 12.3 and 15.4 minutes. There was negligible change in the peak area for the drug. Effect of light on dofetilide indicates the stability of the drug solution till 4 hours with degradation within limits. The results are shown in the figure 231 to 232.

The % degradation observed in all the condition upto 8 hours are presented in the table 127.

7.4.3.5 Application of RP-HPLC to impurity of dofetilide

Separation of process impurity of dofetilide

The chromatogram of dofetilide, impurities and their mixtures is shown in figure 233 to 235.The validation and system suitability parameters ares shown in the table 126. The RT of dofetilide and impurities were 9.35minutes and 8.05 minutes respectively. The resolution between them was 2. The tailing factor was within the limit. The impurity was spiked at three levels with the capsule admixture of dofetilide. The chromatograms are shown in figure 236 to 238. The peak area and % recovery of dofetilide impurity at three levels are given in table129.

Fig.208 UV Absorption spectrum of Dofetilide

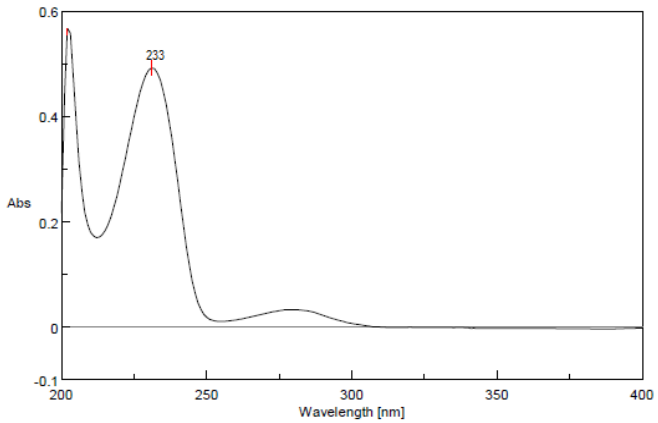


Table 117. Initial chromatographic conditions for Dofetilide

Trial	Mobile phase	mobile phase ratio v/v	Flow Rate ml/min	Injection Volume µl	RT Min.
1	H_2O :MeOH	50:50	0.9	10	15.533
2	H_2O :MeOH	20:80	0.9	10	3.254
3	H_2O:CAN	50:50	0.9	10	6.318
4	H_2O:ACN: MeOH	50:5:45	0.9	10	12.592

Fig.209 HPLC Chromatogram of Trial 1

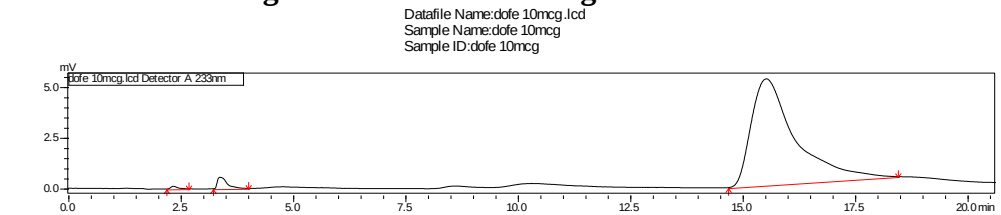


Fig.210 HPLC Chromatogram of Trial 2

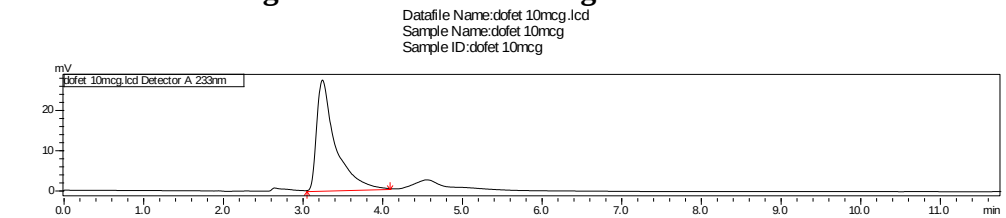


Fig.211-HPLC Chromatogram of Trial 3

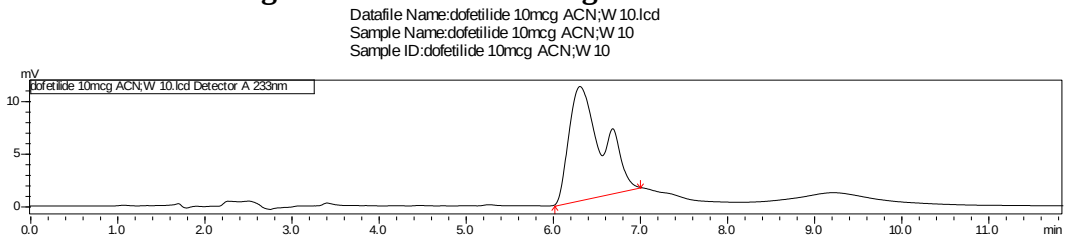


Fig.212 HPLC Chromatogram of Trial 4

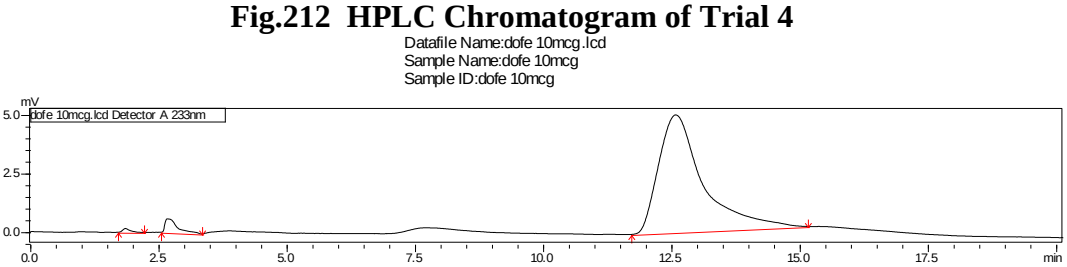


Table 118. Optimization of chromatographic conditions for Dofetilide

Trial	MeOH:ACN:H_2O v/v/v	Flow Rate ml/min	Injection Volume µl	RT Min.
5	10:40:50	1.0	10	7.81
6	10:30:60	1.0	10	15.98
7	10:35:55	1.0	20	9.97

Fig.213 HPLC Chromatogram of Trial 5

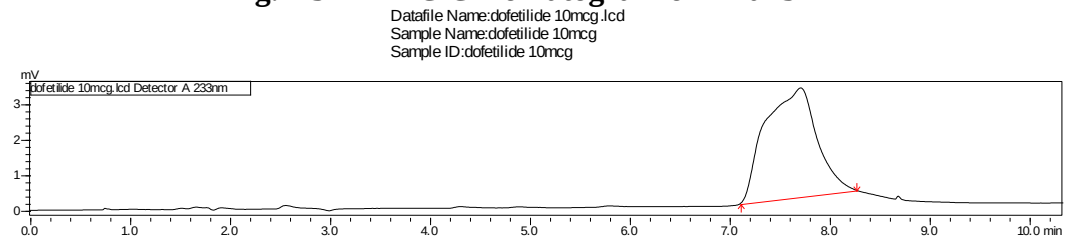


Fig.214 HPLC Chromatogram of Trial 6

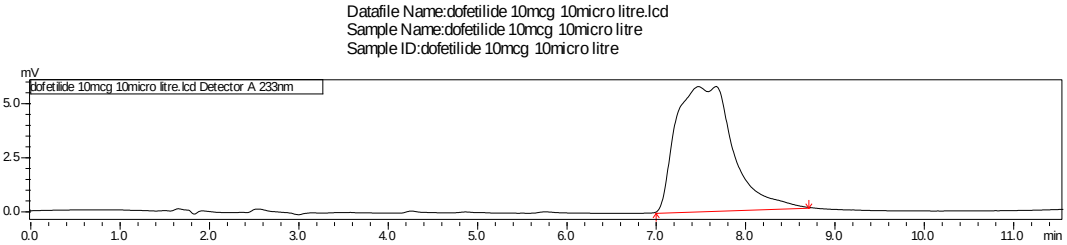


Fig.215 HPLC Chromatogram ofTrial 7

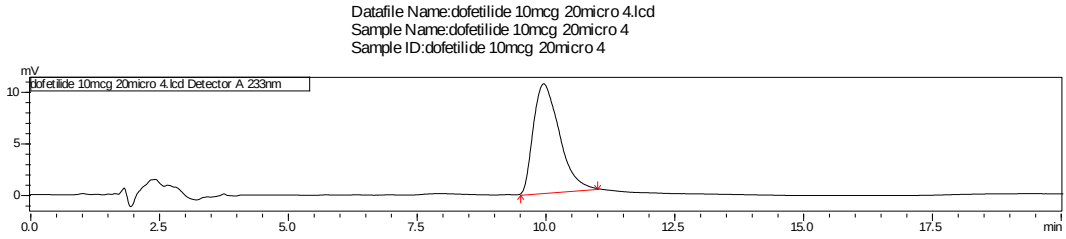


Table 119. Optimized Chromatographic Conditions for Dofetilide

Parameters	Fixed experimental conditions
Column	250 mm x 4.6 id x 5 µ particle size
Wavelength(nm)	233
Mobile phase	H_2O:ACN: MeOH
Ratio of mobile phase(v/v/v)	55:35:10
Flow rate(ml/min)	1
Injection volume(µl)	20
Temperature of column(^oC)	Ambient

Fig.216 Standard chromatogram of Dofetilide (10µg/ml)

Datafile Name:dofetilide 10 mcg.lcd
Sample Name:dofetilide 10 mcg
Sample ID:dofetilide 10 mcg

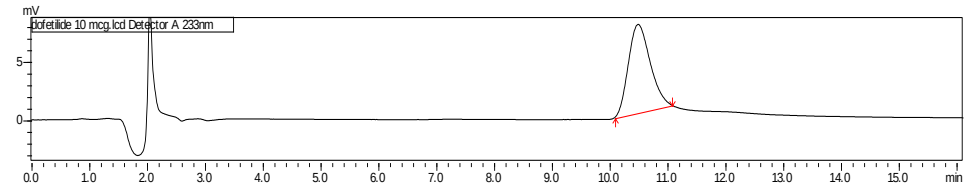


Fig.217 Standard chromatogram of Dofetilide (20µg/ml)

Datafile Name:dofetilide 20 mcg.lcd
Sample Name:dofetilide 20 mcg
Sample ID:dofetilide 20 mcg

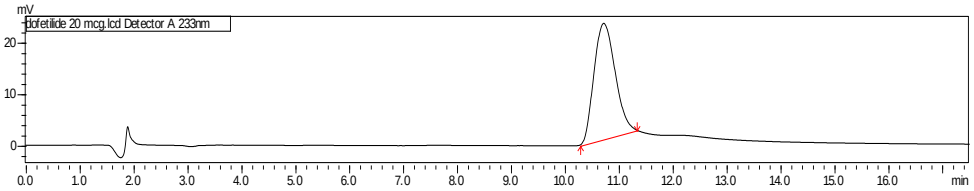


Fig.218 Standard chromatogram of Dofetilide (30µg/ml)

Datafile Name:dofetilide 30 mcg reapt.lcd
Sample Name:dofetilide 30 mcg reapt
Sample ID:dofetilide 30 mcg reapt

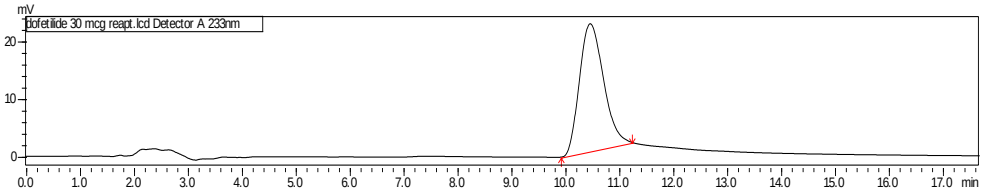


Fig.219 Standard chromatogram ofDofetilide (40µg/ml)

Datafile Name:dofetilide 40 mcg reapt.lcd
Sample Name:dofetilide 40 mcg reapt
Sample ID:dofetilide 40 mcg reapt

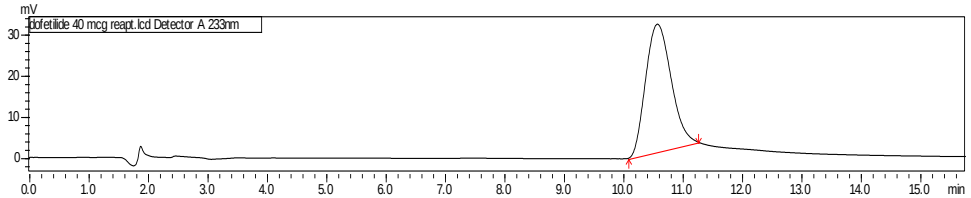


Fig.220 Standard chromatogram of Dofetilide (50µg/ml)

Datafile Name:dofetilide 50 mcg.lcd
Sample Name:dofetilide 50 mcg
Sample ID:dofetilide 50 mcg

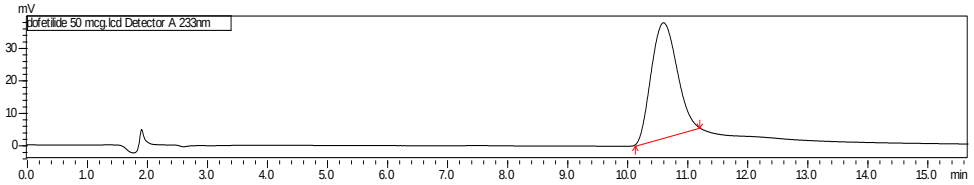


Table 120. Calibration data of Dofetilide

Concentration (mcg/ml)	RT Min.	Peak Area
10	10.498	198880
20	10.622	609918
30	10.468	728697
40	10.582	919381
50	10.612	1166223

Fig.221 Calibration graph of Dofetilide

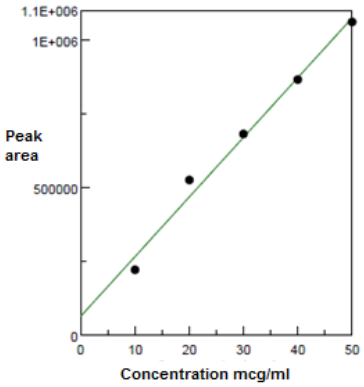


Table 121. Intraday precision studies for Dofetilide

Precision	Peak Area	SD*	%RSD*
Intraday 1	198853	211.63	0.83
Intraday 2	198904		
Intraday 3	198882		
Intraday 4	198799		
Intraday 5	198901		
Intraday 6	198442		

*Average value

Table 122. Interday precision studies for Dofetilide

I Precision	Peak Area*	SD	%RSD
Day 1	198790	98.40	0.91
Day 2	198827	87.52	0.03
Day 3	198842	62.22	0.77

*Mean of six observations

Table 123.Accuracy studies for dofetilide

Level	Amount of Std added mg	Amount of Std* recovered mg	% Recovery of the Std.*
50%	5	5.02	100.4
100%	10	9.86	98.60

*Mean of six observations

Table 124. Stability of dofetilide solution

Room Temperature		Refrigerator	
Time Hours	Peak area	Time Hours	Peak area
0	198696	0	198778
2	198439	24	198545
4	198211	48	197550
8	197201	72	194576
12	193658	96	191154
24	185583	120	184936
36	182495	144	182045
44	178992*	168	179453*
48	154967	192	160032

*Maximum time of stability

Table 125. System suitability parameter of Dofetilide

Tailing factor	Efficiency (N)	Asymmetric factor
0.9059	1585	1.1936

Fig.222 Representative chromatogram of Dofetilide admixture

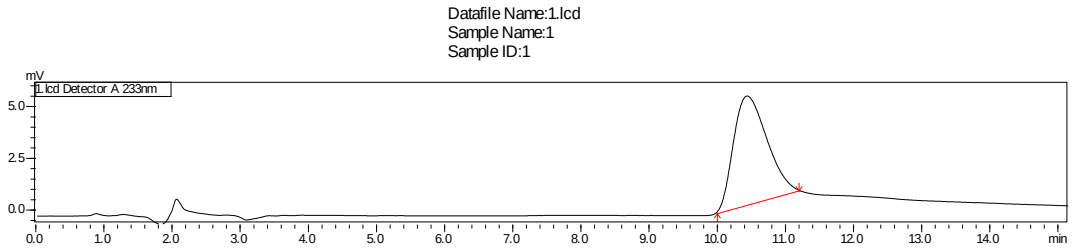


Table 126. Result of analysis of dofetilide admixture

Amount in admixture	Amount of drug found	% Amount of drug found	% RSD*
0.5mg	0.501	100.20	1.4

***Mean of six observations

Fig.223HPLC Chromatogram of Dofetilide (Acid hydrolysis – 0 Hr)

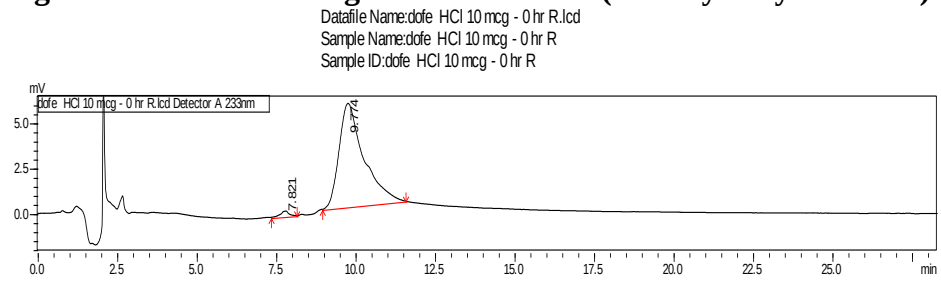


Fig.224 HPLC Chromatogram of Dofetilide (Acid hydrolysis – 8 Hr)

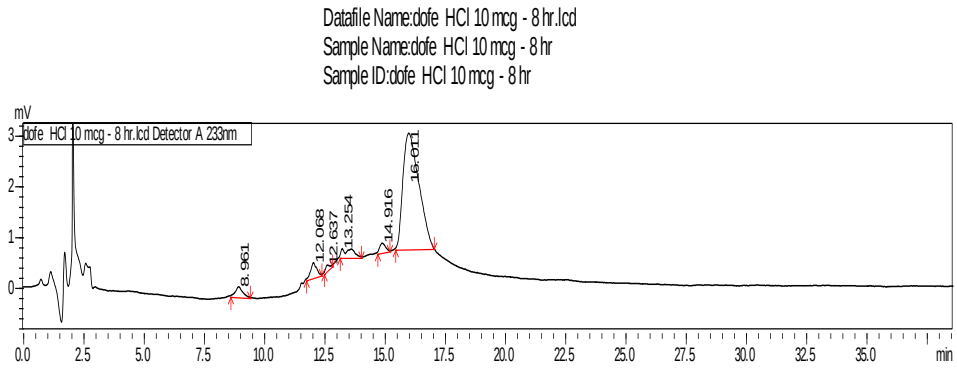


Fig.225 HPLC Chromatogram of Dofetilide (Base hydrolysis – 0 Hr)

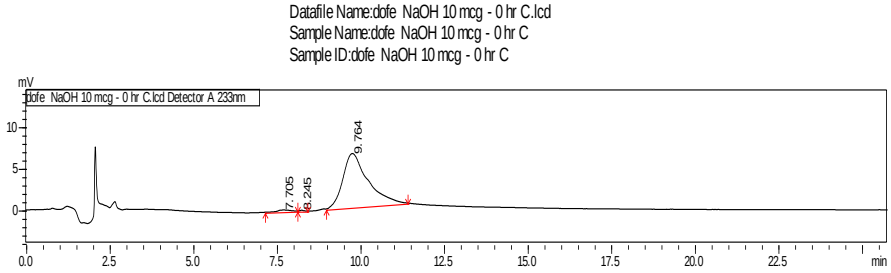


Fig.226HPLC Chromatogram of Dofetilide (Base hydrolysis – 8 Hr)

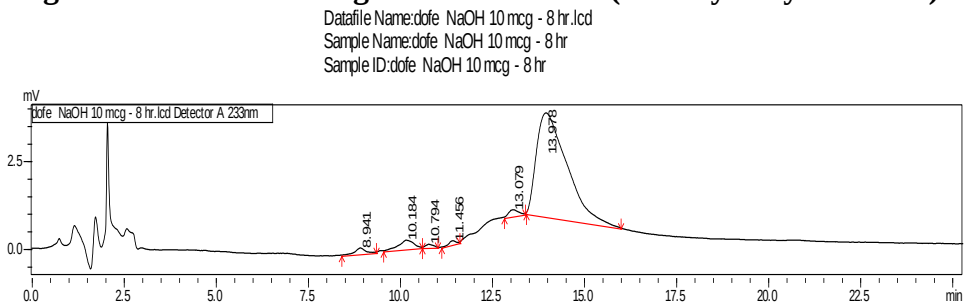


Fig.227 HPLC Chromatogram of Dofetilide (Oxidation – 0 Hr)

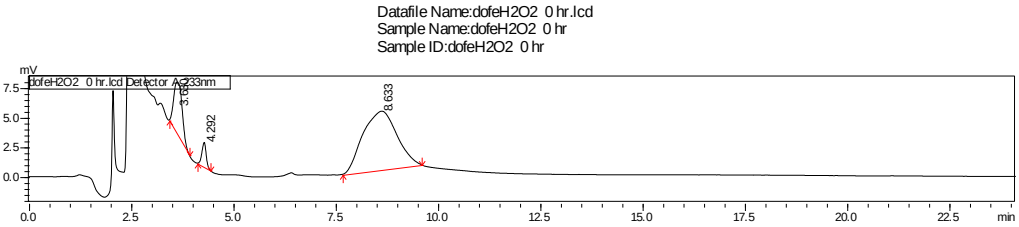


Fig.228 HPLC Chromatogram of Dofetilide (Oxidation – 8 Hr)

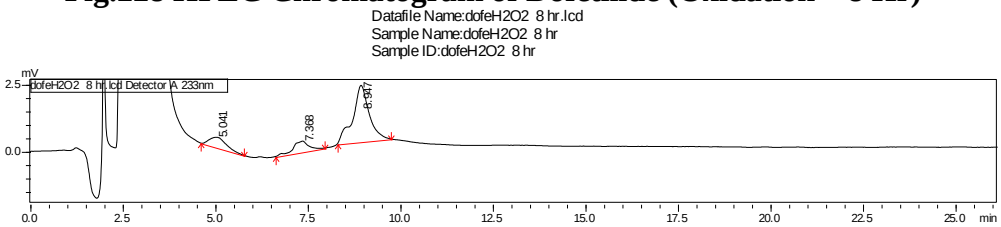


Fig.229 HPLC Chromatogram of Dofetilide (Thermal 80ºC– 0 Hr)

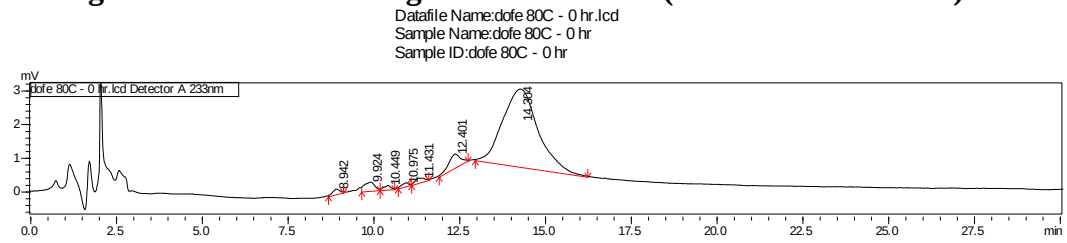


Fig.230 HPLC Chromatogram of Dofetilide (Thermal 80ºC– 8 Hr)

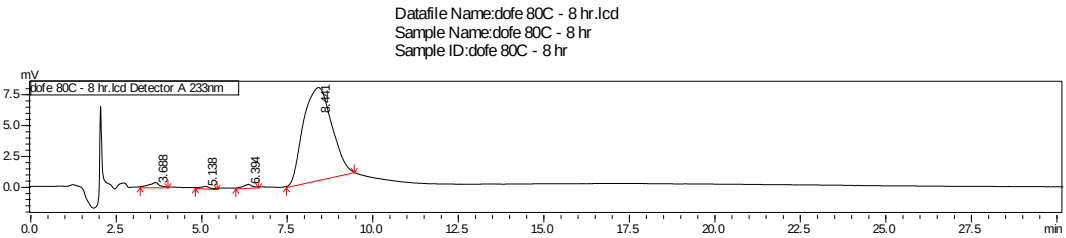


Fig.231 HPLCChromatogram of Dofetilide (Photolysis – 0Hr)

Datafile Name:dofe Light 10 mcg - 0 hr C.lcd
Sample Name:dofe Light 10 mcg - 0 hr C
Sample ID:dofe Light 10 mcg - 0 hr C

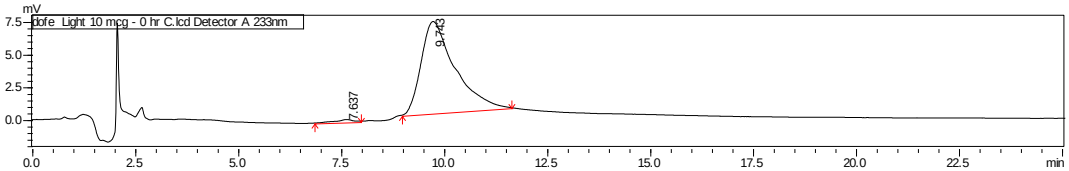


Fig.232HPLC Chromatogram of Dofetilide (Photolysis – 8Hr)

Datafile Name:dofe Light 10 mcg - 8 hr.lcd
Sample Name:dofe Light 10 mcg - 8 hr
Sample ID:dofe Light 10 mcg - 8 hr

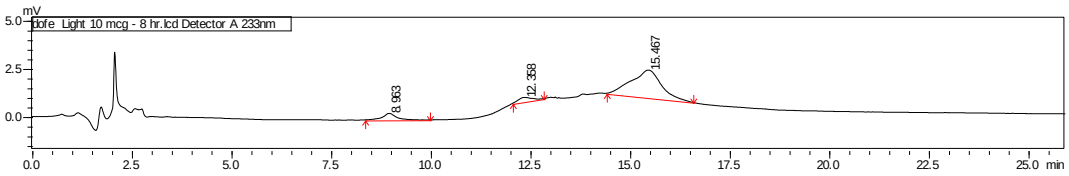


Table 127. Degradation studies of dofetilide by RP-HPLC method

Sample name	Retention time	Peak area	Peak area change %
Acid degradation 0 hrs	7.821 9.774	5119 307506	
Acid degradation 8 hrs	8.961 12.068 12.637 13.254 14.916 16.011	3631 4355 861 4781 2575 103709	**29.06**
Base degradation 0 hrs	7.705 8.245 9.764	4422 643 332953	
Base degradation 8 hrs	8.941 10.184 10.794 11.456 13.079 13.978	2602 6226 1033 1011 2868 168412	**8.97**
Oxidation 0 hrs	3.630 4.292 8.633	63701 14800 283886	
Oxidation 8 hrs	5.041 7.368 8.947	12100 11557 64659	**89.99**
Thermal 80 C 0 hrs	8.942 9.924 10.449 10.975 11.431 12.401 14.304	1724 5124 1352 1094 1908 8320 162826	
Thermal 80 C 8 hrs	3.688 5.138 6.394 8.441	6671 1622 3457 429022	**63.48**
Photolysis 0 hrs	7.637 9.743	5038 396560	
Photolysis 8 hrs	8.963 12.358 15.467	7772 4873 74915	**54.26**

Fig.233 HPLC chromatogram of dofetilide API

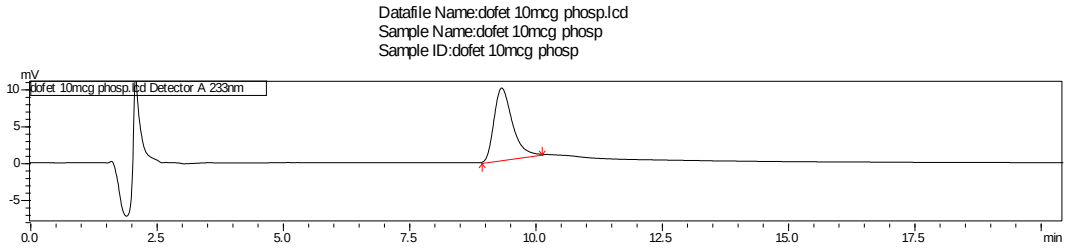


Fig.234 HPLC chromatogram of dofetilide related compound A

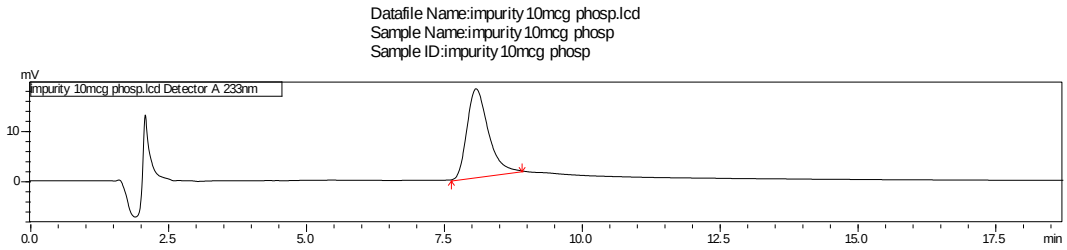


Fig.235 HPLC chromatogram of mixture

(Dofetilide + dofetilide related compound A)

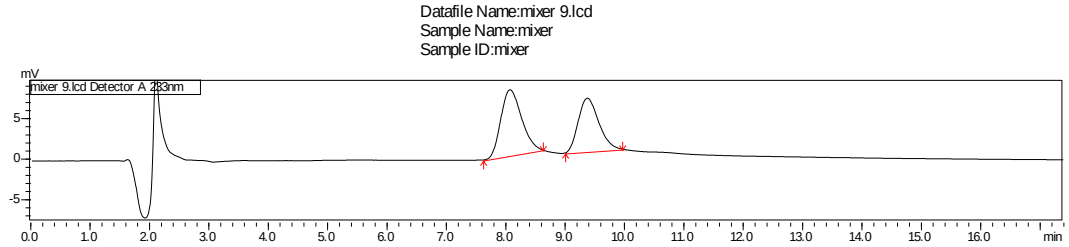


Table 128. Validation and system suitability parameter of Dofetilide

Parameter	Dofetilide	Dofetilide Related Compound A
RT	9.40	8.07
T_f	1.2	1.09
A_s	1.3	1.0
No. of theoretical plates	5516	4192
Linearity (µg/ml)	10-50	0.5-2.5
Precision(%RSD)	0.804	0.996

Fig.236HPLC chromatogram of 50 % recovery of Dofetilide and Dofetilide Related Compound A

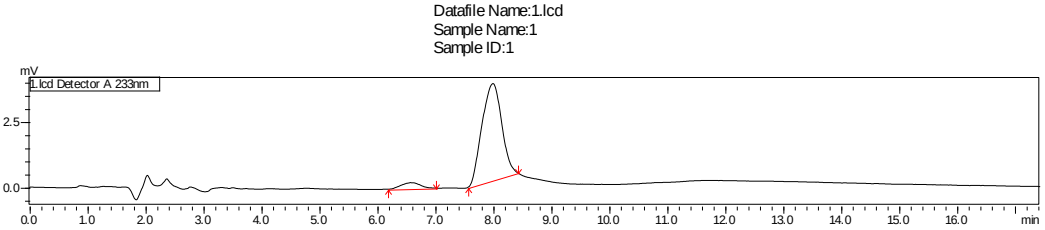


Fig.237 HPLC chromatogram of 100 % recovery of Dofetilide and Dofetilide Related Compound A

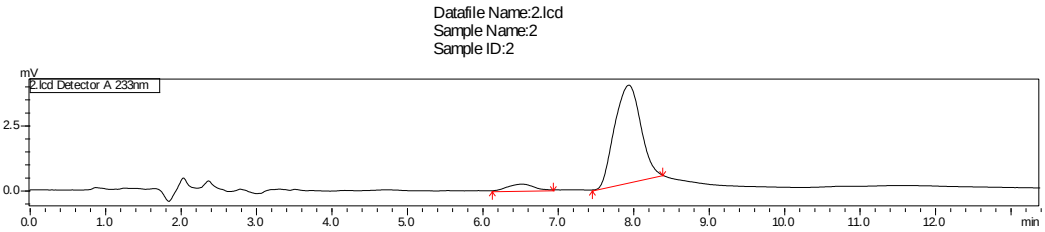


Fig.238 HPLC chromatogram of 150 % recovery of Dofetilide and Dofetilide Related Compound A

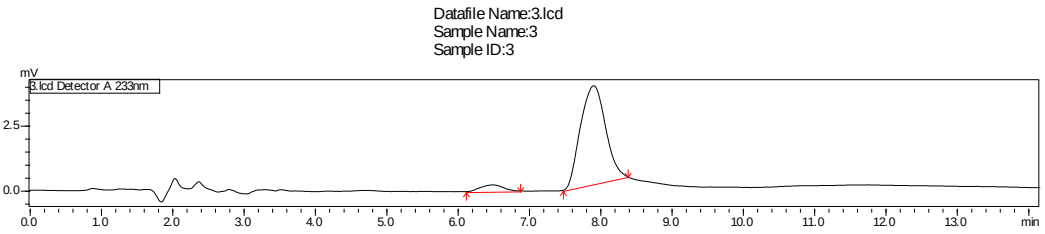


Table 129. Recovery studies of Dofetilide and Dofetilide Related Compound A

% Level	Dofetilide		Dofetilide Related Compound A	
	Peak area	% Recovery	Peak area	% Recovery
50	188776	101.98	1321	101.14
100	190460	99.51	2798	98.14
150	187995	99.44	4405	99.97

*Mean of six observations

8. DISCUSSION

Apixaban, rivaroxaban, ibutilide fumarate and dofetilide are cardiovascular drugs which areused as antiarrhythmic and anticoagulant agents. Till date there are very few reported methods available for the analysis of these drugs .The drugs Ibutilide fumarate, apixaban and rivaroxaban are not official in any pharmacopoeia. The drug dofetilide has been included in the USP 40 fromDecember 2017. The drugs apixaban and rivaroxaban have been approved by the CDSCO under the category of anticoagulants in the year 2012 and 2010 respectively.

The development of UV spectroscopy for the selected drugs was based on the presence of chromophore system present in the structure and its absorption pattern of ultraviolet radiation.The three drugs namely apixaban, ibutilide fumarate and dofetilide are having conjugated chromophore system which enabled the development for the UV method for them.

The selection of solvents for UV Spectroscopic method was based on the solubility, UV transparency, smoothness of the spectrum and absorbance of drugs. Methanol was selected as solvent for apixaban, ibutilide fumarate and dofetilide since it fulfilled the above criteria. In the wavelength chosen for all three drugs the highest absorbance was found. Since the instrumental parameters such as scan speed ,bandwidth and slit width affects the absorbance, they were also optimized. While the scan speed, band width and slit width increased, absorbance was found to be increased, whereas the smoothness of the spectra was lost hence optimization was carried out.The UV method was validated as per the Q1 R2 guidelines linearity, accuracy, precision , specificity, LOD, LOQ, robustness and ruggedness.

The HPTLCmethod were developed for all four drugs based on the polarity and solubility nature of the drugs. A mobile phase system that would retain the drugs under study with an optimum R_f value and compact peaks were chosen for the study after various trials.

A solvent system consisting of Toluene:Dicloromethane:Tetrahydrofuran in the ratio of 2:2:6 was used for the drug Apixaban. The ratio of 5:3:2 v/v/v of TLN:EA:MeOH has been used as mobile phase for the separation of Rivaroxaban. For ibutilide fumarate, two peaks at 0.33 and 0.74 were observed in the selective mobile phase system. The mobile phase fixed for ibutilide fumarate was 8:1:1 v/v/v of MeOH:EA:IPA.It was identified that the peak corresponding to R_f value of 0.74 is due to Fumaric acid which was confirmed by running the Fumaric acid individually. Dofetilide was separated with the mobile phase system composition of MeOH:TLN:ACN (4:2:4 v/v/v) giving a single compact spot at R_f value of 0.53.

The validation the HPTLC methods were carried out for the four drugs before application to formulations. It was observed that HPTLC method could identify drugs in the nanogram level of the selected drugs. The RP-HPLC methods were developed for selected cardiovascular drugs using a C18 column with an appropriate mobile phase which was fixed after a number of trials. The mobile phase systems used to separate these drugs do not involve any buffer and hence the methods are column friendly.

In Analysis of all these selected drugs, the runtime was less than 10 minutes. All the system suitability parameters in the selected chromatographic conditions were found within acceptable criteria.

The methods developed for the drug apixaban and rivaroxaban were used for the estimation from their dosage forms. For Ibutilide fumarate a proposition of drug solution was prepared as mentioned in the leaflet of Corvert injection and analysed by the UV, HPLC and HPTLC methods. An admixture of capsule excipients along with dofetilide was prepared and estimated by the newly developed methods. The amount present and the percentage RSD were calculated were found to be in acceptable limit.

The degradation studies of Apixaban by the HPLC method was carried out using two mobile phase systems. The degradants were identified at separate retention times and calculated for the percentage degradation based on the responses. The drug was found to be stable under conditions of oxidation and light till 4 hours.

Rivaroxaban is subjected to various stress conditions according to the ICH guidelines of acid, base, oxidation, thermal and light. The drug was found to be susceptible to degradation under all the conditions in addition degradation peaks identified at separate retention time. The percentage of degradation was found to be more than 10%.

The degradation of ibutilide fumarate was performed under the above stated conditions and was stable at thermal conditions of 40°C. The level of degradation was more than 10% under the various stress condition adopted as per the guidelines.

The drug dofetilide was found to be stable under the conditions of acid, base, thermal at 40°C and oxidation till 4 hrs after which there was considerable change in the peak area in all these conditions. The drug was found to have more 10% of change in response under thermal condition of 80°C and photolysis.

The impurity profiling was performed with tandem mass spectral identification of selected impurity and the degradation products of rivaroxaban with the m/z value at the respective retention time. The impurity was identified with the transitions into the respective positive ion peak and the structure was compared with the reported literature.

The selected process impurity of rivaroxaban with a M+1 peak at m/z 292 carries the significance of being identified at all the degradation studies with the spectral match of notable intensity. Mass spectrum reveals the presence of selected impurity with m/z of 292 at 10.8 minutes in acid hydrolysis, 10.7 minutes under base hydrolysis, 11.2 minutes in oxidation and 11.4 minutes under photolysis

The other degradants were identified by matching their m/z value. Transition of 237 was identified at 9.6 minutes under oxidation. The positive ion of 193 is present in the condition of base hydrolysis at 12.9 minutes, 9.1 minutes in photolysis. The degradant of m/z 205 was identified at 8.9 minutes under base hydrolysis. The molecular ion peak and M+1 peak of 273 was identified in acid condition at 13.9 minutes and light at 9.4 minutes.

The interacting drug verapamil was selected based on the metabolic pathway. It was reported to be a moderate inhibitor of the CYP3A4 enzymatic activity.As per the literature reports, the IV infusion of Ibutilide fumarate in the dose of 0.1 mg/ml produces a dose related prolongation of the QT interval, which is a lead to be associated with the antiarrhythmic activity.

The metabolism based *in vitro* drug-drug interaction studies of ibutilide fumarate was carried out under simulated condition and in rat liver microsomes. The metabolism was assessed in presence and absence of selected co-administered drug verapamil.

There is no reported pharmacokinetic study for Ibutilide fumarate till date. The current research work on *In vitro* drug-drug interaction studies of Ibutilide fumarate was an investigational study to predict the metabolic interaction with verapamil.

The drug Ibutilide in presence of liver microsomal enzymes was found to decrease linearily to a concentration of 8 % at the end of the study, whereas in presence of verapamil the concentration of 91 % was found remaining in the enzyme pool. It may be due to interference of verapamil in the metabolic pathway of ibutilide fumarate. This was found to be a significant outcome of *in vitro* drug-drug interaction studies of ibutilide fumarate.

The various analytical techniques developed are effective methods for the analysis of selected cardiovascular drugs in their formulations. The applications of the method for the degradation studies for all the four drugs are versatile techniques indicative of the stability of the selected drugs. The impurity profiling studies for Rivaroxaban by the hyphenated method LC-MS/MS in the multiple reaction monitoring mode was applied for the identification of the selected impurity (m/z 291) and degradants of selective transitions. The *in vitro* drug-drug interaction studies between Ibutilide fumarate and verapamil is a valuable insight into the pharmacokinetics behavior of the drug when co administered.

9. SUMMARY AND CONCLUSION

9.1 Development of validated Analytical Methods for the determination of Apixaban

Various analytical methods were developed and validated for the determination of Apixaban such as UV spectrophotometry, HPTLC and HPLC. The UV absorption spectra of Apixaban in methanol revealed its λmax at 287 nm.

In UV spectroscopic method an absorption spectra of apixaban was recorded in methanol from which a wavelength of 289 nm was choosen for analysis. At this wavelength the absorbance was found to be maximum.

The linear absorbance was observed in concentration range between 2 to 20mcg/ml of Apixaban. The LOD and LOQ of Apixaban were found to be 0.53 µg/ml and 1.62 µg/ml respectively. The percentage recoveries of apixaban were found between 101-102. The molar absorptivity of apixaban was found to be $1.041x10^4$. The results of method validation demonstrate the precision, accuracy and suitability of the proposed UV spectrophotometric technique.

The HPTLC method used precoated silica gel $G_{60}F_{254}$ plates as stationary phase and 2:2:6 v/v/v of Toluene: dichoromethane: Tetrahydrofuran as the mobile phase. The separation of apixaban was achieved with an ideal R_f value of 0.55. Linearity was observed in the concentration ranging between 200-2000 ng/band. The LOD and LOQ values of Apixaban were found to be 256.87 and 838.92. The validation results demonstrate that the method is accurate, repeatable, precise, specific and robustness of the developed method was proven during the validation process.

The RP-HPLC method used C_{18} Phenomenex column as stationary phase and 10:90 v/v of water and methanol as mobile phase for the separation of apixaban with the flow rate of 1ml/min and injection volume of 10 µl. The Linearity was observed in the concentration range of 2-20 µg/ml. The percentage recoveries of apixaban was found to be 99.65 and 98.80. The LOD and LOQ values were found to be 0.0013µg/ml and 0.0039µg/ml.

The developed UV spectroscopic and chromatographic methods were successfully applied to the tablets containing 2.5 mg of apixaban. Twenty tablets were taken, pulverised and a quantity of powder equivalent to 10 mg of apixaban was added with methanol. After sonication, the volume was made up and filtered. The aliquot was analysed by respective methods. The amount present per tablet and % label claim were calculated and found to be close to 100. The precision of the methods were proven from %RSD values which are less than 2. The specificity of the methods were proven by studying the response of tablet excipients by proposed methods.

The HPLC method was employed for the degradation study of apixaban.As a result of degradation studies, the stability of apixaban found to be good for oxidation,photolysis and thermal degradation. Whereas it is susceptible for the acid and base hydrolysis.The additional peaks due to degradants were well resolved from apixaban.Application of the HPLC method to the degradation studies proves the selectivity of method as it can quantify apixaban in presence of degradants peaks found. The decreasing order of degradation of apixaban is as below:

Base hydrolysis> acid hydrolysis > Thermal degradation > oxidation> photolysis.

The data comparison between the methods developed for apixaban has been shown in the table 130.The sensitivity of the method in comparison with the reported method has been given in table 131. The statistical evaluation of the percentage assay in the tablets is mentioned in table 132 with the F-test.

Table 130. Comparison between the methods UV Spectroscopy, HPTLC and HPLC of apixaban

Parameter	**UV Spectroscopy**	**HPTLC method**	**HPLC method**
Detection wavelength	287 nm		
Linearity	2- 20mcg/ml	200-2000 ng/ml	2-20 mcg/ml
Precision studies(% RSD)	0.71	0.06	0.66
Accuracy studies(%) 50% level 100% level	102.62 101.73	100.33 101.05	99.65 98.80
LOD(mcg/ml)	0.53	50	0.0013
LOQ (mcg/ml)	1.62	100	0.0039
Applications	Analysis of tablet formulations		• Analysis of tablet formulations • Degradation studies of Apixaban

Table 131.Comparison of UV spectroscopy reported methods and proposed method for apixaban

Parameter	Results		Observation
	Reported method[10]	**Proposed method**	
Detection wavelength	280	287	Developed method is more sensitive than the reported method
Linearity (mcg/ml)	5-50	2-20	
Regression equation(y=ax+b)	y = 0.040x + 0.009	Y=0.024434x+0.00621	
LOD(mcg/ml)	0.73	0.53	
LOQ (mcg/ml)	2.21	1.62	
Stability of solution (hours) Room Temperature Refrigeration	 Not Found Not Found	 12 72	

Table 132. Statistical comparison of the percentage assay determined in the validated methods of UV Spectroscopy, HPTLC and HPLC of apixaban

Parameters	UV Spectroscopy	HPTLC	HPLC
No of Determinations	6		
Mean	99.20	99.28	99.60
SD	0.003	0.400	0.620
Lower 95% CL	99.49	99.70	99.25
Upper 95% CL	99.45	99.72	99.10
F -test	0.996		

9.2 Development of validated Analytical Methods for the determination of Rivaroxaban

For the determination of rivaroxaban in the bulk drug and formulation. Two chromatographic methods were developed vise HPTLC and HPLC . The HPTLC method used precoated silica gel $G_{60}F_{254}$ plates as stationary phase and mobile phase composition of MeOH:EA:H_2O(4:2:4 v/v/v). The separation of Rivaroxaban was achieved with an R_f value of 0.36. The wavelength was selected from the absorption spectra of Rivaroxaban where the λ_{max} was found to be 249 nm. Linearity was observed in the concentration ranging between 120 – 1000 ng/band. The LOD and LOQ values of Rivaroxaban were found to be 8 ng/band and 28 ng/band respectively. The results of validation parameters indicates that the method is accurate, repeatable, precise, specific and robustness.

The RP-HPLC method used C_{18} Phenomenex column as stationary phase and mobile phase system made up of methanol:acetonitrile:water in the proposition of 45:10:45 v/v/v for the separation of Rivaroxaban with 1ml/min as the flow rate and injection volume of 20 µl. The Linearity was observed in the concentration range of 5-30 µg/ml. The percentage recovery at 50% and 100% level of Rivaroxaban was found to be 99.80 and 101.1 respectively. The LOD and LOQ values were found to be 0.00957 µg/ml and 0.029 µg/mlrespectively.

The two chromatographic methods developed and validated were applied to the tablet strength of 10 mg of Rivaroxaban. Tablet powder equivalent to 10 mg was taken and to this 1 ml of DMSO added and 3 ml of acetonitrile was added. After sonication, the volume was made up with acetonitrile and filtered. The aliquot was analysed by two chromatographic methods. The amount present in the tablet and %label claim were calculated and found to be close to 100. The HPLC method was employed for the degradation study of Rivaroxaban. The %RSD values of the interday and intraday precision was found to be less than 2.The degradation of Rivaroxaban in study conditions took place in the decreasing order as mentioned below:

Base hydrolysis> acid hydrolysis > photolysis > oxidation >Thermal degradation

The degradation was prompt in hydrolytic study, where at zero hour itself more than 10% degradation was found. Where as it was quite stable at thermal and oxidative degradation.The HPLC method validated has been applied for the impurity profiling studies. The hyphenated technique LC-MS/MS to identify the impurity selected for the study with the other degradants by the selected m/z values. The structure of the degradants identified in comparison with the reported literature[16].

Comparison between the developed methods for Rivaroxaban have been shown in the table 133. The statistical evaluation for the percentage assay of tablets is mentioned in table 134 with the F-test.

Table 133. Comparison between the HPTLC and HPLC methods of Rivaroxaban

Parameter	HPTLC	HPLC
Solvent	Methanol :acetonitrile (1:1.5 v/v)	DMSO:CAN (1:9 v/v)
Detection wavelength	249 nm	
Linearity	120-1000(ng/band)	5-30 (mcg/ml)
Accuracy studies(%) 50% level 100% level	99.87 98.93	98.8 101.1
LOD	8 (ng/band)	0.001(mcg/ml)
LOQ	28(ng/band)	0.003(mcg/ml)
Applications	Analysis of tablet formulations	Analysis of tablet formulations Degradation studies Impurity profiling of Rivaroxaban Identification of degradants by LC-MS/MS technique.

Table 134. Statistical comparison of the percentage assay of Rivaroxaban by HPTLC and HPLC methods

Parameters	**HPTLC**	**HPLC**
No of Determinations	6	6
Mean	100.6	100.2
SD	0.187	0.283
Lower 95% CL	100.5	101.6
Upper 95% CL	99.97	99.88
F-test	0.854	

9.3 Development of validated Analytical Methods for the determination of Ibutilide fumarate

The spectroscopic and chromatographic methods developed and validated for the determination of Ibutilide fumarate were in accordance with the ICH guidelines validation parameters. It includes UV spectrophotometry, High performance thin layer chromatography and High performance liquid chromatography. The scanning of Ibutilide fumarate in ultraviolet region was found to show λmax at 228 nm. The linearity of the drug absorbance was observed in concentration range between 2 to 14 mcg/ml of Ibutilide fumarate. The Limit of detection and Limit of quantification of Ibutilide fumarate were found to be 0.0624 µg/ml and 0.1892 µg/ml respectively. The molar absorptivity, Sandell's sensitivity and E(1%,1cm) of Ibutilide fumarate was found to be $1.687x10^{5,}$ 0.0261 and 381.8 respectively. The outcomes of the UV spectroscopic method for Ibutilide fumarate was suggestive of the simplicity, specificity, linearity, accuracy, precision, robustness and ruggedness of the method

Precoated silica gel $G_{60}F_{254}$ plates were used as stationary phase for the HPTLC analysis of Ibutilide fumarate. The solvent system made up of MeOH:EA:IPA(8:1:1 v/v/v) with a chamber saturation time of 20 minutes was found to result in acceptable peak characteristics with good resolution between the drug peak and fumaric acid peak. The separation of Ibutilide fumarate and fumaric acid was achieved with an ideal R_f value of 0.36 and 0.74 respectively. Linearity was observed in the concentration ranging between 1000-9000ng/band. The LOD and LOQ values of Ibutilide fumarate were found to be 100ng/band and 400ng/band respectively. The method was found to be specific, precise, accurate, sensitive, robust and suitable for the analysis of formulation.

The C_{18} Shim pack column(250mm x 4.6id x 5µ) was used as stationary phase for the analysis of Ibutilide fumarate. The ideal resolution between the peaks of ibutilide base and fumaric acid was achieved with a mobile phase consisting of 55:45 v/v of Methanol:water. The flow rate of mobile phase was 0.9 ml/min and injection volume of drug solution was 10µl. The Linearity was observed in the concentration range of 2-16 µg/ml with good correlation between the range selected. The % RSD

values of the interday precision,intraday precision and analysis of drug solution was found to be below 1The LOD and LOQ values were found to be 0.005µg/ml and 0.0084µg/ml respectively.The comparison between the methods developed for Ibutilide fumarate has been shown in the table 134.

Application of these analytical methods for the drug solution prepared as per the product monograph of the commercial injection marketed was found to be highly specific for the drug substance. The amount of Ibutilide present was also found to be within the range of 98-102%. The repeatability and reproducibility results for the precision of the three methods were observed with the %RSD values of less than1.

The HPLC method was employed for the degradation study of Ibutilide fumarate for every 30 minutes till 240 minutes. The degradant peak were isolated from Ibutilide and thus evaluation was carried out successfully. Drug solution was stable at 40^{o}C with negligible change of peak area below 3% in study conditions. Ibutilide fumarate was found to be most stable in photolysis condition than any other condition. The descending order of degradation of Ibutilide fumarate at the end of degradation study is as below:

Thermal 60^{o}C >oxidation>Base hydrolysis> acid hydrolysis > photolysis>Thermal 40^{o}C

The HPLC method developed and validated for the necessary parameters and applied for the *in vitro* drug drug interaction studies. The distribution of the sample molecule in the mobile phase selected plays an important role in the separation of the peaks. Hence the peaks of ibutilide fumarate and verapamil were resolved effectively to have a resolution factor of more than 5 and applied for the study .

The interaction studies under simulated conditions of temperature and pH was carried to check the behavior of the drug. The chromatograms were observed for the presence of any additional peaks or any other changes. The enzymatic study of the drug ibutilide fumarate in the concentration of 10 mcg/ml, interacting drug verapamil 10 mcg/ml and mixture of both the drugs in the same concentration were conducted. The

responses of simulated condition were considered suitable for predicting the changes observed in enzymatic study. When the drugs were individually treated with the enzyme there was gradual decrease in the peak area of the two drugs. However there is considerable decrease in the metabolism of Ibutilide fumarate in presence of verapamil.

The sensitivity of the method in comparison with the reported method has been given in table 135. The statistical evaluation of the percentage drug in the drug solution is listed in the table 136 with the F-test.

Table 135. Comparison between the methods UV Spectroscopy, HPTLC and HPLC of Ibutilide fumarate

Parameter	**UV Spectroscopy**	**HPTLC method**	**HPLC method**
Solvent	Methanol		
Detection wavelength	228 nm		
Linearity	2- 14mcg/ml	1000-9000 ng/band	2-16 mcg/ml
Precision studies (% RSD)	0.09	0.35	0.42
Accuracy studies 50% level 100% level	 102.62 101.73	 100.33 101.05	 99.65 98.80
LOD(mcg/ml)	0.0624	100 ng/band	0.0051
LOQ (mcg/ml)	0.1892	400 ng/band	0.0084
Applications	Analysis of drug solution of Ibutilide fumarate(Composition as mentioned in Corvert injection leaflet)[21,22]		• Analysis of drug solution of Ibutilide fumarate • Degradation studies • Interaction studies

Table 136. Comparison of UV spectroscopy reported methods and proposed method for Ibutilide fumarate

Parameter	Results		Observation
	Reported[16] method	**Proposed method**	
Detection wavelength	485	228	Developed method is more simple, sensitive, precise and accurate than the reported method.
Linearity (mcg/ml)	2.5-20	2-14	
LOD(mcg/ml)	Not found	0.0624	
LOQ (mcg/ml)	0.561	0.1892	
Stability of solution (hours) Room Temperature Refrigeration	 Not found Not found	 4 72	

Table 137. Statistical comparison of the percentage drug in the drug solution determined in the validated methods of UV Spectroscopy, HPTLC and HPLC of Ibutilide fumarate

Parameters	UV Spectroscopy	HPTLC	HPLC
No of Determinations	6		
Mean	97.20	99.00	100.5
SD	0.005	0.357	0.859
Lower 95% CL	97.01	98.76	99.97
Upper 95% CL	97.84	99.82	100.8
F –test	0.986		

9.4 Development of validated Analytical Methods for the determination of Dofetilide

Analysis of dofetilide by the validated methods according to the regulatory body ICH was carried out with utmost concern and applied for the admixture and degradation studies. The UV spectrum of dofetilide solution in methanol was identified for the λmax value at 231 nm. Standard drug solution of dofetilide was found to show similar incremental absorbance between 2-18 mcg/ml. The mathematically derived values from the absorbance for molar absorptivity, Sandell's sensitivity and E(1%,1cm) are $3.5x10^5$, 0.0123 and 810.3 respectively. Amount of drug detectable and quantifiable in the UV Spectroscpy method was found to be 0.0591 µg/ml and 0.1791 µg/ml respectively. The observations of the UV spectroscopic method for Dofetilide shows the accuracy, precision, simplicity, specificity, robustness and ruggedness of the method.

HPTLC analysis of Dofetilide was performed using precoated plates of silica gel $G_{60}F_{254}$. The solvent system made up of MeOH:TLN:ACN in the ratio of 4:2:4 v/v/v with a chamber saturation time of 20 minutes was found to be the most suitable with an Rf value of 0.55. Linearity was observed in the concentration ranging between 50-500 ng/band. The LOD and LOQ values of Dofetilide were found to be 20 ng/band and 40 ng/band respectively. The developed method was found to be specific, precise, accurate, sensitive, robust and suitable for the analysis of formulation of dofetilide.

The stationary phase used for the analysis of Dofetilide was C_{18} Shim pack column (250mm x 4.6id x 5µ). After a number of trials with various solvent system, the most ideal composition was found to be 10:35:55 v/v/v of methanol:acetonitrile:water. The flow rate of mobile phase was 1.0 ml/min and injection volume of 20 µl. The Linearity was observed in the concentration range of 10-50 µg/ml. The % RSD values of the precision studies and analysis of drug admixture was found to be below 1The LOD and LOQ values were found to be 0.0042µg/ml and 0.0130µg/ml respectively.

The UV spectroscopy, HPTLC and HPLC methods were applied for the determination of the admixture prepared with the excipients present in the capsule marketed commercially. The amount present in the admixture and the accuracy studies at two levels of 50% and 100% signifies the reliability of the method. The validated

HPLC method was employed for the degradation studiesof Dofetilide till 8 hours. Dofetilide in the solution form is stable at 40^{o}C, under hydrolytic, photolytic and oxidation condition till 4 hours with negligible change of peak area below 10%. By the time of 8 hours in the drug solution the degradation was more than 10% under acid, base, oxidation, light and thermal of 40^{o}C and 80^{o}C. The method could separate few degradant peaks in each of the study, which were well resolved from the drug peak and not interfered with the quantification of the drug. The increasing order of degradation of Dofetilide is as below:

Base hydrolysis<acid hydrolysis <photolysis <Thermal 80^{o}C <Oxidation

The identification of the selected impurity Dofetilide related compound has been studied according to the specification mentioned in the official monograph USP 40. The allowable level of Dofetilide related compound A is 0.5%. The admixture was spiked at 50% level below and above the limit and analysed with the peak eluted at the specific retention time for the Dofetilide related compound A.

The drug dofetilide and selected impurity had good resolution and recovery at three levels and as the drug was well resolved from the impurity enabled the effective quantification.

The comparison between the methods developed for Dofetilide has been shown in the table 138. The statistical evaluation of the percentage drug in the drug solution is listed in the table 139 with the F-test.

Table 138. Comparison between the UV Spectroscopy, HPTLC and HPLC methods of Dofetilide

Parameter	**UV Spectroscopy**	**HPTLC method**	**HPLC method**
Solvent	Methanol		
Detection wavelength	233 nm		
Linearity	2- 18mcg/ml	50-500 ng/band	10-50 mcg/ml
Precision studies (% RSD)	0.2031	0.0427	0.635
Accuracy studies(%) 50% level 100% level	 102.0 100.8	 104.4 100.6	 100.4 98.60
LOD	0.0591(mcg/ml)	100 ng/band	0.0051(mcg/ml)
LOQ	0.1791(mcg/ml)	400 ng/band	0.0084(mcg/ml)
Applications	Application to admixture		• Application to admixture • Degradation studies • Impurity separation and quantification

Table 139. Statistical comparison of the percentage drug in the admixture determined by the validated methods of UV Spectroscopy, HPTLC and HPLC of Dofetilide

Parameters	**UV Spectroscopy**	**HPTLC**	**HPLC**
No of Determinations	6		
Mean	100.40	98.60	100.24
SD	0.29	0.84	0.38
Lower 95% CL	99.85	98.05	99.98
Upper 95% CL	100.44	99.06	100.65
F –test	0.864		

CONCLUSION

In the current research work UV Spectroscopy and chromatographic methods were developed for the four drugs namely apixaban, rivaroxaban, ibutilide fumarate and dofetilide. These methods provide the availability of the simple way of analysis with strategic and segmented working procedure adopted. Each of the analytical method is economical, time saving, and easily adaptive.

The UV Spectroscopic method for apixaban, ibutilide fumarate and dofetilide is advantageous as there is minimum steps involved the sample preparation, time of analysis and procedure.

The HPTLC method developed and validated for the four drugs offers a very selective, sensitive and speedy analysis of the samples. Sensitivity of the method is proven with the results obtained in the nanogram level for the drugs. Among all the three methods, the HPTLC method is highly sensitive .

The HPLC method was found to be highly selective that they effectively quantified the selected drugs without any interference of matrix(excipients), impurity or degradants. The isocratic mode of operation is efficient and advantageous with only aqueous &organic solvents used in the mobile phase without any buffer system.

There is no reported pharmacokinetics data available till date for ibutilide fumarate. The interaction studies developed and validated is a model for the metabolic pathway of ibutilide fumarate as the rat liver microsomes consists of cytochrome 450 enzymes.

The steady state concentration level of ibutilide fumarate when present with verapamil till four hours depicts the interference of verapamil in the metabolic pathway of ibutilide .The results implify a concern on the co-administration of ibutilide fumarate and verapamil. Hence the co administration of ibutilide fumarate and verapamil has to be given serious consideration.

The analytical methods developed and validated can be applied for the routine analysis of the drug substance in the bulk and dosage forms. The applications of the methods adopted signifies the stability of the drugs under the degradation conditions. The interaction studies is a basic framework for the valuable results on co-administration of ibutilide fumarate with verapamil during multiple drug therapy. The impurity profiling is a valuable tool for the identification of impurity in drug substance for rivaroxaban and in drug product for dofetilide respectively.

10. IMPACT OF THE STUDY

Analytical methods are the versatile tool for the identification, quantification and structural elucidation of the drug molecules.

The spectroscopic and chromatographic methods of analysis developed and validated in this work are simple, time efficient and reliable to quantify the selected drugs.

The impurity profiling of rivaroxaban and dofetilide is the sensitive technique to identify the impurity which will affect the potency of the drug.

The degradation studies of ibutilide fumarate and dofetilide are useful for proving the stability of these two drugs under various stress conditions.

The *in vitro* drug-drug interaction studies is an insight on how the Ibutilide drug responds to the enzymes present in the liver when co-administered with verapamil. The method developed and validated for the *in vitro* drug-drug interaction studies of Ibutilide fumarate with verapamil will be useful in the clinical studies of these drugs.

The analytical methods developed and validated for the selected drugs can be adopted for the routine quality control of these drugs in bulk and formulations.

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