HPLC METHOD DEVELOPMENT AND QUANTITATION FOR PHARMACEUTICAL COMPOUNDS

A

THESIS

SUBMITTED TO THE

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IN

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BY

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UNDER THE GUIDANCE

OF

Dr. H. S. Joshi

DEPARTMENT OF CHEMISTRY

(DST-FUNDED, UGC-SAP SPONSORED)

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Statement under o. Ph. D. 7 of Saurashtra University

The work included in the thesis is my own work under the supervision of ***Dr. H. S. Joshi*** and leads to some contribution in chemistry subsidized by a number of references.

Date : 2008
Place : Rajkot

Hitesh B. Patel

This is to certify that the present work submitted for the Ph.D. Degree of Saurashtra University by ***Hitesh B. Patel*** is his own work and leads to advancement in the knowledge of chemistry. The thesis has been prepared under my supervision.

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CONTENT

PART-[B]:

CHIRAL SEPARATION AND METHOD DEVELOPMENT OF API BY HPLC

PART-[C]:

METHOD DEVELOPMENT AND QUANTITATION OF FORMULATION BY UPLC

SYNOPSIS

A comprehensive summary of the work to be incorporated in the thesis entitled "**HPLC method development and quantitation for pharmaceutical compounds**" has been described as under.

PART-[A] HPLC METHOD DEVELOPMENT AND QUANTITATION OF API AND FORMULATION

PART-[B] CHIRAL SEPARATION AND METHOD DEVELOPMENT OF API BY HPLC

PART-[C] METHOD DEVELOPMENT AND QUANTITATION OF FORMULATION BY UPLC

Experimental approach based on several considerations for each section mentioned as under:

Development of analytical method	**Validation parameters for analytical method**
Separation goal and information	Specificity
Selection of mobile phase	Linearity and range
Selection of stationary phase	Precision and intermediate precision
Selection of flow-mode and flow rate	Accuracy
Selection of wave-length	Robustness
Selection of diluent	Solution stability
Injection volume / Concentration	Limit of detection and limit of quantification

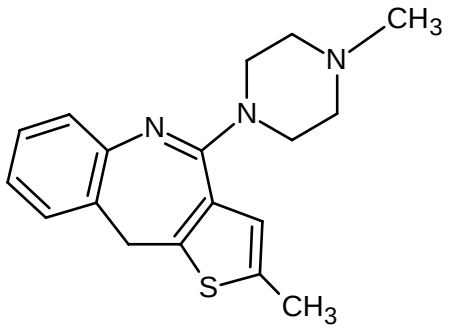
PART-[A]:

HPLC METHOD DEVELOPMENT AND QUANTITATION OF API AND FORMULATION

Aim of present studies

The research work undertaken in these studies in the area of pharmaceuticals and mainly addresses method development for determination of assay of pharmaceutical compound and determination of related impurities in active pharmaceutical substances as well as in its products by HPLC. Each developed analytical method was validated for its intended application as per ICH guideline.

Section-I: HPLC Method development and validation for determination of related impurities in Olanzapine OD tablets

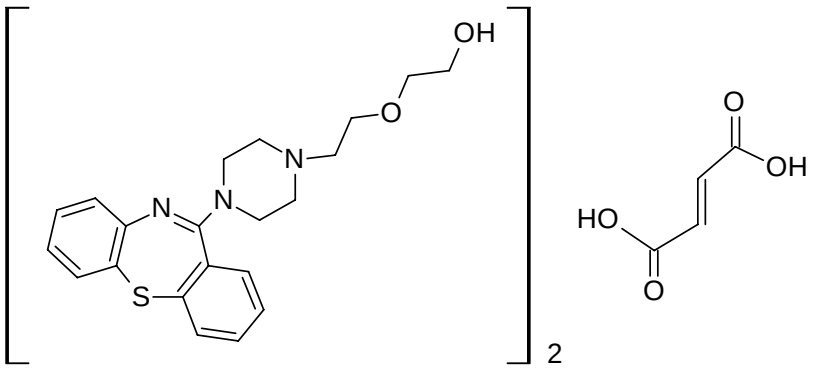


Olanzapine, 2-methyl-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3-*b*] [1,5] benzodiazepine

Developed chromatographic conditions

Buffer	Buffer, 6.24 gm sodium dihydrogen orthophosphate dehydrate in 800 ml of water, pH was adjusted to 6.8 with dilute sodium hydroxide solution, filtered through 0.45µm membrane		
Mobile phase-A	Buffer : Acetonitrile: Methanol, 500:200:300 (v/v/v)		
Mobile phase-B	Water : Acetonitrile, 20:80 (v/v)		
Diluent	Water : Acetonitrile, 20:80 (v/v)		
Column oven temp.	27°C		
Detection	UV at 250 nm		
Injection volume	20 µl		
Gradient programme	**Time (min)**	**Mobile phase-A (%)**	**Mobile phase-B (%)**
	0.01	100	0
	20	100	0
	25	70	30
	30	40	60
	35	20	80
	40	10	90
	45	10	90
	50	40	60
	55	100	0
	60	100	0

Section-II: HPLC Method development and validation for determination of related impurities in Quetiapine fumarate

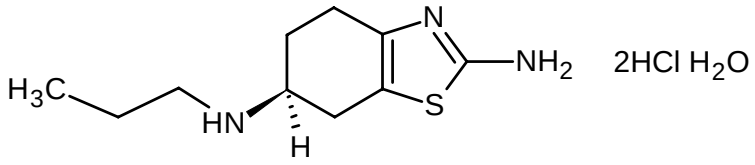


Quetiapine fumarate

Developed chromatographic conditions

Mobile phase-A	Buffer : Acetonitrile : Methanol, 70: 13: 17 (v/v/v) (Buffer, 40 mM ammonium acetate, pH was adjusted with ammonia solution to 9.2, filtered through 0.45µm PVDF membrane)		
Mobile phase-B	Acetonitrile		
Column	X terra RP -8 ,150 mm X 4.6mm 5µm		
Column oven temp	45°C		
Detection	UV at 250 nm		
Flow rate	1.5ml/min		
Injection volume	20µl		
Run time	80 min		
Gradient program	**Time (min)**	**Mobile phase-A (%)**	**Mobile phase-B (%)**
	0.0	100	0
	40	100	0
	65	50	50
	75	50	50
	76	100	0
	80	100	0
Diluent	0.1ml of perchloric acid (70%) in 1000 ml of mixture of water and Acetonitrile in ratio of 20:80		

Section-III: HPLC Method development and validation for assay of Pramipexole in Pramipexole tablets



S-isomer

Pramipexole, (S)-2-amino-4,5,6,7-tetrahydro-6-(propylamino)benzothiazole as 2HCl H_2O

Developed chromatographic conditions

Mobile phase	0.1% ammonia solution:acetonitrile:methanol,70:15:15 (v/v/v)
Column	X terra RP -8 ,150 mm X 4.6mm 5µm
Column oven temp	25°C
Detection	UV at 260 nm
Flow rate	1.0 ml/min
Injection volume	10µl
Run time	10 min
Diluent	Water: Methanol, 50:50 (v/v)

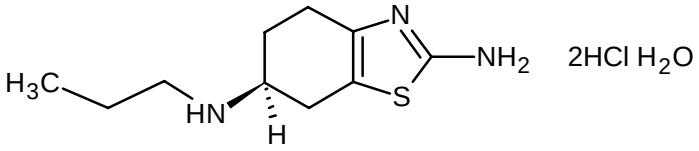
PART-[B]:

CHIRAL SEPARATION AND METHOD DEVELOPMENT OF API BY HPLC

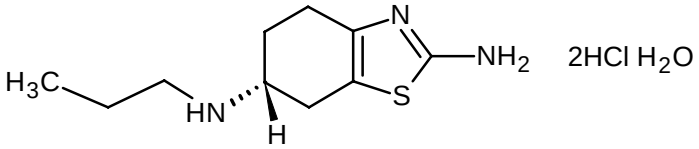
Aim of study

The research work undertaken in this study in the area of pharmaceuticals and addresses method development for determination chiral purity or determination of R-isomer as an impurity for active pharmaceutical substances by HPLC. Developed analytical method was validated for its intended application as per ICH guideline.

Section-I: Chiral RP-HPLC method development and validation for determination of R-isomer in Pramipexole



S-Isomer of Pramipexole (API)



R-Isomer of Pramipexole (Impurity)

Developed chromatographic conditions

Mobile phase	Buffer: Acetonitrile in ratio of 70: 30 (v/v)
Column	CHIRAL PACK AD-RH 150 mm x 4.6 mm with 10 x 4 mm guard cartridge as of same CSP in gourd holder
Column oven temp	25°C
Detection	UV at 260 nm
Flow rate	0.6 ml/min
Injection volume	20µl
Run time	45 min
Diluent	40:60, Methanol: 0.1% v/v ammonia solution

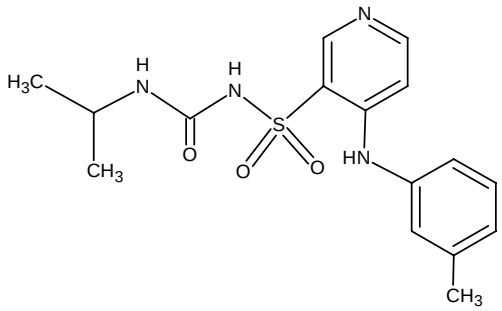
PART-[C]:

METHOD DEVELOPMENT AND QUANTITATION OF FORMULATION BY UPLC

Aim of study

The research work undertaken in these studies in the area of pharmaceuticals and mainly addresses method development for determination of related impurities in drug products by UPLC. Developed analytical method was validated for its intended application as per ICH guideline.

Section-I: UPLC Method development and validation for determination of related impurities in Torsemide tablets



Torsemide, 1-isopropyl-3-[(4-m-toludino-3-pyridyl)sulfonyl]urea

Developed chromatographic conditions

Buffer	20 mM potassium dihydrogen phosphate, pH adjusted to 3.5 with orthophosphoric acid, filtered through 0.22µm		
Mobile phase-A	Buffer : Acetonitrile, 90:10 (v/v)		
Mobile phase-B	Buffer : Acetonitrile, 50:50 (v/v)		
Diluent	Buffer : Acetonitrile : Methanol, 25:25:50 (v/v/v)		
Column oven temp.	30°C		
Detection	UV at 288 nm		
Injection volume	6 µl		
Gradient programme	**Time (min)**	**Mobile phase-A (%)**	**Mobile phase-B (%)**
	0.0	75	25
	2.5	65	35
	4.0	25	75
	4.6	25	75
	5.0	75	25
	6.0	75	25

The developed procedure has been evaluated for the specificity, linearity, accuracy, precision, limit of detection, limit of quantification and robustness. It has been proved that it was specific, linear, precise, accurate and robust and stability indicating. Hence, the method could be of use for routine quality control analysis and also for analysis of stability samples.

Hitesh B. Patel
Place :
Date :

Dr. H.S. Joshi
Place :
Date :

GENERAL INTRODUCTION

History of chromatography

In 1903 a Russian botanist Mikhail Tswett produced a colorful separation of plant pigments through calcium carbonate column. Chromatography word came from Greek language chroma = color and graphein = to write i.e. color writing or chromatography [1-2].

History of HPLC

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times of compounds being isolated by column chromatography. However, flow rates were inconsistent, and the question of whether it was better to have constant flow rate or constant pressure was debated [3].

High pressure liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds.

By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above the previous techniques. Computers and automation added to the convenience of HPLC. Improvements in type of columns and thus reproducibility were made as such terms as micro-column, affinity columns, and Fast HPLC began to immerge.

By the 2000 very fast development was undertaken in the area of column material with small particle size technology and other specialized columns. The dimensions of the

typical HPLC column are 100-300 mm in length with an internal diameter between 3-5 mm. The usual diameter of micro-columns, or capillary columns, ranges from 3 μm to 200 μm [4].

In this decade sub 2 micron particle size technology (column material packed with silica particles of < 2μm size) with modified or improved HPLC instrumentation becomes a popular with different instrument brand name like UPLC (Ultra Performance Liquid Chromatography) of Waters and RRLC (Rapid Resolution Liquid Chromatography) of Agilent.

Modern High Performance Liquid Chromatography (HPLC)

The highly sophisticated reliable and fast liquid chromatographic (LC) separation techniques are become a requirement in many industries like pharmaceuticals, agrochemicals, dyes, petrochemicals, natural products and others. Early LC used gravity fed open tubular columns with particles 100s of microns in size; the human eye was used for a detector and separations often took hours (days?) to develop. Today's HPLC requires very special apparatus which includes the following.

1. Extremely precise gradient mixers.
2. HPLC high pressure pumps with very constant flow.
3. Unique high accuracy, low dispersion, HPLC sample valves.
4. Very high efficiency HPLC columns with inert packing materials.
5. High sensitivity low dispersion HPLC detectors.
6. High speed data acquisition systems.
7. Low dispersion connecting tubes for valve to column and column to detector.

HPLC Gradient mixtures

HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. This can be complicated in HPLC by the small elution volumes required by many systems. It is much more difficult to produce a constant gradient when mixing small volumes then when mixing large volumes. For low pressure systems this requires great precision in the operation of the miniature mixing

valves used and low dispersion flows throughout the mixer. For multi-pump high pressure systems it requires a very precise control of the flow rate while making very small changes of the flow rate.

HPLC Pumps

Because of the small particles used in modern HPLC, modern LC pumps need to operate reliably and precisely at pressures of 10,000 p.s.i. or at least 6,000 p.s.i. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical purposes HPLC pumps should have flow rates that range from 0 to 10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. It is extremely difficult to provide a very constant flow rate at very low flow rates. If 1% is considered acceptable then for 1ml/min a flow variation of less then 10µl/min is required. This level of constancy is required because most HPLC detectors are flow sensitive and errors in quantization will result from change in flow rate.

HPLC Sample Valves

Since sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 p.s.i. For analytical HPLC, the sample volume should be selectable from sub micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml. To maintain system efficiency the sample valve must be designed to have very low dispersion characteristics, this is true not only for flow dispersion but also for the less obvious problems of dispersion caused by sample adsorption/desorption on valve surfaces and diffusion of sample into and out of the mating surfaces between valve moving parts. It goes without saying that the valves must deliver a very constant sample size but this is usually attained by the use of a constant size sample loop.

HPLC Columns

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the

high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. Packing these small particles into the column is a difficult technical problem but even with good packing a great amount of care must be given to the column end fittings and the inlet and outlet connection to keep dispersion to a minimum. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized because of the much lower quantities of both which are required. In particular very expensive optically pure compounds can be used to make Chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents.

HPLC Detectors

In order to give an accurate chromatographic profile the detector sampling (cell) volume must be a small fraction of the solute elution volume. If the detector volume were larger then the elution volume then you would have peaks that appeared with flat tops as the whole peak would be resident in the detector at the same time. This means that as column volumes decrease and system efficiencies increase the volume of the detector cell volume must also decrease. It is odds for the requirement for detector to maintain high sensitivity as this is usually dependant on having a larger cell volume. Again, this requires the very careful design of modern detectors. Many types of detectors can use with HPLC system like UV-Visible or PDA (Photo Diode Array), RI (Refractive Index), Fluorescence, ECD (Electro Chemical Detector), ELSD (Evaporative Light Scattering Detector), and many others hyphenated techniques like MS, MS/MS and NMR as well as evaporative IR.

HPLC Data acquisition

In HPLC data acquisition system the higher sampling rate needed for the rapidly eluting narrow peaks of the HPLC chromatogram. Although the theoretical number of samples needed for good quantization are actually quite small, for real systems a hundred samples or more per peak is recommended; thus, for a 4 sec wide peak, a rate of 25

samples per second may be required. The same data analysis and reporting software can be used as in ordinary LC.

Conclusion

HPLC is probably the most universal type of analytical procedure; its application areas include quality control, process control, forensic analysis, environmental monitoring and clinical testing. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with a constant incorporation of new highly selective column packings.

(I) EFFECT OF VARIABLES ON COLUMN EFFICIENCY

A mathematical approximation of the behavior of chromatographic column efficiency is obtained from the Van Deemter equation:

$$H = A + \frac{B}{u} + C \cdot u$$

Where, H is the plate height, u is the linear velocity of the mobile phase, A is the eddy diffusion term, B is the longitudinal diffusion coefficient, and C is the coefficient of the mass transfer term. The lower the value of H, the more efficient the column.

The eddy diffusion term A, represents the multitude of pathways by which a component finds its way through the column. In a poorly packed column, the retention time for molecules of the same component can vary significantly depending on the numerous flow paths that could be taken. This effect results in band-broadening. The mobile phase velocity also affects the eddy diffusion parameter, and at moderate to high flow rates the zone-broadening is greater. At low flow rates, the molecules of a component are not significantly dispersed by multi-channeling; diffusion averaging results in this case.

The longitudinal diffusion term, B/u, describes a band-broadening process that is inversely related to the mobile phase velocity. The analyte is in the column for a shorter

time when the flow rate is high; hence the diffusion term is less. In HPLC, this term is negligible since diffusion coefficients of liquids are very small relative to gases (as in GC).

The mass transfer term, C.u, describes the time available for equilibrium of an analyte to be established between the mobile and stationary phases. At high mobile flow rates there is less time for this equilibrium to take place and a contribution to the broadening effect is observed.

(II) SEPARATION MECHANISMS

A useful classification of the various LC techniques is based on the type of distribution (or equilibrium) that is responsible for the separation. The common interaction mechanisms encountered in LC are classified as adsorption, partition, ion-exchange, gel permeation or size exclusion, and chiral interaction. In practice, most LC separations are the result of mixed mechanisms. A brief description of the separation mechanisms is as under.

Adsorption

When the stationary phase in HPLC is a solid, the type of equilibrium between this phase and the liquid mobile phase is termed *adsorption*. All of the pioneering work in chromatography was based upon adsorption methods, in which the stationary phase is a finely divided polar solid that contains surface sites for retention of analytes. The composition of the mobile phase is the main variable that affects the partitioning of analytes. Silica and alumina are the only stationary phases used, the former being preferred for most applications. Applications of adsorption chromatography include the separation of relatively non-polar water-insoluble organic compounds. Because of the polar nature of the stationary phase and the impact of slight variations in mobile phase composition on the retention time, adsorption chromatography is very useful for the separation of isomers in a mixture.

Partition

The equilibrium between the mobile phase and a stationary phase comprising of either a liquid adsorbed on a solid or an organic species bonded to a solid is described as

partition. The predominant type of separation in HPLC today is based on partition using bonded stationary phases. Bonded stationary phases are prepared by reaction of organochlorosilane with the reactive hydroxyl groups on silica. The organic functional group is often a straight chain octyl (C-8) or octyldecyl (C-18); in some cases a polar functional group such as cyano, diol, or amino may be part of the siloxane structure. Two types of partition chromatography may be distinguished, based on the relative polarities of the phases.

When the stationary phase is polar and the mobile phase relatively less polar (n-hexane, ethyl ether, chloroform), this type of chromatography is referred to as *normal-phase chromatography*. For this reason, the use of silica as the stationary phase (as in adsorption chromatography) is also considered to be a normal phase separation method. When the mobile phase is more polar than the stationary phase (which may be a C-8 or C-18 bonded phase), this type of chromatography is called *reversed-phase chromatography*. Reversed-phase chromatography separations are carried out using a polar aqueous-based mobile phase mixture that contains an organic polar solvent such as methanol or acetonitrile. Because of its versatility and wide range of applicability, reversed-phased chromatography is the most frequently used HPLC method. Applications include non-ionic compounds, polar compounds, and in certain cases ionic compounds.

Ion-exchange

Ion-exchange separations are carried out using a stationary phase that is an ion-exchange resin. Packing materials are based either on chemically modified silica or on styrene-divinylbenzene copolymers, onto which ionic side groups are introduced. Examples of the ionic groups include (a) Sulfonic acid (strong cation exchanger), (b) Carboxylic acid (weak cation exchanger), (c) Quaternary ammonium groups (strong anion exchanger), and (d) Tertiary amine group (weak anion exchanger). The most important parameters that govern the retention are the type of counter-ion, the ionic strength, pH of the mobile phase, and temperature. Ion chromatography is the term applied for the chromatographic separation of inorganic anions/cations, low molecular weight organic acids, drugs, serums, preservatives, vitamins, sugars, ionic chelates, and certain organometallic compounds.

The separation can be based on ion-exchange, ion-exclusion effects, or ion pairing. Conductivity detectors in ion chromatography provide universal and sensitive detection of charged species. The employment of some form of ion-suppression immediately after the analytical column eliminates the limitation of high background signal from the mobile phase in conductivity detection.

Size Exclusion

High molecular weight solutes (>10,000) are typically separated using size exclusion chromatography – gel filtration or gel permeation. In size-exclusion LC, the components of a mixture are separated according to their ability to penetrate into the pores of the stationary phase material. Packing materials used are wide-pore silica gel, polysaccharides, and synthetic polymers like polyacrylamide or styrene-divinylbenzene copolymer. In gel filtration the mobile phase is aqueous and the packing material is hydrophilic, while in gel permeation an organic mobile phase is used and the stationary phase is hydrophobic. Size-exclusion applications include the separation of large molecular weight biomolecules, and molecular weight distribution studies of large polymers and natural products. For a homologous series of oligomers, the retention time (volume) can be related to the logarithm of the molecular mass.

Chiral Interaction

Chiral compounds or enantiomers have identical molecular structures that are non superposable mirror images of each other. Rapid and accurate stereochemical resolution of enantiomers is a challenge in the field of pharmaceuticals and drug discovery. A chiral stationary phase contains one form of an enantiomeric compound immobilized on the surface of the support material. Typically, derivatives of optically active polysaccharides that are chemically bonded to silica form the packing material. A chiral separation is based on differing degrees of stereochemical interaction between the components of an enantiomeric sample mixture and the stationary phase.

Method development

Method development should be based on several considerations. It is preferable to have maximum sample information to make development fast and desired for intended

analytical method application, physical and chemical properties are most preferable as primary information. Moreover, separation goal needs to define at beginning so; appropriate method can be developed for the purpose. An LC method development is very huge area for even pharmaceuticals with regulatory requirement of international standards. So, prior to method validation and usage at quality control many aspects need to focus as per ICH guideline. Method development can be based on sample and goal as well as available resources for chromatography but few basic steps for method development are can be discussed as below [5].

Steps in method development

1. Sample information, define separation goals
2. Sample pretreatment, need of special HPLC procedure
3. Selection of detector and detector settings
4. Selection of LC method; preliminary run; estimate best separation conditions
5. Optimize separation conditions
6. Check for problems or requirement for special procedure
7. Method validation

Sample information

1. Number of compounds present
2. Chemical structure of compounds
3. Chemical nature
4. Molecular weight of compounds
5. pKa Value(s) of compounds
6. Sample solubility
7. Sample stability and storage
8. Concentration range of compounds in sample
9. UV spectra of compounds or properties for detection of compounds

Separation goals

Goal	Comment
Resolution	Precise and rugged quantitative analysis requires that resolution be greater than 1.5
Separation time	< 5-10 minutes is desirable for routine procedure (e.g. Dissolution profile)
Quantitation	< 2% RSD for assays
Pump pressure	< 150 Bar is desirable, < 200 bar is usually essential (For UPLC-waters and RRLC-agilent these values are 5 fold and 3 fold respectively)
Peak height	Narrow peaks are desirable for large signal/noise ratio
Solvent consumption	Minimum mobile phase use per run is desirable

Sample preparation

Select the optimum sample amount
Determine the diluent that gives the best; -Solution stability -Solubility for sample or it's component (s)
Investigate the effect of diluent in terms of -Organic and aqueous solvent ratio -pH -Extraction volume -Extraction procedure and time -Chromatographic changes like peak shape and resolution
Note: As a diluent mobile phase is preferable with consideration of above points for better baseline
Solution may require dilution or buffering
Determine sample concentration which gives LOQ below the identification threshold in case of related substances
Sample may require sample pretreatment to remove interferences and/or protect the column and equipment
As a part of sample pretreatment; filter compatibility study is required

Detection

Before the first sample is injected during the HPLC method development we must be reasonably sure that the detector selected will sense all sample components of interest. Normally variable wavelength UV detector is the first choice of the chromatographers, because of their convenience and applicability for most organic samples. UV spectra can be obtained by PDA detector. When the UV response of the sample is inadequate, other detector or derivative HPLC method can be used.

Selection of LC method and mobile phase selection in partition chromatography

Successful chromatography requires a proper balance of the intermolecular forces between the solute, the mobile phase, and the stationary phase. The important criteria to consider for method development are resolution, sensitivity, precision, accuracy, limit of detection, limit of quantitation, linearity, reproducibility, and time of analysis and robustness of the method. In all of these, the column quality plays an important role since the peak shape affects all criteria required for optimum separation. The factors that affect the column efficiency have already been described above.

Column dimensions and particle size affect the speed of analysis, resolution, column backpressure, detection limit, and solvent consumption. HPLC methods have traditionally been developed using columns measuring 10, 15 or 25 cm in length and 4.6 mm ID. Short columns of 5 cm or less in length and 1 or 2 mm ID are now available; when packed with particles of size 5 micron or less, very high efficiency columns are obtained. The advantages of using shorter columns are lower backpressures, dramatic solvent savings, greater sensitivity, reduced analysis time, and applicability to small sample quantities - all achieved without compromising resolution. Using these columns, gradient methods may be used to achieve very rapid analyses of samples that contain a wide polarity range of analytes. The future of reversed-phase HPLC method development will involve a significant increase in the use of use narrow-bore and micro-bore columns.

Often in choosing a column for partition chromatography, the polarity of the stationary phase is matched roughly to that of the analytes in the sample; a mobile phase of different polarity is used for elution. The analytes must be soluble in the mobile phase and the solvent must be compatible with the analytical method.

As a general guide, use normal phase chromatography for the separation of polar compounds and reversed-phase chromatography for components that are in the moderately polar to non-polar range.

Normal phase chromatography commonly involves the use of silica, aminopropyl, diol, and cyanopropyl stationary phases. These columns may be used to separate polar compounds such as amines, anilines, nitroaromatics, phenols, and pesticides.

Isocratic elution in reversed-phase chromatography is typically accomplished using a mobile phase mixture of water and another solvent of lower eluting strength (acetonitrile, methanol). In cases where the time of analysis is compromised or when the resolution is poor, gradient elution using 2 or 3 different solvents is recommended.

The relative polarity of a solvent is a useful guide to solvent selection in partition chromatography. The relative polarities of the listed solvents may differ slightly depending on the literature source, since the scale used to measure polarity may be different. The following should suffice as a general reference for relative solvent polarity.

1	Fluoroalkanes (least polar)	10	Acetone
2	Hexane	11	Ethyl acetate
3	Isooctane	12	Dioxane
4	Carbon tetrachloride	13	Isopropanol
5	Toluene	14	Ethanol
6	Diethyl ether (ether)	15	Acetic acid
7	Chloroform	16	Methanol
8	Methylene chloride	17	Acetonitrile
9	Tetrahydrofuran (THF)	18	Water (most polar)

There is a strong dependence of the retention time on the mobile phase composition, and the retention parameter may be easily altered by variation of solvent polarity. This is the easiest way to improve chromatographic resolution of two

overlapping species or to decrease overall separation time for components with widely differing retention values. A good starting point is a mixture of water and a polar organic solvent (methanol or acetonitrile). The effect of mobile phase polarity on elution time can be tested at a few different solvent proportions. If greater selectivity is required, a mobile phase comprising of 3-4 solvents may be used. Theoretical calculations have indicated that a mobile phase mixture of water, THF, methanol, and acetonitrile may be used to resolve most reversed-phase applications within a reasonable length of time.

The various analytes to be separated may also be arranged based on the polarities of their functional groups. A general guide to relative solute polarity going from non-polar to the most polar group is as follows:

1	Hydocarbons (least polar)	6	Amides
2	Ethers	7	Amines
3	Esters	8	Alcohols
4	Ketones	9	Water (most polar)
5	Aldehydes		

Stationary Phases:

Many types of stationary phases available commercially with different column material chemistry start from C-18 for reversed phase to silica for Normal phase chromatography. Chromatographers may needs to consider many aspects before selection column.

Type of column	Remark
C-18 ("octadecyl", "ODS")	Rugged; retentive; widely used
C-8 ("octyl")	Similar to C18, but slightly less retentive
C-3 and C-4	Less retentive; less stable; used mainly for peptides and proteins
C-1 ("trimethylsilyl")	Least retentive; least stable
Phenyl and phenethyl	Moderately retentive; selectivity change
CN ("cyano")	Moderately retentive; also normal-phase
NH_2 ("amino")	Weakly retentive; more often used for normal-phase; less stable
Polystyrene	Stable for $1 < pH < 13$; good peak shape and lifetime ; selectivity change; selectivity change; often less efficient

The most widely used HPLC packings are the long-chain alkyls such as C18 or C8. These differ somewhat in their overall retentivity, although most separations can be carried out on either material. Shorter-chain alkyl packings are less retentive, but are also less stable. The silyl ether bonds are labile to hydrolysis at low pH. With long-chain packings, the hydrophobicity of the chain limits the rate of hydrolysis as well as protecting the underlying silica from dissolution in basic solution.

Aromatic bonded phases typically have an overall retentivity comparable to that of C8 material, but with an added selectivity for samples which can differ in their interactions with aromatic groups. More polar groups, such as cyano or amino can also be bonded to silica to provide selectivity differences compared to the alkyl phases. The resulting packings are more commonly used for normal- phase than for reversed-phase LC.

Finally, polystyrene based packings provide a viable alternative to silica for applications in which silanol interactions must be avoided altogether or for which high-pH operation is required. Because such packings have no silanol groups, their selectivity can be quite different from that of silica-based materials.

Beyond the very tenuous guidelines given above, there is no way to make sweeping generalizations concerning initial column choices for particular samples. Because selectivity is based on differences is molecular structure and depends on secondary interactions, the only effective way to establish suitability of a particular column for a particular sample is empirical: try it.

The most common type of HPLC column is the C18 or C8 bonded phase silica. These provide a good compromise among retention, selectivity, lifetime, operating pressure, etc. In most applications, either a C8 or C18 will do equally well. There is often more selectivity difference between the “same” columns from different manufacturers than between “different” columns from the same source.

Silica columns are the most commonly used type because they tend to be more efficient and reproducible than their polymeric equivalents. The latter can be quite useful when an extended pH range is required, or when silanol interactions give rise to excessive tailing on silica based column.

Variables that affect N

Flow rate, column length, particle size, column quality and operating temperature, these variables also affect the run time and pressure.

Because HPLC hardware is limited to operating pressures of about 5,000 psi, it is rarely feasible to generate extremely large column plate numbers. HPLC columns today provide 5,000 - 15,000 plates for well behaved samples. This number has remained roughly constant for over twenty years, but the column length needed to achieve it has decreased by a factor of about 5, with a corresponding decrease in run time.

A rough approximation of the plate number expected from a given column is provided by: $N = 3000\ L/dp$ where, L is the column length in cm and dp is the packing particle size in μm.

The column in current routine practice consists of a 5-μm bonded-phase silica in a 10 or 15-cm column. 3-μm packings in shorter columns give faster analysis but are not as rugged as their 5-μm counterparts. Either configuration can generate the 10,000 or so plates required for general-purpose HPLC method development.
Column dimensions can be arbitrarily divided into the following categories based on internal diameter (this terminology is not universal):

Preparative ≥10 mm	Analytical 3-5 mm	Microbore ≤ 1 mm
Semi-preparative 5 - 10 mm	Narrow bore 2-3 mm	

Standard analytical scale columns are typically available in 5, 10, 15, and 25-cm lengths.

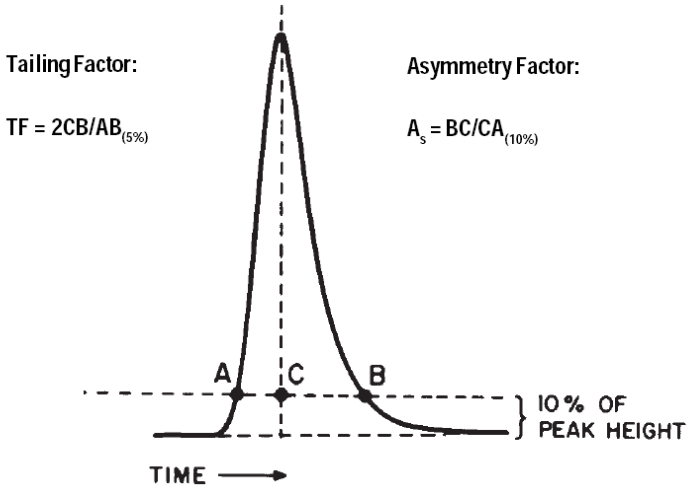
Through most of our discussion, we have assumed symmetrical, Gaussian peak shape. Real chromatographic peaks, however, are rarely symmetrical. Although mathematically elegant general expressions for quantifying peak symmetry can been developed, practical difficulties have led chromatographers to the “rough and ready” definition of asymmetry shown here.10% of peak height is typically but not universally

used for the measurement. The asymmetry factor generally increases as the measurement is made further down the peak.

USP (pharmacopoeial) methods commonly specify a tailing factor of the peak measured by formula:

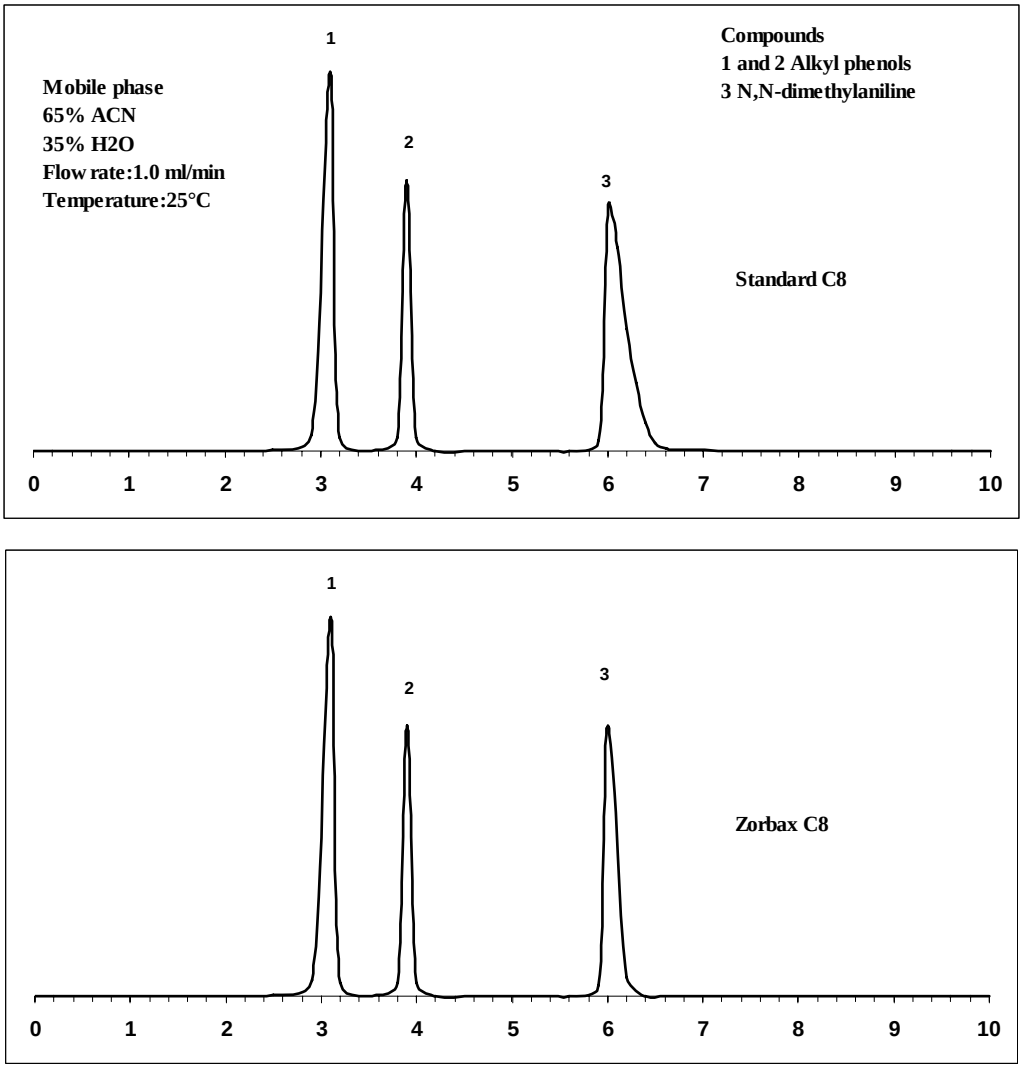
$$Tailing\ \ factor = \frac{2\ CB}{AB}$$

It is measured at 5% of peak height. Tailing factor at 5% and asymmetry factor at 10% give (very) roughly equivalent numbers overall, but may be quite different in specific cases (depending on the exact shape of the peak).



A bad column can lead to tailing bands in one of two ways. A plugged frit or a void will cause tailing for all bands in the chromatogram. A column packed with "acidic silica" particles will cause tailing of basic (amine) components of the sample. In this example, compound #3 is basic (dimethylaniline), and it is seen to tail in the top chromatogram — but not the bottom. The reason is that the top separation is carried out with an acidic silica packing.

By far the major contributor to peak tailing is the existence of secondary retention effects. On silica based columns, these come primarily from interactions with underivatized silanol groups.



Some silanols are quite acidic (pKa's can range down to 3.5 or lower) which means that they can interact via ion exchange at most reasonable pH values. Even at low pH (or with neutral silanols) , a sufficiently aggressive base can remove the proton to generate an ion-exchange interaction. Just to make life interesting, there is a non-bonding electron pair on the oxygen that can also interact with acids via hydrogen bonding.

Actually, the problem is not interactions with silanols, but rather hindered interactions with silanols such that some sample molecules become strongly held via a two-point interaction (ion exchange + hydrophobic). The characteristics required for tailing are the existence of a low concentration of highly retentive active sites. As a result, these sites are quickly overloaded and attachment / release from these sites may be slow. Each of these latter effects can result in peak tailing.

Unfortunately, theoretical predictions of mobile phase and stationary phase interactions with a given set of sample components are not always accurate, but they do help to narrow down the choices for method development. The separation scientist must usually perform a series of trial-and-error experiments with different mobile phase compositions until a satisfactory separation is achieved [6-7].

Method Validation

A developed analytical method for routine use needs to be validated as per ICH guideline for following validation parameters based on its intended application [8]:

1. Specificity
2. Linearity & Range
3. Precision
   (A) Method precision (Repeatability)
   (B) Intermediate precision (Ruggedness)
4. Accuracy (Recovery)
5. Robustness
6. Solution stability
7. System suitability

Method validation is vast area which includes many validation parameters with different approaches for different level of requirement based on intended use of analytical method, criticality and regulatory requirements. Validated method also can give the unpredicted or unknown problem during the course of routine usage, because validated method is also limited confidence, as method was validated for known or predicted variable parameters or every method can fail sooner or later [9]. But still after method development it needs to be validated as per requirement which gives certain level of confidence for its intended use.

References

[1] http:// www. umich.edu/~orgolab/Chroma/chromahis.html

[2] From Wikipedia, the free encyclopedia

[3] http:// kerouac.pharm.uky.edu/asrg/hplc/history.html

[4] http:// www. laballiance.com/la_info%5Csupport%5Chplc3.htm

[5] Lloyed R. Snyder, Joseph J. Krikland, Joseph L. Glajch, 2nd ed., John wiley & Sons, INC. 1997 p.1-20

[6] http:// www. chromatography-online.org/HPLC.html

[7] http:// elchem.kaist.ac.kr/vt/chem-ed/sep/lc/graphics/hplc-sch.gif

[8] ICH guideline, Q2 (R1) step 4, Validation of Analytical Procedures: Text and Methodology (2005)

[9] Johan W. Dolan, LCGC North America 24-4(2006) 374

Section-I: HPLC Method development and validation for determination of related impurities in Olanzapine OD tablets

1. Introduction to Olanzapine

Olanzapine is chemically 2-methyl-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3-*b*] [1,5] benzodiazepine, a thienobenzodiazepine derivative [Fig.1], belongs to class of second generation derivative antipsychotic agents, the so-called atypical antipsychotics. They have greater affinity for erotonin 5-HT_2 serotonin receptors than for dopamine D_2 receptors and cause fewer extra pyramidal symptoms (EPS) in contrast with classical antipsychotics (e.g. haloperidol). The efficacy and safety of olanzapine has been demonstrated in randomised, placebo-controlled and comparative trials in positive and negative symptoms of schizophrenia, and also as monotherapy or in combination with mood stabilizers in the treatment of acute manic or mixed episodes associated with bipolar disorder [1].

Its molecular weight is 312.43 g/mole with molecular formula of $C_{17}H_{20}N_4S$. Its dissociation constants (pKa) 4.69 and 7.37 are reported. Its melting point is 190°C to 195°C [2].

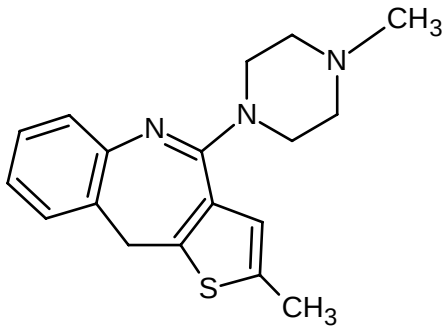


Fig.1: Olanzapine

Strengths: Olanzapine OD tablets are available in four strengths as 2.5 mg, 5 mg, 7.5 mg, 10 mg and 20 mg of Olanzapine as an active ingredient for oral administration.

1.1 Active substance

The active substance olanzapine is a yellow crystalline powder. Olanzapine can exist as five possible polymorphic forms (I, II, III, IV and V). The control of polymorphism has been achieved and was found to be stable during stability studies. The structure has been

fully elucidated by ^{1}H-NMR, UV, IR and mass spectroscopy, and elemental analysis. Physico-chemical characterization includes appearance, solubility, loss on drying, tapped density, DSC scan and X-ray diffraction to identify polymorphic form. The active substance has no chiral centres and no isomerism could be found in the literature [3].

2. Literature review

The literature reviews regarding Olanzapine suggest that various analytical methods were reported for drug substance as well as in pharmaceutical formulation and in various biological fluids. The literature reviews for analysis of Olanzapine are as under.

2.1 Pierre M. Llorca, François Coudore, Christophe Corpelet, Aurelie Buyens, Monique Hoareau and Alain Eschalier have developed a method for Olanzapine in blood plasma using the Dionex HPLC system used consisted of a Dionex injector (Model ASI-100), an isocratic pump (Model P-580A), and a photodiode array detector (Model UVD-170S). Chromatograms were processed using the Chromeleon™ chromatographic data collection and analysis system and Ultremex silica column [250 x 4.6 mm (i.d.); Phenomenex]. The mobile phase consisted of a methanol-deionized water mixture (70:30 by volume) containing 0.110 mL/L butylamine. The mobile phase was filtered through a 0.22 μm filter and degassed before use. The chromatography was carried out at ambient temperature at a flow rate of 1 mL/min. Peaks were monitored at 273 nm [4].

2.2 M. A. Raggi, G. Casamenti, R. Mandrioli, S. Fanali, D. De Ronchi and V. Volterra have developed a sensitive high performance liquid chromatograpic method for the analysis of the novel antipsychotic drug, olanzapine, in human plasma. Chromatography was performed on a reversed-phase column (C8, 150×4.6 mm i.d., 5 μm) with acetonitrile-phosphate buffer (pH=2.5) as the mobile phase. The detection voltage was +900 mV and the cell and column temperature were 50°C. The flow rate was 1 mL min^{-1}. The method provides a linear response over an olanzapine concentration range of 2–100 ng mL^{-1}. Isolation of olanzapine from plasma was accomplished by a solid phase extraction procedure, using C8 cartridges, which gave high extraction yield and needed a small amount of human plasma (only 0.25 mL). The results obtained analysing plasma samples from patients in therapy with

olanzapine were satisfactory in terms of precision and sensitivity, and were compared to those obtained by means of a method based on HPLC with UV detection [5].

2.3 B. Venkateswara Reddy et al. have developed a rapid, specific reversed phase HPLC method for simultaneous determination of olanzapine and fluoxetine in their formulations. Chromatographic separation of these two pharmaceuticals was carried out on an Inertsil C_{18} reversed phase column (150 mm × 4.6 mm, 5 μm) with a 40:30:30 (*v*/*v*/*v*) mixture of 9.5 mM sodium dihydrogen phosphate (pH adjusted to 6.8 ± 0.1 with triethylamine), acetonitrile and methanol as mobile phase. The flow rate 1.2 mL min^{-1} and the analytes are monitored at 225 nm. Paroxetine was used as internal standard. The assay results were linear from 25 to 75 μg mL^{-1} for olanzapine ($r^2 \geq 0.995$) and 100–300 μg mL^{-1} for fluoxetine ($r^2 \geq 0.995$), showed intra- and inter-day precision less than 1.0%, and accuracy of 97.7–99.1% and 97.9–99.0%. LOQ was 0.005 and 0.001 μg mL^{-1} for olanzapine and fluoxetine, respectively. Separation was complete in less than 10 min. Validation of the method showed it to be robust, precise, accurate and linear over the range of analysis [6].

2.4 Catlow J. T., Barton R. D., Clemens M., Gillespie T. A., Goodwin M., Swanson S. P. have developed a sensitive reversed-phase HPLC method for the analysis of olanzapine in human plasma is described. Isolation of olanzapine from plasma was accomplished by solid-phase extraction utilizing an ion-exchange/reversed-phase cartridge designed for basic drug extraction. The drug was subsequently separated by reversed-phase HPLC and monitored by electrochemical detection (ED). Electrochemical analysis was used to detect olanzapine due to its uniquely low oxidative potential. Ascorbic acid was added to prevent oxidation during extraction. The limit of quantitation for the assay was established at 0.25 ng/ml utilizing a 1-ml human plasma sample. The average inter-day accuracy was 96.6% with a average precision (%C.V.) of 3.22% over the concentration range of 0.25 to 100 ng/ml. This method was applied to human plasma samples from human clinical trials with olanzapine. The HPLC-ED method compared favorably with a negative chemical ionization GC-MS method previously utilized for analysis of olanzapine in human plasma [7].

2.5 Dasandi B., Shah P., Gandhi C., Bhat K. M., Shivprakash have developed a new method for the estimation of olanzapine in human plasma and validated. They utilized

RP column and amperometric detector. The mobile phase consisted of 75mM sodium dihydrogen phosphate buffer of pH 7.0, acetonitrile and methanol in 55:22.5:22.5 %v/v ratios in an isocratic mode at 1.5ml_{-1} flow rate. The drug is extracted in to 3ml each of Tertiary Butyl Methyl Ether (TBME) twice from plasma, evaporated and the residue dissolved in mobile phase. The percentage extraction is found to be more than 79% and the linearity range from 0.5ng to 50.0ng ml_{-1}. The method has been validated extensively and found to have good interday and intraday precision. This method has been applied to study pharmacokinetics in 12 healthy human volunteers [8].

2.6 Jingqi Bao and Brian D. Potts have developed a sensitive assay method for the measurement of Olanzapine in rat brain tissue using HPLC with electrochemical detection. The assay has a lower limit of quantitation of 0.5 ng/ml in tissue homogenate and utilizes a liquid–liquid extraction followed by reversed-phase HPLC for the quantitative analysis of Olanzapine. The method provided a linear response for Olanzapine over a concentration range of 0.5–100 ng/ml with a coefficient of determination (r^2) greater than 0.9995. The extraction efficiencies of Olanzapine and internal standard (LY170158) were greater than 82% in brain tissue. The intra-assay and inter-assay relative errors ranged from −5.38 to 17.60% and −3.25 to 10.53%, respectively. The intra-assay and inter-assay RSD values were in the range of 1.12 to 6.96% and 3.78 to 6.68%. Long-term stability studies showed that brain tissue homogenate samples spiked with Olanzapine and internal standard are stable at −70°C for at least 110 days. However, a room temperature stability study showed that Olanzapine was not stable in brain homogenate if the sample was exposed at 25°C longer than 2 h. This method has been used for the study of the disposition and pharmacokinetics of Olanzapine in male Sprague Dawley rats [9].

2.7 Ramisetti Nageswara Rao and co-workers have developed an RP-HPLC method for the simultaneous separation and determination of olanzapine (OLZ) and its process impurities in bulk drugs and pharmaceutical formulations. The separation was accomplished on Inertsil ODS 3V (4.6 mm×250 mm; particle size 5 μm) column using 0.2 M ammonium acetate (pH = 4.50) and ACN as mobile phase in gradient elution mode. The analytes were monitored by a photo diode array (PDA) detector set at 254 nm and the flow rate was kept at 1.0 mL/min. The chromatographic behavior of all the compounds was examined under variable compositions of different solvents,

buffer concentrations, and pH. The method was validated in terms of accuracy, precision, and linearity. Four unknown process impurities observed consistently during the analysis of different batches of OLZ were isolated and characterized by ESI-MS/MS, ^{1}H NMR, and FT-IR. The proposed RP-HPLC method was successfully applied to the analysis of commercial formulations. The method could be of use not only for rapid and routine evaluation of the quality of OLZ in bulk drug manufacturing units but also for the detection of its impurities in pharmaceutical formulations [10].

2.8 E. J. Harvey, R. J. Flanagan, and D. M. Taylor worked on stability of a suspension of olanzapine in Guy's hospital paediatric formula base for oral administration in hospitals for that they used an HPLC method based on that developed for the measurement of clozapine in Guy's hospital paediatric base.3 A 150 x 4.6mm internal diameter stainless steel column packed with Spherisorb S5 SCX (Phase Separations) was used with methanolic ammonium perchlorate (35mmol/L, pH 6.7) at a flow-rate of 1.5ml/min as eluent. Sample injection was performed using a Perkin-Elmer ISS-101 autosampler and detection was by UV absorption (260nm). Peak heights were recorded using a Hewlett-Packard 3392A integrator. A methanolic stock solution of olanzapine pure compound (LY170053; Eli Lilly, Indianapolis, United States) (50mg olanzapine/50ml, ie, 1g/L) was prepared and stored at 2–8C. Portions of this solution were diluted in methanol:deionised water (93.5+6.5) to give calibration solutions containing 5mg, 10mg and 15mg olanzapine per litre. Portions (100µl) of each of the calibration standards were added to portions (100µl) of internal standard solution (10mg/L methanolic LY170222 [Eli Lilly])4 in autoinjector vials and vortex-mixed for 10 seconds. These solutions (20µl portions) were analysed in duplicate by HPLC as described above. The retention times of the internal standard and of olanzapine were approximately 10.0 min and 11.5 min, respectively. The olanzapine calibration solutions were freshly prepared by dilution from the stock solution on each analysis day [11].

2.9 Chiu J A and Franklin R B have developed a sensitive HPLC assay for measurement of the antipsychotic drug, olanzapine, in plasma. The assay has a limit of quantitation of 1 ng ml^{-1} in plasma and utilizes solid-phase extraction and electrochemical detection. The method provides a linear response for olanzapine over

a concentration range of 1-100 ng ml^{-1} with coefficients of determination greater than 0.9912. The inter-assay precision was 15.9% at the limit of detection and ranged from 7.33% to 8.47% over the range of 5-100 ng ml^{-1}. The intra-assay precision was in the range 0.97%-26.0%. The inter-assay accuracy ranged from 98.9 to 118% and the intra-assay accuracy ranged from 92.5% to 125% of the theoretical value. In addition, the assay was extended to measure the plasma levels of two metabolites of olanzapine, namely the N-desmethyl- and the 2-hydroxymethyl analogs. The utility of the assay was demonstrated following the administration of a single oral dose of 14C-olanzapine to rats where, at several time-points after dosing, the plasma was assayed for total radioactivity, levels of olanzapine, and the two metabolites. Olanzapine and two of its metabolites accounted for less than 50% of the total plasma radiocarbon; olanzapine accounting for approximately 39% at the Cmax, N-desmethyl for 5% and 2-hydroxymethyl for 8% respectively. The plasma elimination half-times for olanzapine and the two metabolites were approximately the same, ranging from 3.3 to 4.4 h [12].

2.10 C. R. Shah, B.N. Suhagia, N.J. Shah, D.R. Patel, N.M. Patel have developed a rapid, selective and stability-indicating high performance thin layer chromatographic method and validated for the simultaneous estimation of olanzapine and fluoxetine in combined tablet dosage form. Olanzapine and fluoxetine were chromatographed on silica gel 60 F_{254} TLC plate using methanol:toluene (4:2 v/v) as the mobile phase and spectrodensitometric scanning-integration was performed at a wavelength of 233 nm using a Camag TLC Scanner III. This system was found to give compact spots for both olanzapine (R_f value of 0.63±0.01) and fluoxetine (R_f value of 0.31±0.01). The polynomial regression data for the calibration plots showed good linear relationship with r^2 =0.9995 in the concentration range of 100-800 ng/spot for olanzapine and 1000-8000 ng/spot for fluoxetine with r^2 =0.9991. The method was validated in terms of linearity, accuracy, precision, recovery and specificity. The limit of detection and the limit of quantification for the olanzapine were found to be 30 and 100 ng/spot, respectively and for fluoxetine 300 and 1000 ng/spot, respectively. Olanzapine and fluoxetine were degraded under acidic, basic and oxidation degradation conditions which showed all the peaks of degraded product were well resolved from the active pharmaceutical ingredient. Both drugs were not further degraded after thermal and

photochemical degradation. The method was found to be reproducible and selective for the simultaneous estimation of olanzapine and fluoxetine. As the method could effectively separate the drugs from their degradation products, it can be employed as a stability-indicating method [13].

2.11 Poornachander Thatipalli, Ramesh Kumar, Chandrasekhar Bulusu, Ramesh Chakka, Pratap R. Padi, Anjaneyulu Yerra, and Satyanarayana Bollikonda reported synthesis and characterization of impurities of an anti-psychotic drug substance, Olanzapine HPLC analysis of olanzapine, a known anti-psychotic drug, showed impurity peaks ranging from 0.05 to 0.15 % during process development. These samples were analyzed by LCMS and the peaks were identified at m/z 230, 341, 511, 326, 361 and 329. All six impurities were synthesized individually and characterized based on their spectral data (IR, NMR and Mass). The structure of these impurities were assigned as 2-methyl-4,9-dihydro-3-thia-4,9- diazabenzo [f]azulen-10-one 4,1-[4-(2-methyl-4H-3-thia-4,9-diazabenzo[f]azulen-10-yl) piperazin-1-yl] ethanone 5,bis-[10-(2-methyl-4H-3-thia-4,9-diazabenzo[f]azulene)]-1,4- piperazine 6, 2,4-dimethyl-10-(4-methylpiperazin-1-yl)-4H-3-thia-4,9-diazabenzo[f]azulene 7, 10-(4-chloromethyl-4-methylpiperazin-1-ylium)-2-methyl-4H-3-thia-4,9-diazabenzo[f] azulene chloride 8 and 2-methyl-10-(4-methyl-N-oxopiperazin-1-yl)-4H-3-thia-4,9-diazabenzo[f]azulene 9 respectively. The formation, synthesis and characterisation of the olanzapine impurities are discussed [14].

2.12 R. Ravichandran briefing in pharmacopeial forum about method for Olanzapine drug substance using Zorbax RX-C8 or Zorbax SB-C8 brand of L7 column manufactured by Agilent Technologies, RT of Olanzapine in Related substances is 13 minute in gradient elution while 6.7 minute in Assay test for isocratic mode [15].

3. Aim of present study

Literature reveals that the Olanzapine OD tablet is official in any pharmacopeia [16-21]. Many methods for Olanzapine assay for drug substance and formulation as well as related substances for drug substances were reported. Few methods for Olanzapine in biological fluids also reported [4-15]. But none of method was reported for determination of related substances in Olanzapine OD tablets. The present communication describes

method for determination of related impurities for Olanzapine OD tablets with established relative response factor (RRF) and relative retention time (RRT) for known impurities. Simple and precise analytical method for determination of related impurities for Olanzapine OD tablets was developed and validated as per ICH guideline [22]. Hence, this HPLC method could be of use for formulation development and stability testing as well as at quality control laboratory for routine use.

The aim and scope of the proposed work are as under

- LC Method development for determination of related impurities in Olanzapine OD tablets formulation
- Forced degradation study under stress condition
- To resolve all known impurities and generated during the force degradation studies
- Perform analytical method validation for the proposed method as per ICH guideline.

4. Experimental

4.1 Materials, chemicals and reagents

Materials, chemicals and reagents were used as mentioned in Table 1 and Table 2

Table 1: Materials

S. No	Material Name	Lot. No./ Batch no.	Mfg. by	Potency/ Purity
1	Olanzapine working standard	OTV/2007/543	TRC, Bhat	99.72%
2	Olanzapine orodispersible tablets 20mg	BA 527001	TPL, Chattral	--
3	Placebo of olanzapine orodispersible Tablets 20mg	P/20/050	TRC, Bhat	--
4	2-methyl -4-(methyl-4-oxide-1-piperazinyl)10H-thieno-[2,3-b] [1,5]-benzodiazepine (Impurity 1)	OTV/2007/1491	TRC, Bhat	99.37%
5	2-methyl-4 oxo-10 H-thieno-[2,3-b] [1,5]-benzodiazepine (Impurity 2)	OLA IMP/XIX/010	TRC, Bhat	88.75%
6	TBD (4-Amino-2-methyl-10H-thieno[2,3-b][15]benzodiazepine HCl)	1311ST03-131700104	TEVA	99.7%

Table 2: Chemicals and reagents

S. No.	Chemical/Reagent	Grade	S. No.	Chemical/Reagent	Grade
1	Acetonitrile	HPLC	5	Sodium hydroxide	AR
2	Sodium dihydrogen ortho phosphate dehydrate	AR	6	Hydrogen peroxide	AR
3	Water	Milli-Q	7	Glacial acetic acid	GR
4	Hydrochloric Acid	AR	8	Methanol	HPLC

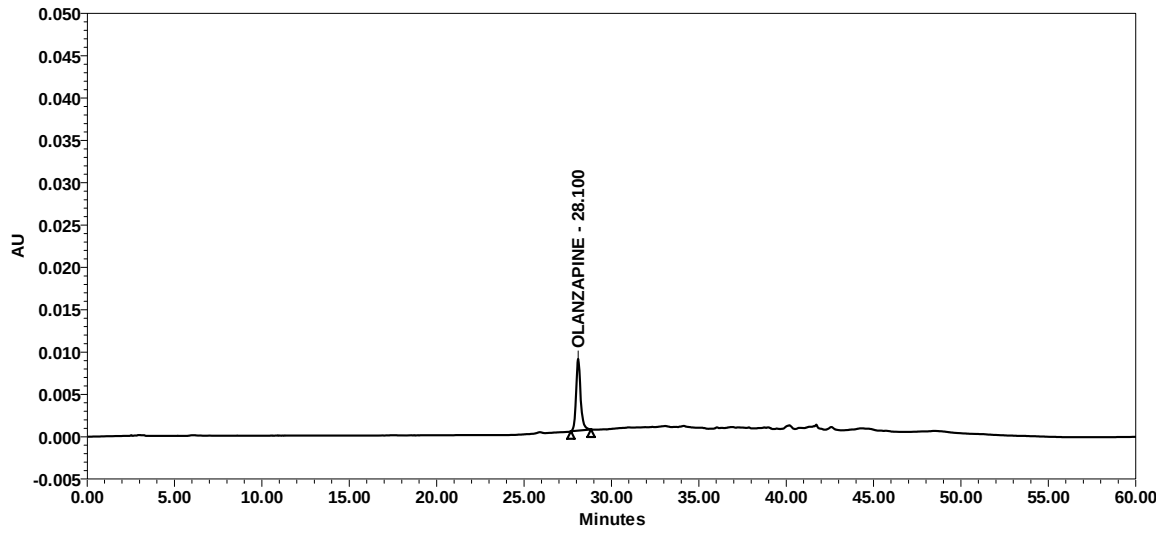
4.2 Instrumentation

The HPLC system of Waters separations module 2695, PDA detector 2996, UV detector 2487 with Empower pro- empower 2 software and Shimadzu LC-2010C HT with LC solution version 1.22 SP1 were used for this entire study and chromatographic separation was achieved on YMC-Pack ODS-A-303 (250 mmX4.6 mm; 5µm) column as stationary phase with binary gradient mode.

4.3 Diluted standard solution:

Accurately weighed and transferred 25 mg of olanzapine reference/working standard in 100ml volumetric flask, 70ml of diluent was added to dissolve with aid of sonication and diluted to volume with diluent. 5ml of this solution was diluted to 50ml with diluent. Further 5ml of this solution was diluted to 50ml with diluent (2.5µg/ml of olanzapine).Fig.2

Fig.2: Chromatogram of diluted standard preparation



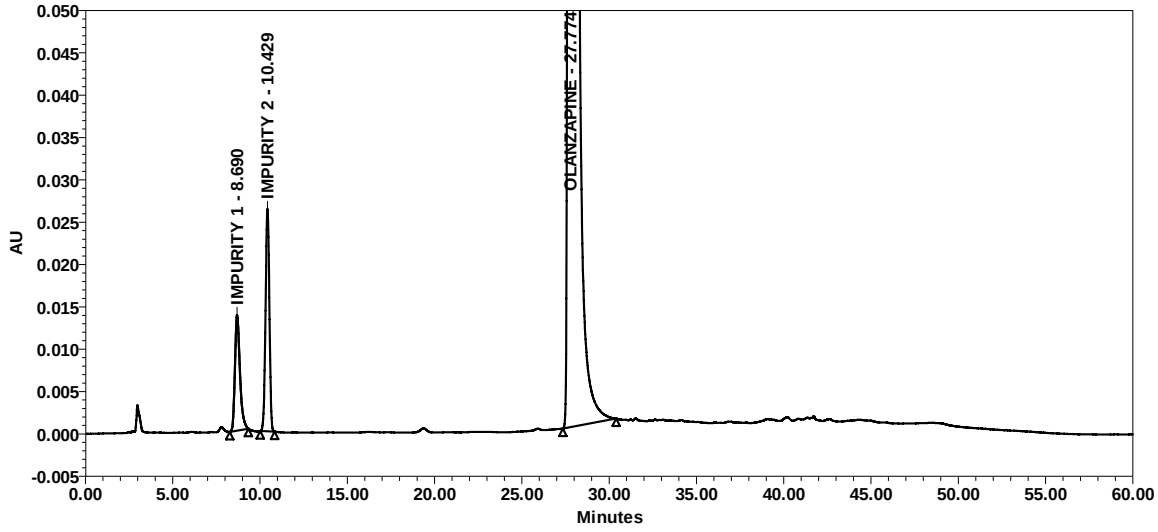
4.4 Impurity-1 stock solution:

Accurately weighed and transferred 5 mg of olanzapine imp-1 standard in 100ml volumetric flask, 70ml of diluent was added to dissolve with aid of sonication and diluted to volume with diluent (50 µg/ml of olanzapine imp-1).

4.5 Resolution solution

Accurately weighed and transferred 25 mg of olanzapine reference/working standard in 50 ml volumetric flask, 30 ml of diluent was added to dissolve with aid of sonication, 5ml of Impurity 1 Stock solution was added and diluted to volume with diluent (500 µg/ml of Olanzapine and 5 µg/ml of olanzapine imp-1) chromatogram shown in fig 3.

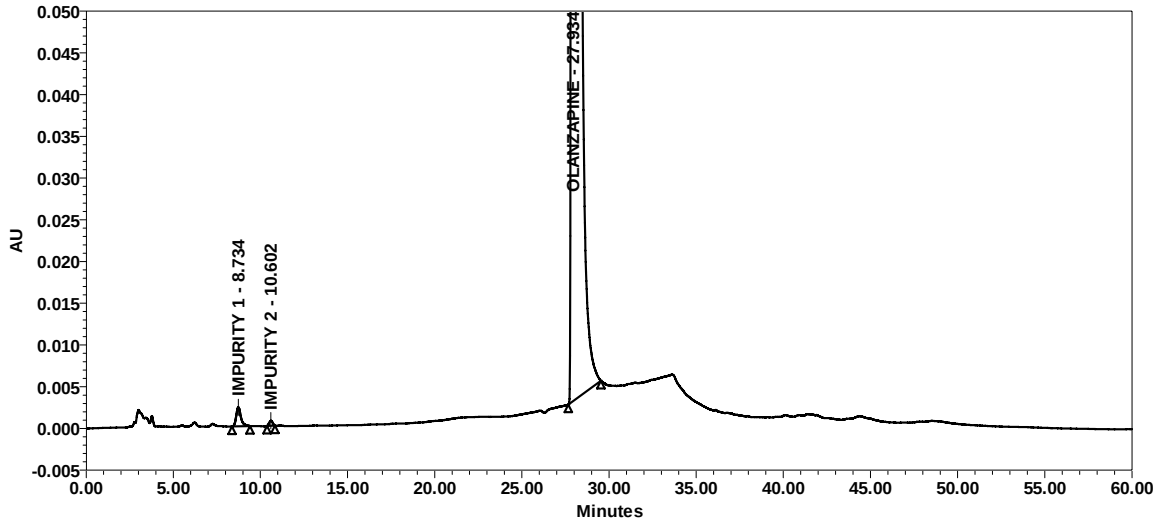
Fig.3: Chromatogram of resolution solution



4.6 Sample solution

Thirty tablets were crushed to fine powder by mortar and pestle. Sample powder equivalent to about 50mg of olanzapine was weighed and transferred to 100 ml volumetric flask, 70ml of diluent was added and sonicated for 30 minute with intermittent swirling, diluted to volume with diluent (500µg/ml of olanzapine) and filtered with 0.45 µm nylon membrane filter. As such sample preparation as per fig 4.

Fig.4: Chromatogram of sample solution (as such preparation)



5. Result and discussion

5.1 Development and impurity identification

Method development was started with olanzapine and olanzapine impurity 1 (Fig 5) but during the coarse of product stability study an unknown impurity was found more than 0.1 % so, as per ICH guideline it needs to identify. It was an unknown but specified unknown by it's RRT in terms of chromatography it was consistently observed at different station of drug product stability in increasing order.

In order to identify unknown specified impurity, primary forced degradation data was focused whether any impurity in forced degradation is forming at the same RRT or not? And answer was yes it was observed that an unknown impurity at same RRT was present in acid as well as peroxide degradation study. Acid degradation was preferred for impurity isolation to identify it. As reaction enriched to about 30% of impurity of interest it was transferred to preparative scale to get pure impurity. Finally it was made as known as impurity 2 (Fig 6) with all required structural confirmation like Mass, NMR, IR and CHNS analysis.

5.2 Separation for known impurities

Impurity stock solution of Olanzapine and its impurity 1 and impurity 2 was used for method development parameters like column selection and gradient optimization (30µg/ml of olanzapine and its related impurity 1 and impurity 2). Once desired

separation for known impurities and main component was achieved forced degradation was performed. Finally proposed method with chromatographic condition as per table 3 was subjected to method validation as per ICH guideline with consideration of sample concentration to achieve LOQ below the reporting threshold of impurities [23].

Table 3: Chromatographic condition

Buffer	Buffer, 6.24 gm sodium dihydrogen orthophosphate dehydrate in 800 ml of water, pH was adjusted to 6.8 with dilute sodium hydroxide solution, filtered through 0.45µm membrane		
Mobile phase-A	Buffer : Acetonitrile: Methanol, 500:200:300 (v/v/v)		
Mobile phase-B	Water : Acetonitrile, 20:80 (v/v)		
Diluent	Water : Acetonitrile, 20:80 (v/v)		
Column oven temp.	27°C		
Detection	UV at 250 nm		
Injection volume	20 µl		
Gradient programme	**Time (min)**	**Mobile phase-A (%)**	**Mobile phase-B (%)**
	0.01	100	0
	20	100	0
	25	70	30
	30	40	60
	35	20	80
	40	10	90
	45	10	90
	50	40	60
	55	100	0
	60	100	0

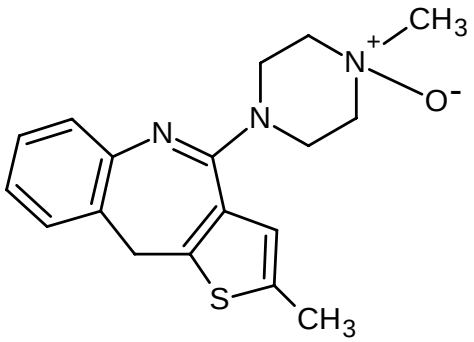


Fig. 5: Structure of olanzapine Impurity-1

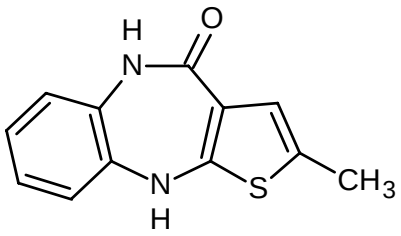


Fig. 6: Structure of olanzapine Impurity-2

5.3 Forced degradation study

Forced degradation was performed on olanzapine OD tablets to achieve desired degradation and placebo as well as olanzapine drug substance were treated with similar conditions as mentioned below, based on development trials optimized forced degradation conditions were established. Final sample concentration was achieved 5400µg/ml of olanzapine with diluent as proposed sample concentration.

5.3.1 Oxidative degradation

Olanzapine OD tablets powder equivalent to 50mg of 0lanzapine was subjected to oxidative stress condition by 5ml of 3% v/v H_2O_2 solution in 100ml volumetric flask and kept at room temperature for 30 min and sample solution was prepared as per procedure.

5.3.2 Acidic degradation

Olanzapine OD tablets powder equivalent to 50mg of Olanzapine was subjected to acidic stress condition by 5ml of 1N HCl solution in 100ml volumetric flask and heated on water bath at 60°C for 60 min and sample was neutralised with 1N NaOH and sample solution was prepared as per procedure.

5.3.3 Base degradation

Olanzapine OD tablets powder equivalent to 50mg of Olanzapine was subjected to alkaline stress condition by 5ml of 1N NaOH solution in 100ml volumetric flask and heated on water bath at 60°C for 60 min and sample was neutralized with 1N HCl and sample solution was prepared as per procedure.

5.3.4 Thermal degradation

Olanzapine OD tablets were kept in temperature controlled oven at 105 °C for 72 hours and sample solution was prepared as per procedure.

5.3.5 Photolytic degradation

Olanzapine OD tablets were kept in photo stability chamber to expose it for 1.2 million lux hours and sample solution was prepared as per procedure.

5.4 Method validation

Method validation approach:

The method for the determination of related impurities for olanzapine OD tablets of 2.5mg, 5mg, 7.5mg, 10mg, 15mg, and 20 mg is same. Sample concentration for all the strengths is same (500 µg/ml of olanzapine). Olanzapine OD tablets 2.5mg, 5mg , 7.5mg,10mg ,15mg , 20mg are weight proportional formulations (scale up-down) so complete validation performed on highest strength (20 mg) only. Linearity was performed by 5 levels of concentration. Recovery was performed by 3 replicates x 3 concentration. Filter media interference study was covered in accuracy study.

5.5.1Specificity

Interference from blank was not observed at any peak of interest and peak purity of all known impurities in spiked sample (as per method precision) observed within the acceptance criteria and forced degradation data as discussed in section 5.3 summarized in table 5 and chromatograms are as shown in fig.7,(A) to 7(O).

Table 5: Forced degradation data

Stress condition	Oxidative degradation	Acid degradation	Base degradation	Thermal degradation	Photolytic degradation
% of degradation	21.6	29.8	8.0	0.2	0.1

Fig.7 (A): Peroxide degradation blank

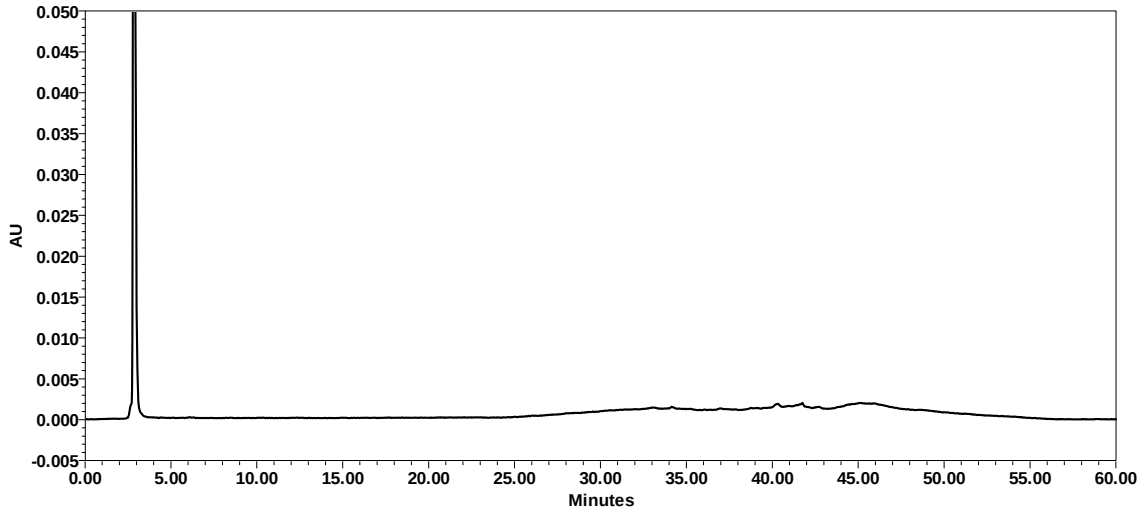


Fig.7 (B): Peroxide degradation placebo

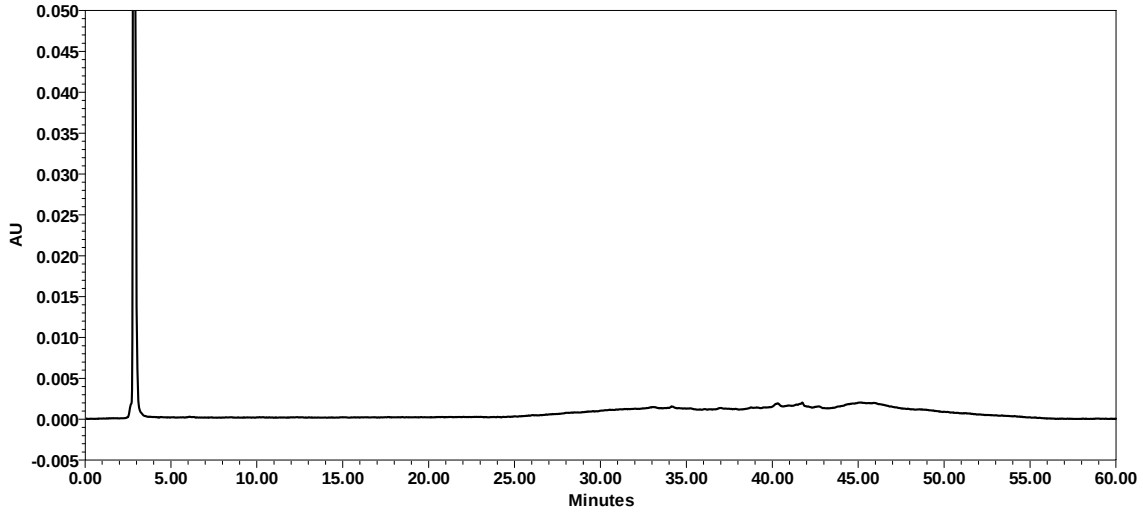


Fig.7 (C): Peroxide degradation sample

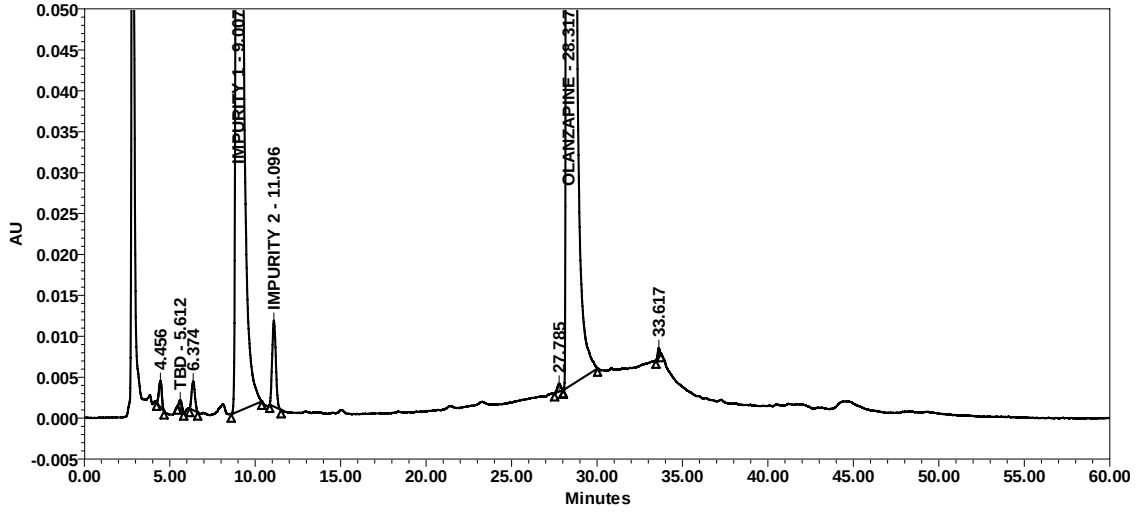


Fig.7 (D): Acid degradation blank

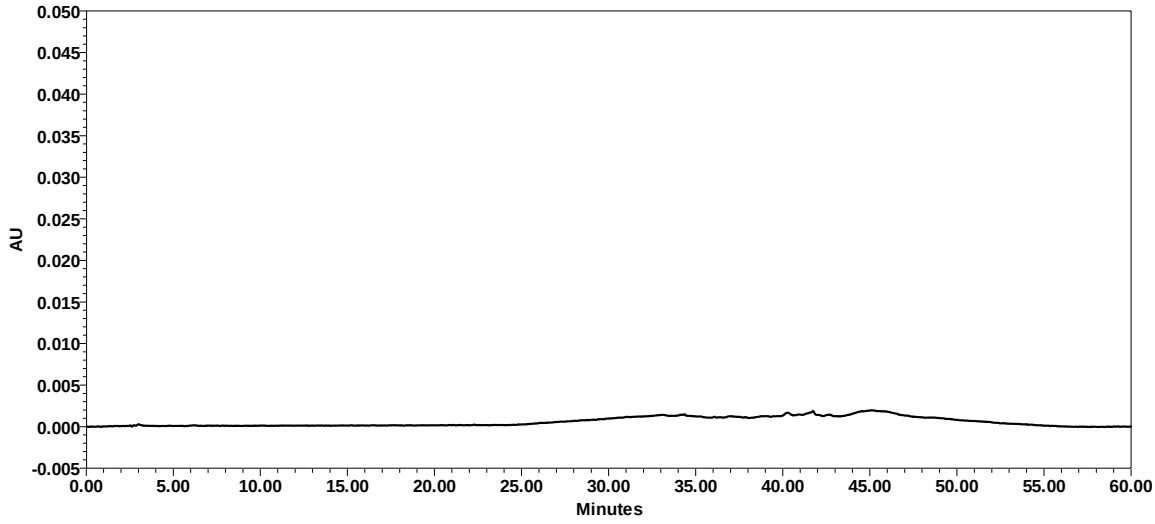


Fig.7 (E): Acid degradation placebo

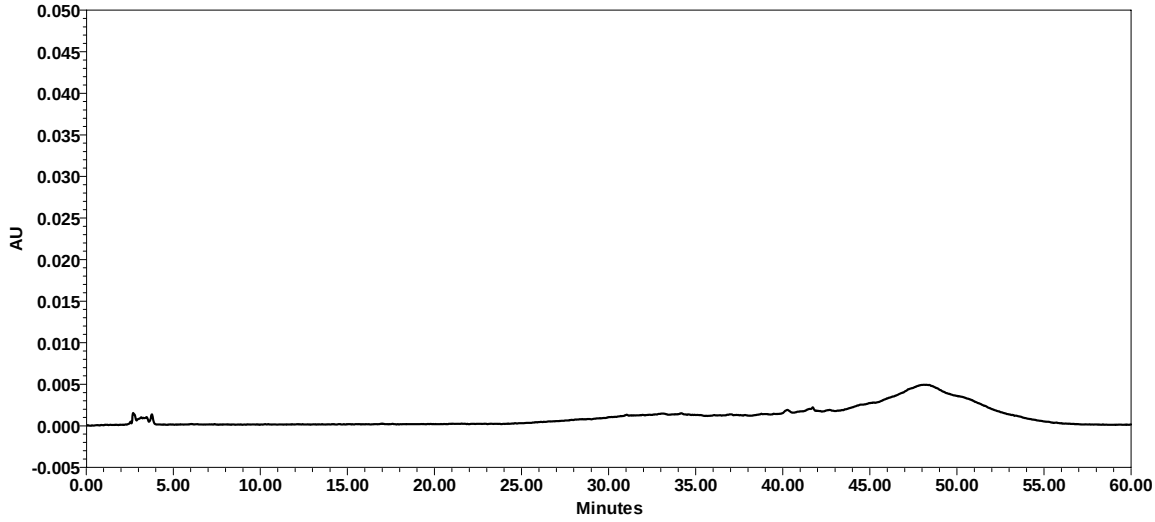


Fig.7 (F): Acid degradation sample

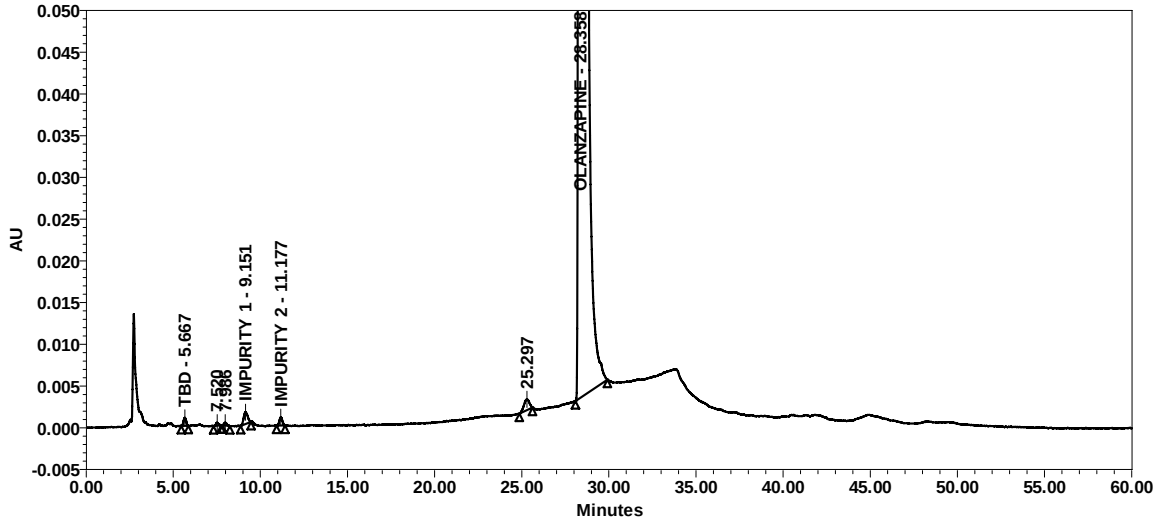


Fig.7 (G): Base degradation blank

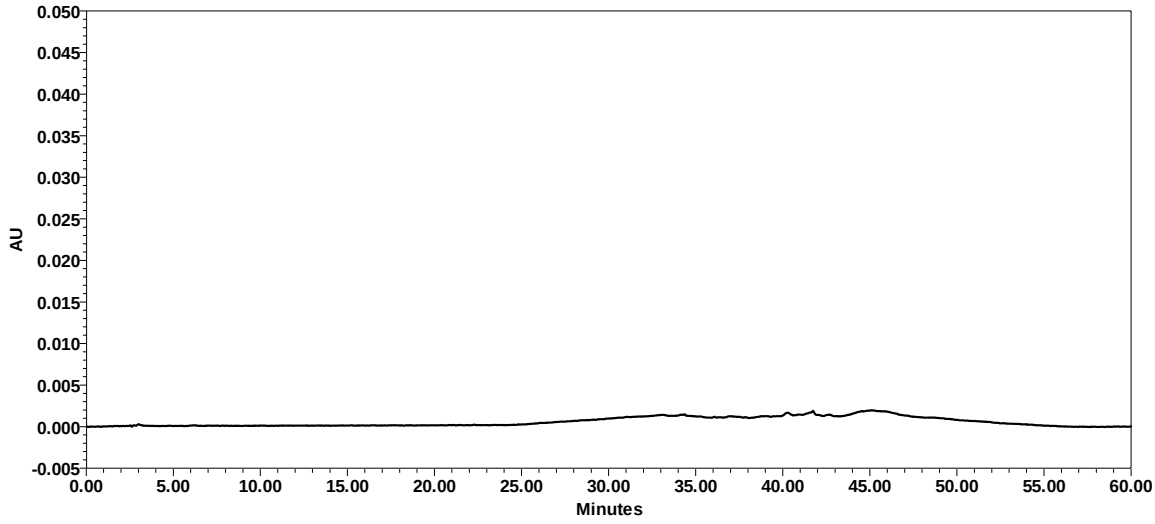


Fig.7 (H): Base degradation placebo

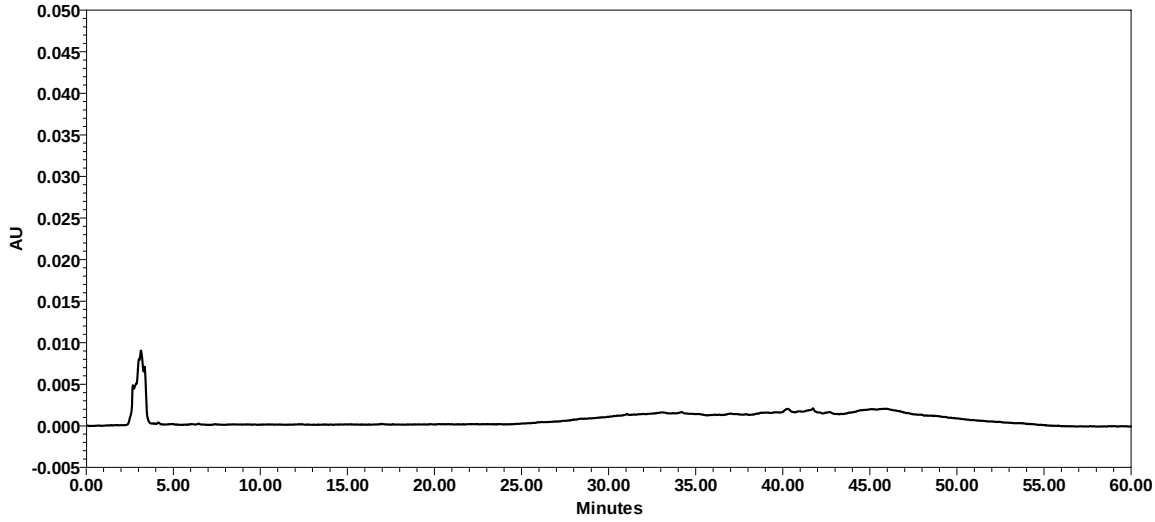


Fig.7 (I): Base degradation sample

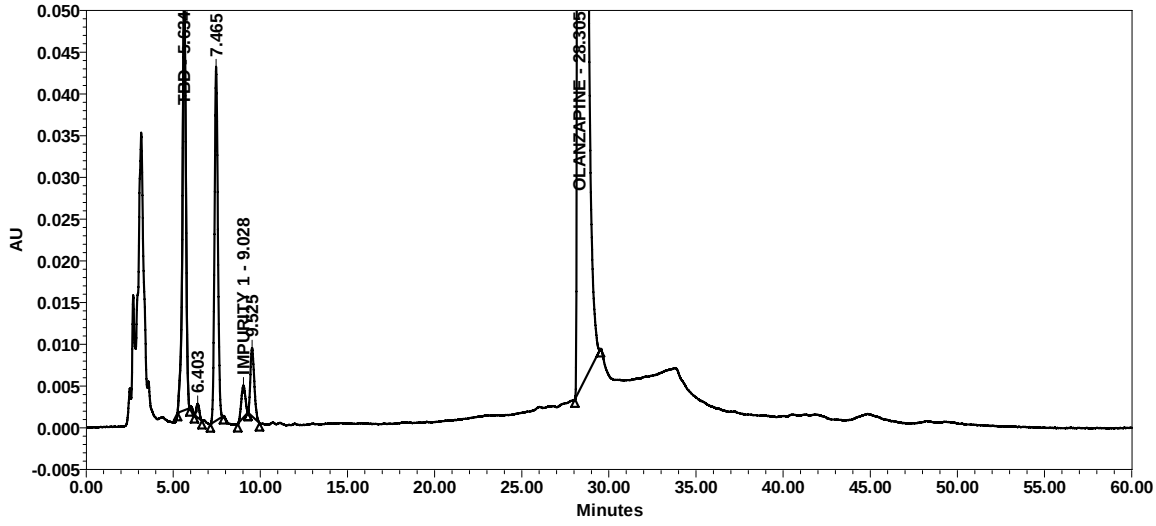


Fig.7 (J): Thermal degradation placebo

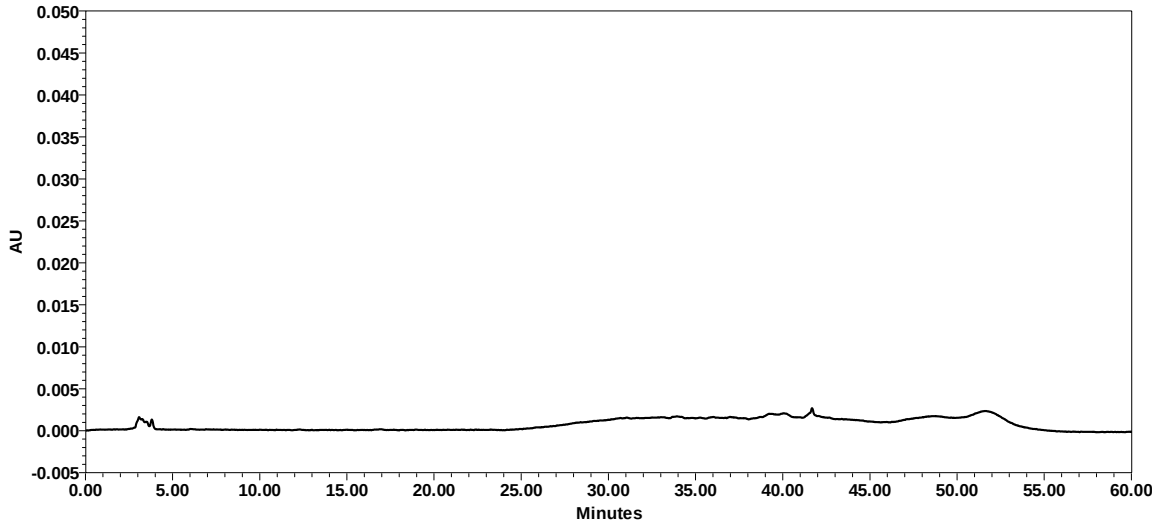


Fig.7 (K): Thermal degradation sample

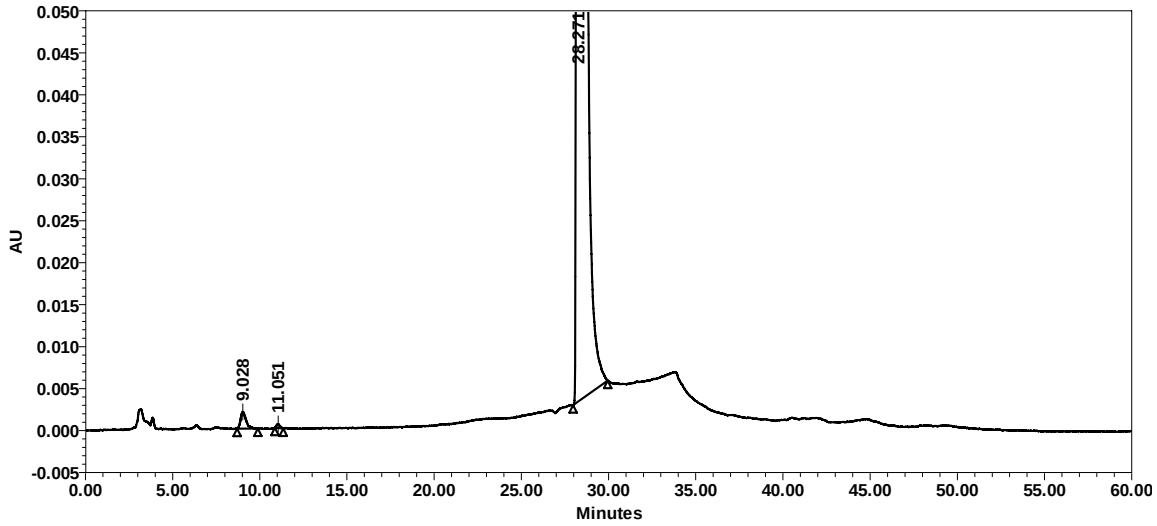


Fig.7 (L): Photo degradation sample

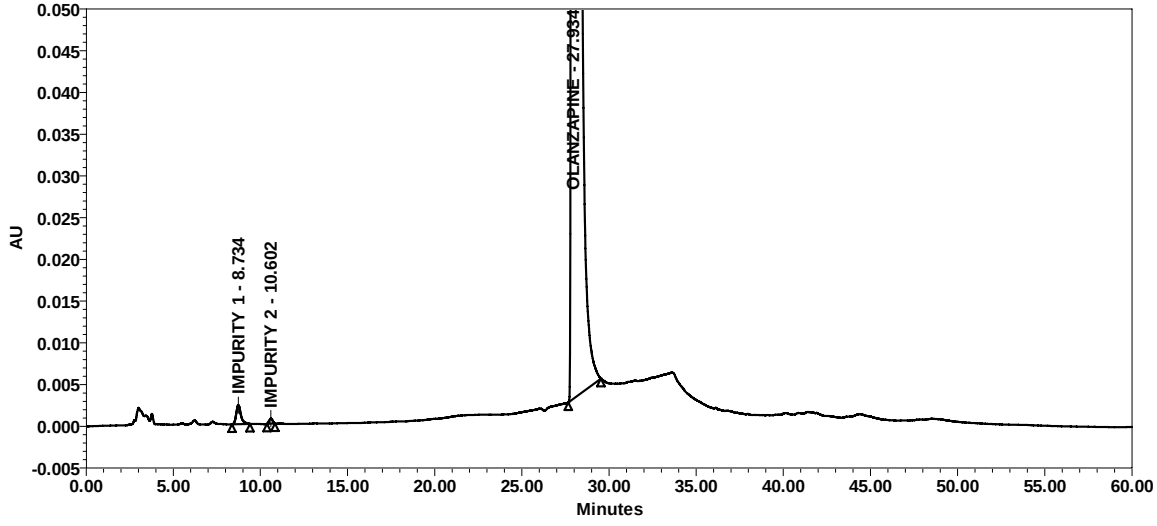


Fig.7 (M): Olanzapine impurity-1

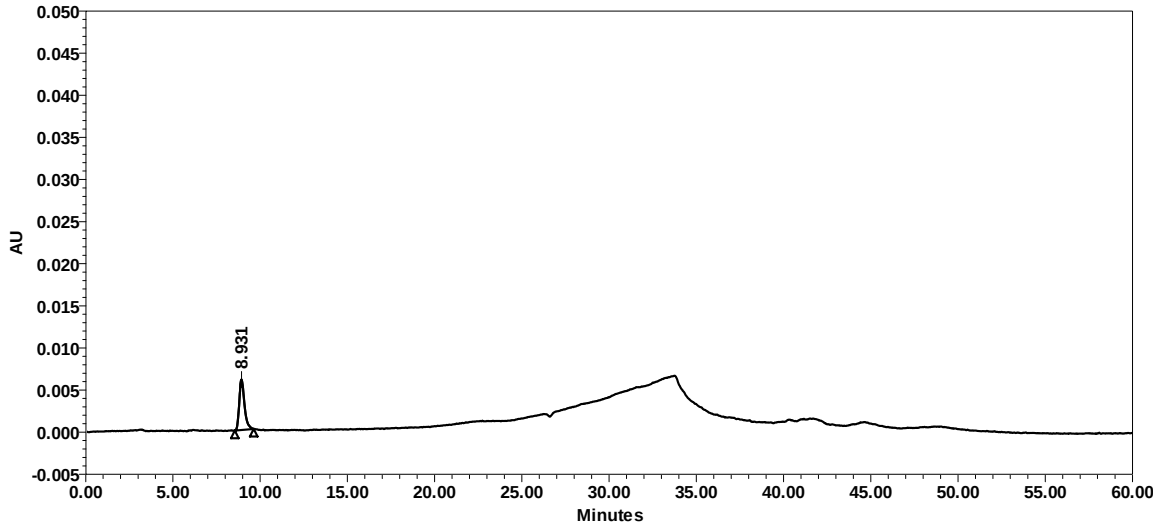


Fig.7 (N): Olanzapine impurity-2

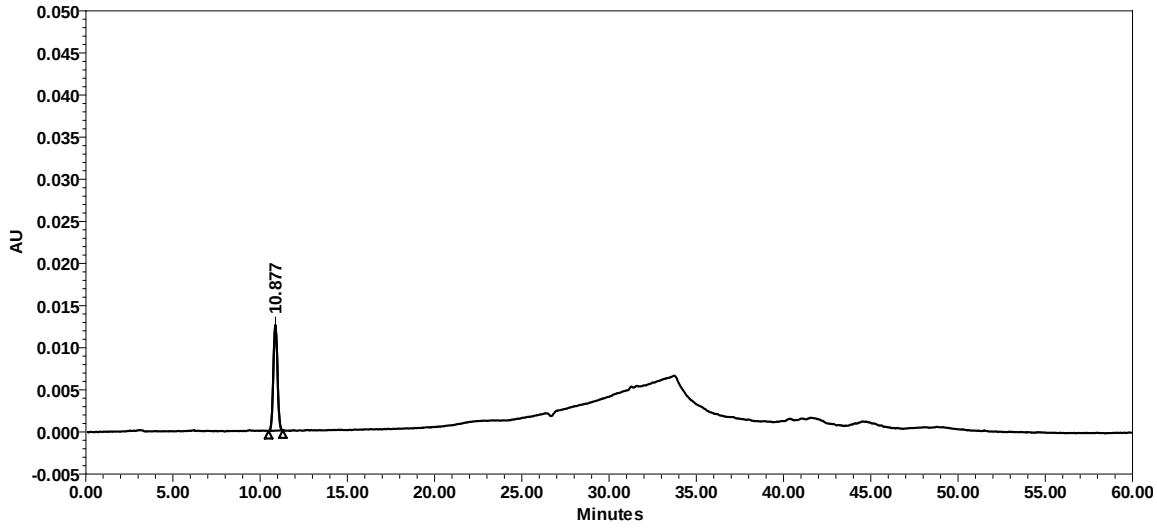
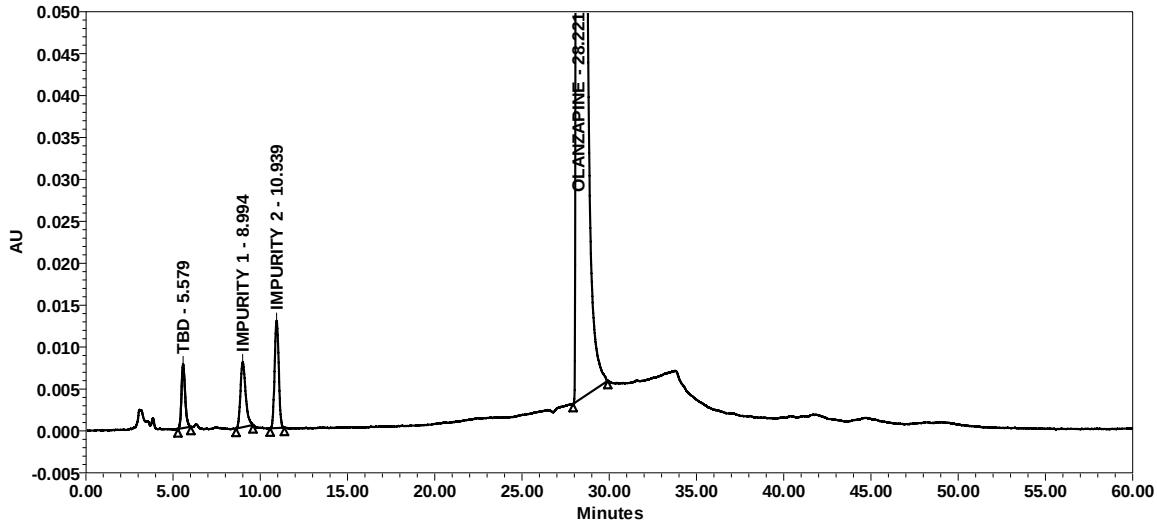


Fig.7 (O): Sample spiked with impurities



5.5.2 Linearity

Linearity and relative response factor determination

The calibration curve for Olanzapine and its impurities were constructed over the absolute concentration range of 0.25-3.75 µg/ml i.e. 0.05% to 0.75% concentration with respect to sample solution concentration in five levels. Peak area of each impurities and Olanzapine versus respective concentration was plotted and linear regression analysis performed on resultant curves. Relative response factor (RRF) was calculated for each known impurities by taking the ratio of impurity slope to Olanzapine slope [24]. Statistical data summarized in table 6 and chromatograms mentioned in Fig.8 (A) to (E).

Table 6: Linearity and RRF

Sr. No.	% Linearity level	Impurity 1		Impurity 2		Olanzapine	
		Conc. (%)	Average area	Conc. (%)	Average area	Conc. (%)	Average area
1.	LOQ	0.05	9990	0.05	21030	0.05	11038
2.	50%	0.26	60541	0.24	107234	0.10	29453
3.	80%	0.42	94993	0.39	175318	0.16	45530
4.	100%	0.53	118357	0.48	214724	0.20	59325
5.	150%	0.79	177964	0.72	316143	0.30	88925
Correlation coefficient (r)		0.99979		0.99980		0.99916	

Slope of regression line	225407	441128	308368
Y-intercept	-47.82	1025.8	-3101.4
Residual sum of squares	6717283	19451918	5887483
RRF	0.72	1.43	---

RRT = Relative retention time

RRF = Relative response factor

Fig.8 (A): Linearity level-1

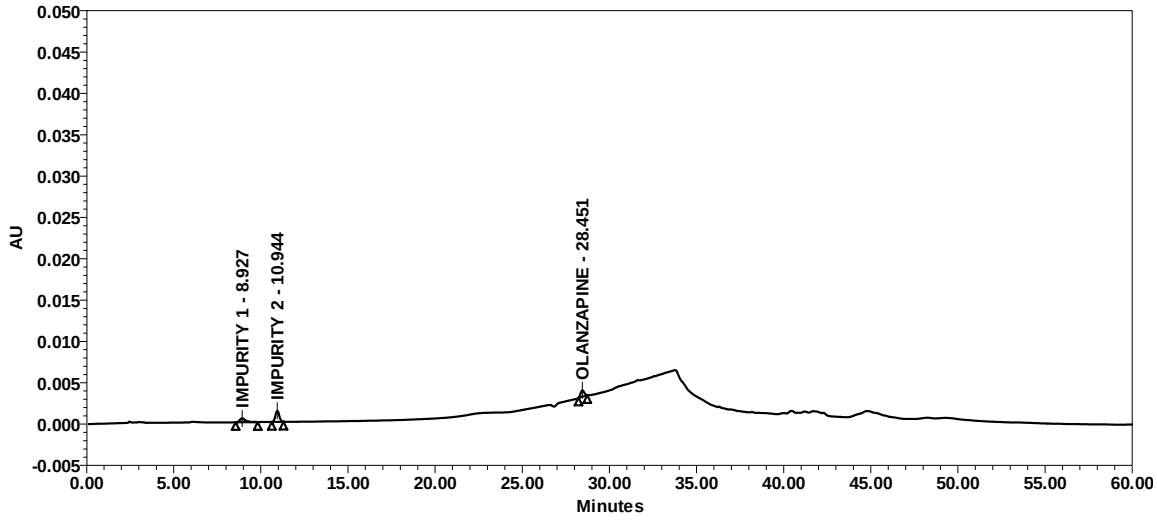


Fig.8 (B): Linearity level-2

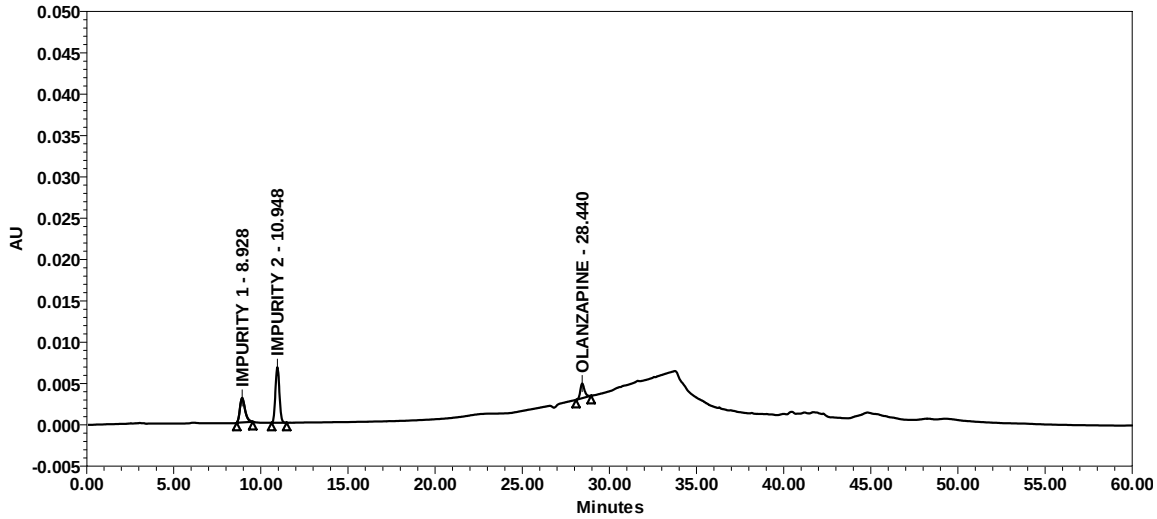


Fig.8 (C): Linearity level-3

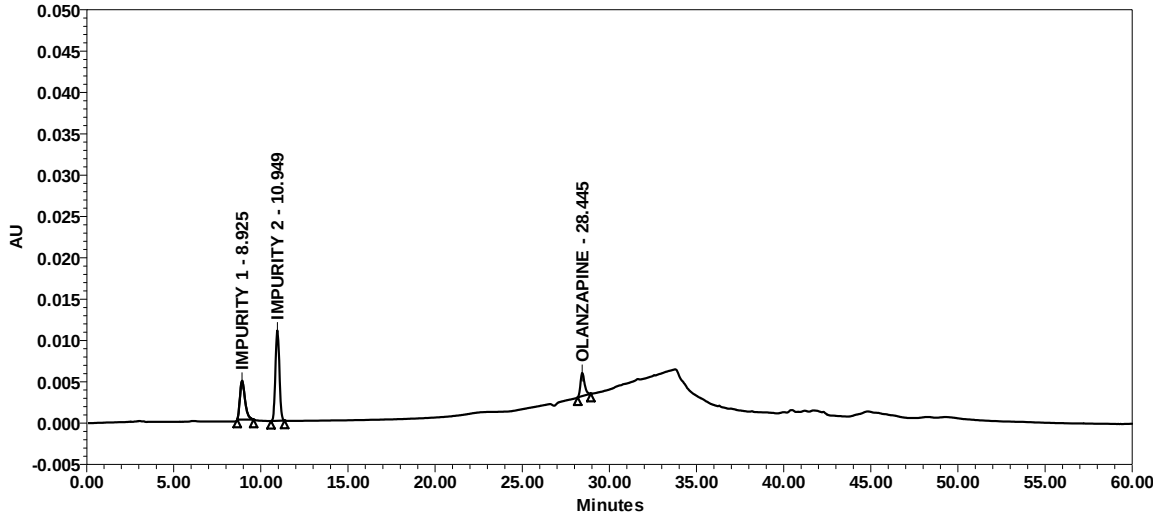


Fig.8 (D): Linearity level-4

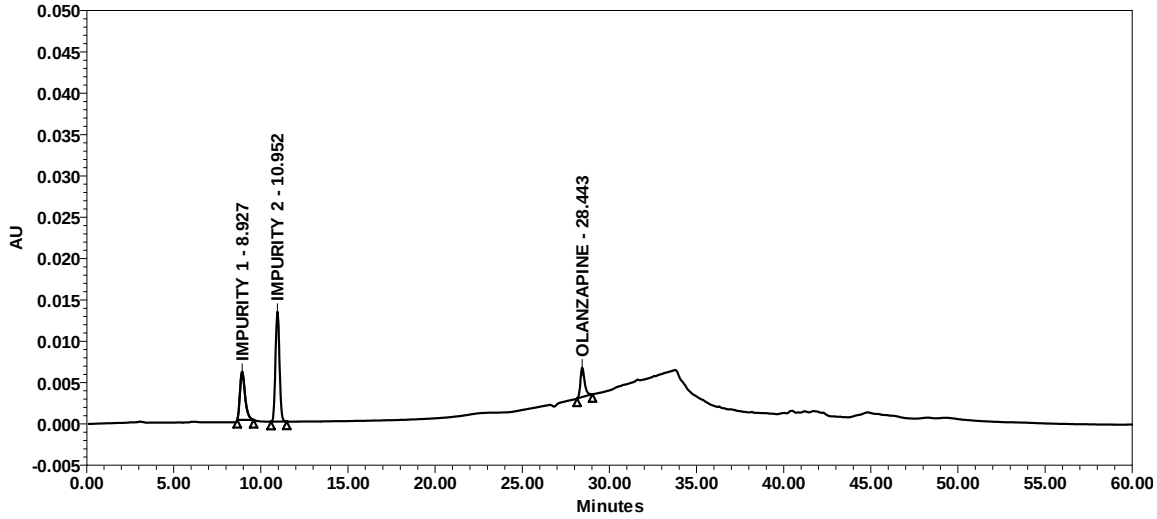
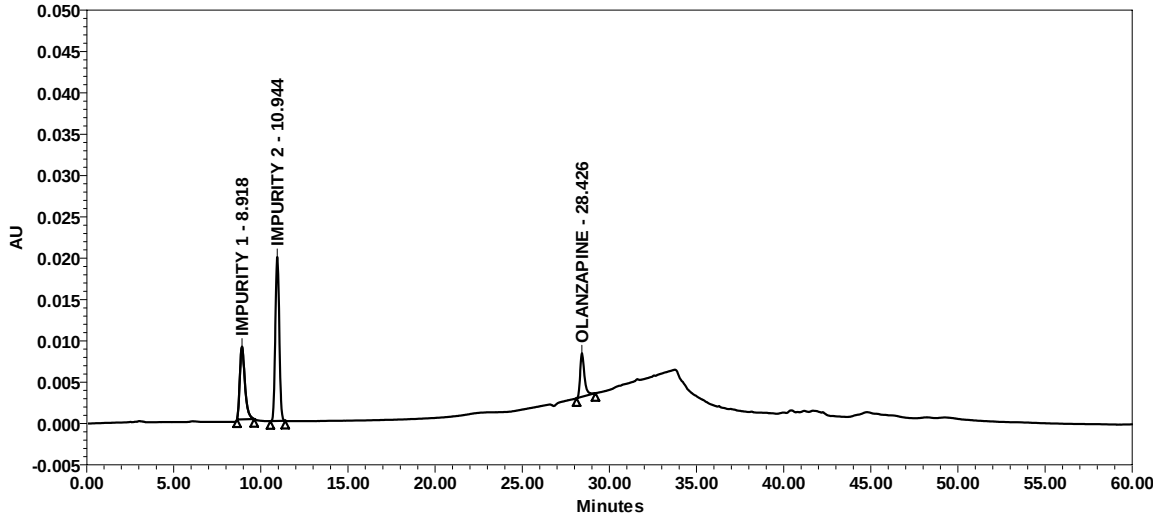


Fig.8 (E): Linearity level-5



5.5.3 Precision at LOQ

The LOQ concentrations for all known impurities were determined 0.25 µg/ml as 0.05 % with respect to sample solution concentration of Olanzapine by signal to noise ratio methodology . The reproducibility at LOQ was determined by six replicate injections of composite mixture of impurities and olanzapine solution as per table 7 and Fig.9.

Fig. 9: Chromatogram of precision at LOQ

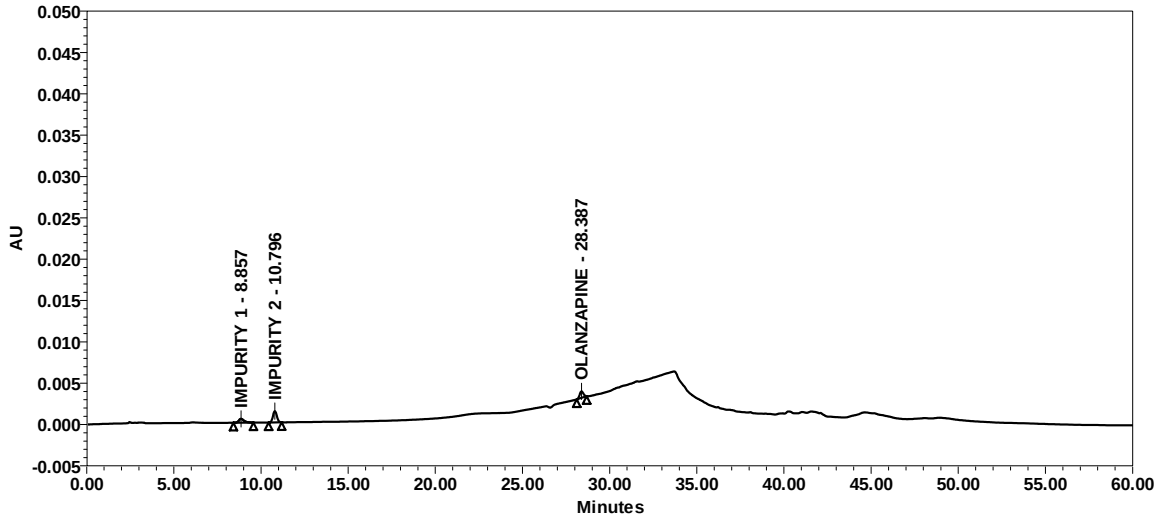


Table 7: Precision at LOQ

Peak Area			
Injection	**Impurity-1**	**Impurity-2**	**Olanzapine**
1	9467	21057	11392
2	9685	21043	11397
3	9646	21152	11873
4	9449	21023	11942
5	9612	21236	11757
6	9542	21056	11711
MEAN	9567	21095	11679
SD	96.7	82.4	234.9

RSD	1.01	0.39	2.01

5.5.4 Precision

Method precision was established by six sets of sample preparation. To demonstrate the method precision olanzapine impurity 2 was spiked at self life specification level to all six sample preparation while olanzapine impurity 1 was above the LOQ so, it was not spiked in the sample preparations as shown in fig 10. Data are summarized in table 8.

Table 8: Summary of precision day-1 and day-2

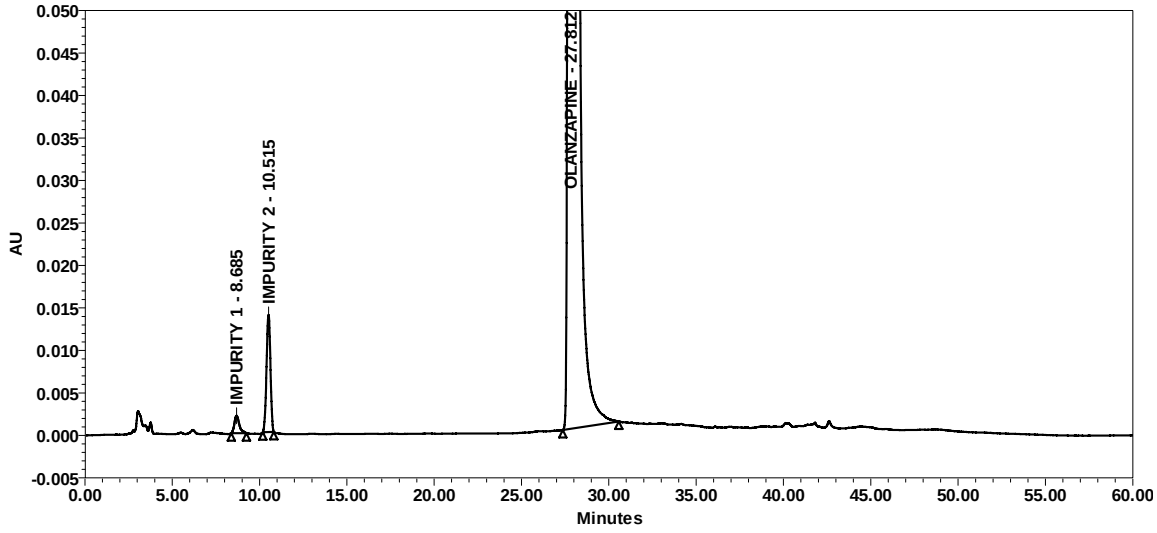
Day-1				
Set No.	Imp-1 (%)	Imp-2 (%)	Unk imp (%)	Total Imp (%)
1	0.18	0.49	BQL	0.67
2	0.18	0.49	BQL	0.67
3	0.18	0.49	BQL	0.67
4	0.18	0.49	BQL	0.67
5	0.18	0.49	BQL	0.67
6	0.18	0.49	BQL	0.67
Mean	0.18	0.49	-	0.67
SD	0.00	0.00	-	0.00
%RSD	0.000	0.000	-	0.000
95% CI	0.000	0.000	-	0.000
Day-2				
Set No.	Imp-1 (%)	Imp-2 (%)	Unk imp (%)	Total Imp (%)
1	0.18	0.50	BQL	0.68
2	0.19	0.50	BQL	0.69
3	0.19	0.51	BQL	0.70
4	0.18	0.50	BQL	0.68
5	0.18	0.50	BQL	0.68
6	0.18	0.51	BQL	0.69

Mean	0.18	0.50	-	0.69
SD	0.005	0.005	-	0.008
%RSD	2.82	1.03	-	1.19
95% CI	±0.005	±0.005	-	±0.009

SD = Standard Deviation, %RSD = Relative Standard Deviation

Unk imp = Unknown impurity, CI = confidence interval

Fig. 10: Chromatogram of method precision



5.5.5 Accuracy (Recovery)

The accuracy was evaluated by the recovery study for method which was established at three levels in the range of 0.25-3.75 µg/ml (0.05% to 0.75% relative to sample concentration, 3 levels x 3 preparations). Calculated amount of known impurities were added in form of solution to the sample preparation to attain lowest to highest level. Data summarized in table 9, chromatograms shown in Fig.11 (A) to (C).

Fig.11 (A): Chromatogram of accuracy level-1

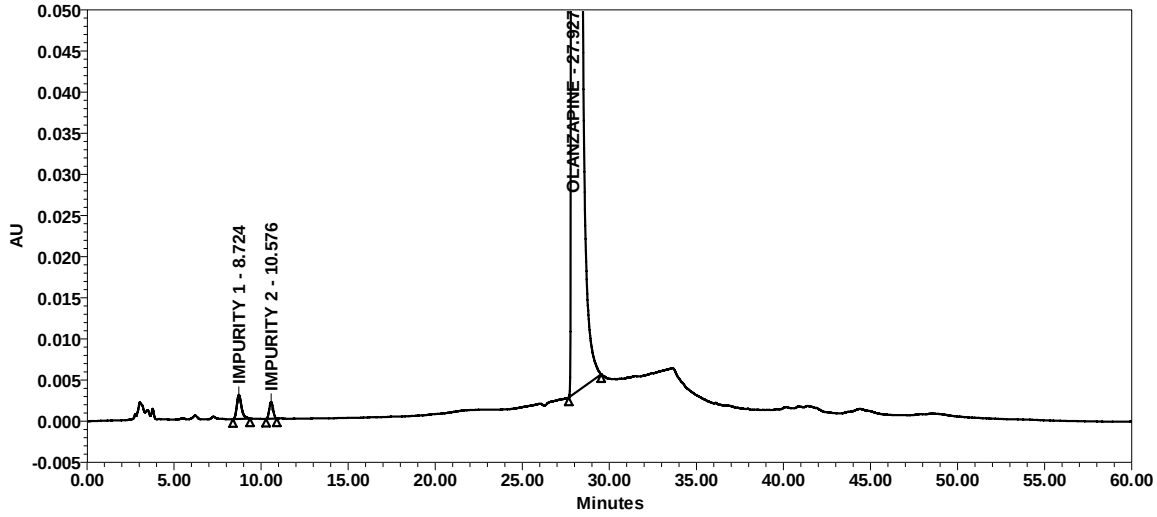


Fig.11 (B): Chromatogram of accuracy level-2

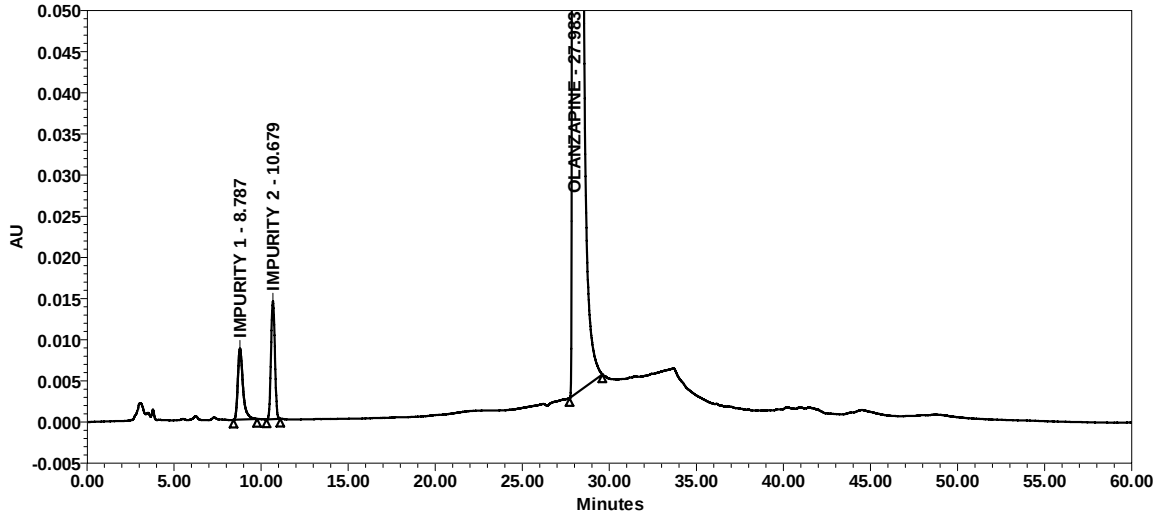


Fig.11 (C): Chromatogram of accuracy level-3

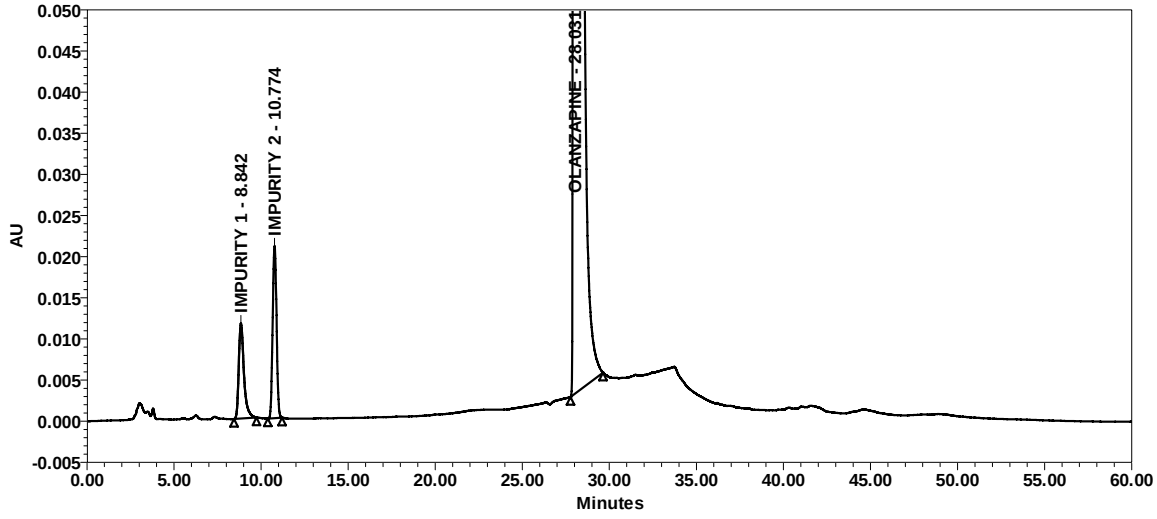


Table 9: Summary of recovery data

Name	Amount added [a] (mcg/ml)	Recovery [a] (%)	% RSD
Impurity-1	0.25	104.2	2.8
	2.5	102.4	1.75
	3.75	102.8	0.39
Impurity-2	0.25	104.8	0.31
	2.5	99.5	0.10
	3.75	99.6	0.15

a = Average of three determinations

5.5.6 Solution stability

The solution stability at 25°C was studied at different time interval against fresh standard preparation at specified time interval. The results obtain for the solution stability study at different time intervals for standard and sample preparation shown in table 10(A) and 10(B). The results obtained are well within the acceptance criteria. Therefore, the diluted standard preparation and sample preparation are stable in solution form up to 59 hours and 56 hours respectively at room temperature.

Table 10(A): Sample solution stability

For sample preparation

Time	Condition	% Impurity	Absolute difference (%)
A) For impurity-1 (%)			
Initial	Room temperature	0.18	Not applicable
After 44 hours	Room temperature	0.19	0.01
After 56 hours	Room temperature	0.19	0.01
B) For impurity -2 (%)			
Initial	Room temperature	0.50	Not applicable
After 44 hours	Room temperature	0.51	0.01
After 56 hours	Room temperature	0.51	0.01
C) For any unknown impurity (%)			
Initial	Room temperature	BQL	Not applicable
After 44 hours	Room temperature	BQL	0.00
After 56 hours	Room temperature	BQL	0.00
D) For total impurities (%)			
Initial	Room temperature	0.68	Not applicable
After 44 hours	Room temperature	0.70	0.02
After 56 hours	Room temperature	0.70	0.02

Table 10(B): Standard solution stability

For diluted standard preparation			
Time	**Condition**	**% Assay**	**Absolute difference (%)**
Initial	Room temperature	100.0	Not applicable
After 47 hours	Room temperature	99.8	0.2
After 59 hours	Room temperature	101.8	1.8

5.3.6 Robustness:

The result of robustness study of the developed method has shown in Table 11(A) and 11(B). The results have shown that during all variance conditions value of related impurities for the test preparation was within the acceptance criteria. It was in accordance with that of accepted true value from method precision study. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

Table 11(A): System suitability summary of robustness study

Condition	System suitability parameters		
	Resolution between imp-1 and imp-2	**Resolution between imp-2 and olanzapine**	**% RSD for standard (NMT 2.0)**
A) Change in flow rate			
Normal condition (1.0 ml per minute)	3.6	38.4	0.27
0.9 ml per minute	3.8	39.0	1.52
1.1 ml per minute	2.8	30.4	0.27
B) Change in column temperature			
Normal condition (27°C)	3.1	33.6	0.33
Oven temperature 32°C	2.4	34.2	0.51
C) Change in buffer pH			
Normal condition pH 6.8	3.7	39.1	0.28
Buffer pH 6.6	4.0	35.7	0.42
Buffer pH 7.0	4.0	43.6	0.94
D) Change in column lot*			
Normal: YMC/OA/250-5/09 Lot No: O425001079(W)	3.1	33.6	0.33
Changed: YMC/OA/250-5/06 Lot No: O42586532(W)	3.6	38.4	0.27

Table 11(B): System suitability summary of robustness study

Condition	% Impurities			
	Imp-1	**Imp-2**	**Any single unknown imp (%)**	**Total impurities (%)**
A) Change in flow rate				
Normal condition (1.0 ml per minute)	0.18	0.50	BQL	0.68
0.9 ml per minute	0.16	0.52	BQL	0.68
Absolute difference from normal condition	0.02	0.02	Not applicable	0.00
Normal condition (1.0 ml per minute)	0.18	0.49	BQL	0.67
1.1 ml per minute	0.19	0.50	BQL	0.69
Absolute difference from normal condition	0.01	0.01	Not applicable	0.02
B) Change in column temperature				
Normal condition 27.0°C	0.18	0.49	BQL	0.67
Oven temperature 32°C	0.18	0.49	BQL	0.67
Absolute difference from normal condition	0.00	0.00	Not applicable	0.00
C) Change in buffer pH				
Normal condition pH-6.8	0.19	0.51	BQL	0.70
Buffer pH 6.6	0.18	0.52	BQL	0.70
Absolute difference from normal condition	0.01	0.01	Not applicable	0.00
Buffer pH 7.0	0.19	0.51	BQL	0.70

Absolute difference from normal condition	0.00	0.00	Not applicable	0.00
D) Change in column lot*				
Lot No: O425001079(W)	0.18	0.49	BQL	0.67
Lot No: O42586532(W)	0.18	0.50	BQL	0.68
Absolute difference from normal condition	0.00	0.01	Not applicable	0.01

6. Quantitation and calculation formulae

6.1 Relative Standard Deviation (%)

Relative Standard Deviation (%) = $\frac{SD \times 100}{\overline{X}}$

6.2 Calculation formulae in linearity and RRF

Correlation coefficient (r) = $\frac{n\ (\sum xy)\ -\ (\sum x)(\sum y)}{\sqrt{\{[n\sum x^2 - (\sum x)^2]\ [n\sum y^2 - (\sum y)^2]\}}}$

Correlation coefficient (r) = CORREL (Array 1, Array 2)

Slope of regression line = $\frac{n\ (\sum xy)\ -\ (\sum x)(\sum y)}{n\sum x^2 - (\sum x)^2}$

Slope of regression line = SLOPE (Array 1, Array 2)

y- intercept = $A = \overline{y} - B\overline{x}$

y- intercept = INTERCEPT (Array 1, Array 2)

Residual sum of squares = $\sum (Y_{original} - Y_{calculated})^2$

Where,

$Y_{calculated} = A + Bx$

n = Number of measurements

x = Individual concentration

y = Individual area

$\bar{x}$ = Average concentration

$\bar{y}$ = Average area

Residual sum of squares = SUMXMY2 (Array 1, TREND (Array 1, Array 2, Array2))

$$\text{Relative Response Factor} = \frac{\text{Slope of regression line for impurity}}{\text{Slope of regression line for standard}}$$

6.3 Accuracy (% Recovery)

$$\% \text{ Recovery} = \frac{\text{Ammount of sbustance (mg) found}}{\text{Ammount of substance (mg) added}} \text{ x } 100$$

6.4 % Impurity in olanzapine Tablets

$$\% \text{ Impurity} = \frac{\text{Asp}}{\text{Astd}} \times \frac{\text{Std. wt}}{\text{Dilution}} \times \frac{\text{Dilution}}{\text{Spl Wt}} \times \frac{\text{Avg Wt.}}{\text{LC}} \times \frac{1}{\text{RRF}} \times \text{Std. assay \% (as such)}$$

e.g.

$$\% \text{ Impurity-1} = \frac{41362}{165232} \times \frac{25.91}{100} \times \frac{5}{50} \times \frac{5}{50} \times \frac{100}{806.21} \times \frac{320.2}{20} \times \frac{1}{0.72} \times 99.72$$

% Impurity-1 = 0.18

$$\% \text{ Impurity-2} = \frac{226953}{165232} \times \frac{25.91}{100} \times \frac{5}{50} \times \frac{5}{50} \times \frac{100}{806.21} \times \frac{320.2}{20} \times \frac{1}{1.43} \times 99.72$$

% Impurity-2 = 0.49

% Total impurities = Total known impurities + Total unknown impurities

% Total impurities = (0.18 + 0.49) + (0.00)

% Total impurities = 0.67

Asp	:	Area of impurity in sample
Astd	:	Mean area of diluted standard
Std. wt	:	Std weight in mg
Spl wt	:	Sample weight in mg
Avg wt	:	Average weight in mg
LC	:	Label claim per tablet (mg)

6.5 95% Confidence Interval (CI)

95% Confidence Interval (CI) = $\pm \frac{t\alpha \times SD}{\sqrt{n}}$

Where,

SD = Standard Deviation

$\overline{X}$ = Average concentration

n = Number of measurements

t_α = t value at n-1 from t table (two tail)

95% Confidence Interval (CI) = SD (2.57058)/SQRT (6)

References

[1] Merck Index 14th edition, Merck & Co.INC. 2006 p.1175

[2] Physicians' Desk Reference, Thomson Healthcare INC., PDR 62 ed 2008 p.1855-1866

[3] http:// www.emea.europa.eu/humandocs/PDFs/EPAR/Zyprexa/064696cs1.pdf

[4] Pierre M. Llorca, François Coudore, Christophe Corpelet, Aurelie Buyens, Monique Hoareau and Alain Eschalier, Clinical chemistry, 47(2001) 1719-1721

[5] M. A. Raggi, G. Casamenti, R. Mandrioli, S. Fanali, D. De Ronchi and V. Volterra Chromatographia 51(2000)562

[6] B. Venkateswara Reddy, K. V. N. Suresh Reddy, J. Sreeramulu, G. V. Kanumula, Chromatographia 66(2007)111-114

[7] Catlow J. T., Barton R. D., Clemens M., Gillespie T. A., Goodwin M., Swanson S. P. Journal of chromatography. B, Biomedical applications, 668(1995) 85-90

[8] Dasandi B., Shah P., Gandhi C., Bhat K. M., Shivprakash, Indian Drugs, 40(2003)350-354

[9] Jingqi Bao and Brian D. Potts, Journal of chromatography. B, Biomedical applications, 752(2001)61-67

[10] Ramisetti Nageswara Rao, Ale Narasa Raju, Ramaram Narsimha, Ganduri Raveendra Babu, Journal of High Resolution Chromatography, 31(2008)107-118

[11] E. J. Harvey, R. J. Flanagan, and D. M. Taylor, the Pharmaceutical Journal 265(2000) 275-276

[12] Chiu J A and Franklin R B, Journal of Pharmaceuticals and Biomedical analysis 14(1996)609-615

[13] C. R. Shah, B.N. Suhagia, N.J. Shah, D.R. Patel, N.M. Patel, Indian Journal of Pharmaceutical Science 70(2008)251-255

[14] Poornachander Thatipalli, Ramesh Kumar, Chandrasekhar Bulusu, Ramesh Chakka, Pratap R. Padi, Anjaneyulu Yerra, and Satyanarayana Bollikonda, Arkivoc 11(2008) 195-201

[15] MDPP R. Ravichandran, US Pharmacopeial Forum 34-3(2008)641-643

[16] US Pharmacopeia, USP 31 NF 26, 2008

[17] European Pharmacopeia, 6th edition, 2008

[18] British Pharmacopeia, 2008

[19] Indian Pharmacopeia, 2007

[20] Japanese Pharmacopeia, 15th edition (2006)

[21] Pharmacopeia of the people's republic of china (2005)

[22] ICH guideline, Q2 (R1) step 4, Validation of Analytical Procedures: Text and Methodology (2005)

[23] ICH guideline, Q3B (R2) step 4, Impurities in new drug products (2006)

[24] Behnam Davani, Karen A. Russo, Andrzej Wilk, Lokesh Bhattacharya USP PF 31(2) (2005) 626-636

Section-II: HPLC Method development and validation for determination of related impurities in Quetiapine fumarate

1. Introduction to quetiapine fumarate

Quetiapine is chemically 2-[2-(4-dibenzo[b,f]thiazepin-11-yl-1-piperazinyl) ethoxy]ethanol (Fig.1A) is a dibenzothiazepine atypical antiscychotic and reported to have affinity for serotonin (5-HT_2), histamine (H_1), and adrenergic (α_1 and α_2) receptors as well as dopamine D_2 receptors. Quetiapine is used in treatment of schizophrenia and of mania associated with bipolar disorder. Molecular formula of quetiapine fumarate (Fig. 1B) is $(C_{21}H_{25}N_3O_2S)_2\,C_4H_4O_4$ with molecular weight 883.1 g/mole [1]. Its melting point is 172°C to 173°C [2-3]. Quetiapine fumarate is a weak acid with dissociation constant (pKa) 3.3 and 6.8 with moderate pH dependent solubility, 94.3 mg/mL to 2.37 mg/mL at pH values from 1 to 9 reported [4].

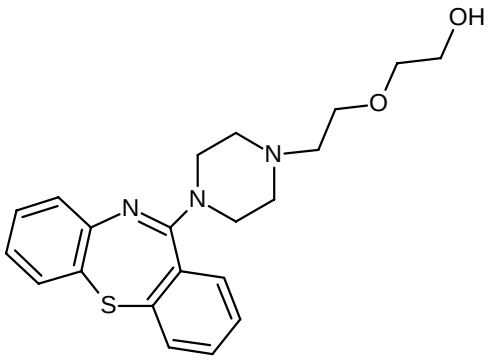


Fig.1A: Quetiapine

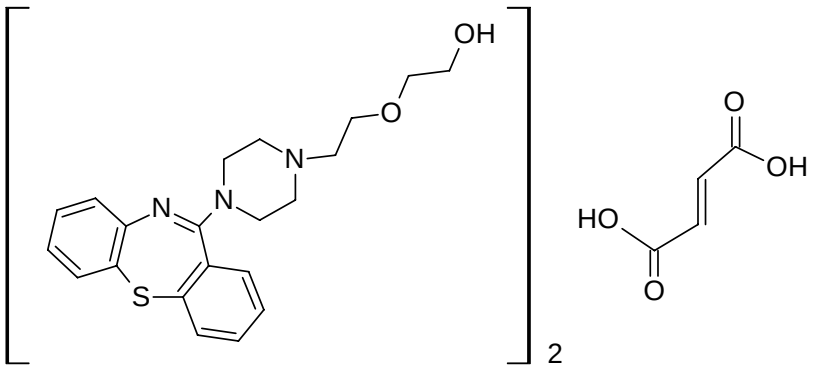


Fig.1B: Quetiapine fumarate

Strengths: Quetiapine tablets are available in six strengths equivalent to 25 mg, 50 mg, 100 mg, 150 mg, 200 mg, and 300 mg of Quetiapine in form of quetiapine fumarate as an active ingredient for oral administration.

Innovator: SEROQUEL tablets, AstraZeneca

PHARMACOLOGY

Pharmacodynamics

Quetiapine is an atypical antipsychotic agent. Quetiapine and the human plasma metabolite, N-desalkyl quetiapine, interact with a broad range of neurotransmitter receptors. Quetiapine and N-desalkyl quetiapine exhibit affinity for brain serotonin (5HT2) and dopamine D_1 and D_2 receptors. It is this combination of receptor antagonism with a higher selectivity for 5HT2 relative to D2 receptors which is believed to contribute to the clinical antipsychotic properties and low extrapyramidal side effects (EPS) liability of SEROQUEL. Additionally, N-desalkyl quetiapine has high affinity at serotonin 5HT1 receptors. Quetiapine and Ndesalkyl quetiapine also have high affinity at histaminergic and adrenergic α_1 receptors, with a lower affinity at adrenergic α_2 receptors. Quetiapine has no appreciable affinity at cholinergic muscarinic or benzodiazepine receptors. Quetiapine is active in tests for antipsychotic activity, such as conditioned avoidance. It also reverses the action of dopamine agonists, measured either behaviourally or lectrophysiologically, and elevates dopamine metabolite concentrations, a neurochemical index of D2 receptor blockade. The extent to which the N-desalkyl quetiapine metabolite contributes to the pharmacological activity of SEROQUEL in humans is not known. In pre-clinical tests predictive of EPS, quetiapine is unlike standard antipsychotics and has an atypical profile. Quetiapine does not produce dopamine D2 receptor supersensitivity after chronic administration. Quetiapine produces only weak catalepsy at effective dopamine D2 receptor blocking doses. Quetiapine demonstrates selectivity for the limbic system by producing depolarization blockade of the mesolimbic but not the nigrostriatal dopamine-containing neurons following chronic administration. Quetiapine exhibits minimal dystonic liability in haloperidol-sensitised or drug-naive Cebus monkeys after acute and chronic administration. The results of these tests predict that SEROQUEL should have minimal EPS liability, and it has been hypothesised that agents with a lower EPS liability may also have a lower liability to produce tardive dyskinesia.

Pharmacokinetics

Absorption

Quetiapine is well absorbed and the bioavailability of quetiapine is not significantly affected by administration with food [5].

Distribution

The elimination half-lives of quetiapine and N-desalkyl quetiapine are approximately 7 and 12 hours respectively. Quetiapine is approximately 83% bound to plasma proteins. Steady state peak molar concentrations of the active metabolite N-desalkyl quetiapine are 35% of that observed for quetiapine. The pharmacokinetics of quetiapine and N-desalkyl quetiapine are linear across the approved dosage range. The kinetics of quetiapine do not differ between men and women.

Metabolism

Quetiapine is extensively metabolised by the liver following oral administration, with parent compound accounting for less than 5% of unchanged drug related material in the urine or faeces, following the administration of radiolabelled quetiapine. The average molar dose fraction of free quetiapine and the active human plasma metabolite N-desalkyl quetiapine is <5% excreted in the urine. In vitro investigations established that CYP3A4 is likely to be the primary enzyme responsible for cytochrome P450 mediated metabolism of quetiapine. N-desalkyl quetiapine is primarily formed and eliminated via CYP3A4. CYP2D6 and CYP2C9 are also involved in quetiapine metabolism.

Quetiapine and several of its metabolites (including N-desalkyl quetiapine) were found to be weak to modest inhibitors of human cytochrome P450 3A4, 2C19,2D6, 1A2 and 2C9 ctivities in vitro. In vitro CYP inhibition is observed only at concentrations approximately 5 to 50-fold higher than those observed at a dose range of 300 to 800 mg/day in humans. Based on these in vitro results, it is unlikely that coadministration of quetiapine with other drugs will result in clinically significant drug inhibition of cytochrome P450 mediated metabolism of the other drug. From animal studies it appears that quetiapine can induce cytochrome P450 enzymes. In a specific interaction study in

psychotic patients, however, no increase in the cytochrome P450 activity was found after administration of quetiapine.

1. The mean clearance of quetiapine in the elderly is approximately 30 to 50% lower than that seen in adults aged 18 to 65 years.
2. Excretion
3. Approximately 73% of the radioactivity is excreted in the urine and 21% in the
4. Faeces.

Use in renal impairment

The mean plasma clearance of quetiapine was reduced by approximately 25% in subjects with severe renal impairment (creatinine clearance less than 30 mL/min/1.73m2), but the individual clearance values are within the range for normal subjects.

Contraindications

SEROQUEL is contraindicated in patients who are hypersensitive to any component of this product.

2. Literature review

The literature review regarding quetiapine fumarate suggest that various analytical methods were reported for drug substance as well as in pharmaceutical formulation and in various biological fluids. The literature reviews for analysis of Quetiapine are as under.

2.1 Vijaya Kumar M., Muley have developed a stability indicating HPLC method for determination of quetiapine fumarate in bulk drug and solid dosage forms. In this method Puroshere STAR RP18e (C18 250mm x 4.6 mm, 5µm) column with mobile phase consisting of Methanol: Water: TEA in the ratio of 73:27:0.4 % v/v/v pH 3.0 adjusted with phosphoric acid in isocratic mode. The detection wavelength is 289 nm and flow rate is 1.0 ml/min. In the range of 10-300 µg/ml, linearity of quetiapine shows a regression co efficient of 0.9996. The proposed method is sufficient selective to distinguish the parent drug and degradation products after hydrolysis, photolysis, or chemical oxidation and from excipients [6].

2.2 Bharathi C.H., Prabahar K. J., Prasad C. H. S., Srinivasa Rao M. Trinadhachary G. N., Handa V. K., Dandala R., Naidu A have documented identification, isolation, synthesis and characterization of impurities of quetiapine fumarate and six unknown impurities and one known impurity (intermediate) were identified ranging from 0.05-0.15% by reverse-phase HPLC. These impurities were isolated from crude samples using reverse-phase preparative HPLC. Based on the spectral data, the impurities were characterized as 2-[4-dibenzo[b,f][1,4]thiazepine-11-yl-1-piperazinyl]1-2-ethanol (impurity I, desethanol quetiapine), 11-[(N-formyl)-1-piperazinyl]-dibenzo[b,f][1,4]thiazepine (impurity II, N-formyl piperazinyl thiazepine), 2-(2-hydroxy ethoxy)ethyl-2-[2-[4-dibenzo[b,f][1,4]thiazepine-11-piperazinyl-1-carboxylate (impurity III, quetiapine carboxylate), 11-[4-ethyl-1-piperazinyl]dibenzo[b,f][1,4] thiazepine (impurity IV, ethylpiperazinyl thiazepine), 2-[2-(4-dibenzo[6,/][1,4]thiazepin-11-yl-1-piperazinyl)ethoxy]1 -ethyl ethanol [impurity V, ethyl quetiapine), 1,4-bis[dibenzo[b,f][1,4]thiazepine-11-yl] piperazine [impurity VI, bis(dibenzo)piperazine]. The known impurity was an intermediate, 11-piperazinyldibenzo [b,f][1,4]thiazepine (piperazinyl thiazepine). The structures were established unambiguously by independent synthesis and co-injection in HPLC to confirm the retention times. To the best of our knowledge, these impurities have not been reported before. Structural elucidation of all impurities by spectral data (^{1}H NMR, ^{13}C NMR, MS and IR), synthesis and formation of these impurities are discussed in detail [7].

2.3 Julia Sachse, Johannes Köller Sebastian Härtter and Christoph Hiemke have developed an HPLC method with column switching is described for the determination of quetiapine, clozapine, perazine, olanzapine and metabolites in blood serum. After clean-up on silica C8 material (20 μm particle size) drugs were separated on ODS Hypersil C18 material (5 μm; column size 250 mm × 4.6 mm i.d.) within 25 min and quantified by ultraviolet (UV) detection at 254 nm. The limit of quantification ranged between 10 and 50 ng/ml. At therapeutic concentrations of the drugs, the inter-assay reproducibility was below 10%. Analyses of drug concentrations in serum of 75– 295 patients treated with therapeutic doses of the antipsychotic drugs revealed

mean ± S.D. steady state concentrations of 139 ± 136 ng/ml for quetiapine, 328 ± 195 ng/ml for clozapine, 48 ± 27 ng/ml for olanzapine and 71 ± 52 ng/ml for perazine The method was thus suitable for routine therapeutic drug monitoring and may be extended to other drugs [8].

2.4 B. Barrett and co-workers have developed validated HPLC–MS/MS method for determination of quetiapine in human plasma highly sensitive and selective high-pressure liquid chromatography–tandem mass spectrometry (LC–MS/MS) methodwas developed for the quantitative determination of quetiapine (QUE) in human Na2EDTA plasma with mass spectrometry (MS) detection. Clozapine (CLO) was employed as an internal standard. Samples were extracted using solid phase extraction (SPE). Oasis HLB cartridges and the concentration of quetiapine was determined by isocratic HPLC–MS/MS. The SRM mode was used for MS/MS detection. The method was validated over a concentration range of 1.0–382.2 ng/mL. Inter- and intra-day precision and accuracy of the proposed method were characterized by relative standard deviation (R.S.D.) and the percentage of deviation, respectively; both were lower than 8%. The developed method was employed in the pharmacokinetic study of quetiapine [9].

2.5 Mandrioli R.; Fanali S.; Ferranti A.; Raggi M. A have developed **a** precise and feasible high- performance liquid chromatographic (HPLC) method for the analysis of the novel antipsychotic drug quetiapine in plasma. The analysis was carried out on a C8 (150 x 4.6 mm i.d., 5 μm) reversed-phase column, using a mixture of acetonitrile, methanol and pH 1.9 phosphate buffer as the mobile phase; triprolidine was used as the internal standard. Careful pretreatment of the biological samples was implemented by means of solid- phase extraction (SPE). A good linearity was found in the 4-400 ng ml^{-1} quetiapine plasma concentration range. The application to some plasma samples of patients treated with Seroquel® tablets gave satisfactory results. The accuracy was good (quetiapine mean recovery = 92%), as well as the precision (mean RSD = 3.3%). The method seems to be suitable for the clinical monitoring of patients treated with quetiapine [10].

2.6 Vasil N, Atanasov, Kamen P. Kanev and Mariana Io. Mitewa have developed a method for detection and identification of atypical quetiapine metabolite in urine, Quetiapine fumarate (Seroquel®) is an atypical antipsychotic dibenzothiazepine derivative. Due to its extensive hepatic metabolism and low level of unchanged excretion (< 1%) the routine toxicological drug-screening analyses of urine often leads to false negative results. In the present study, we report that a newly identified metabolite of quetiapine, N-desalkylquetiapine, can be used as an indicative marker of quetiapine-intake in urine using common GC-MS screening procedure. The structure of the mentioned metabolite was solved from the mass-spectrum obtained and the quetiapine presence was proved by consequent HPLC plasma analysis [11].

2.7 Davis PC and Wong J Gefvert O have developed a method for sensitive and specific HPLC assay for the measurement of the antipsychotic compound quetiapine in human plasma and validated. The assay employs a three-step liquid liquid extraction of quetiapine and its 7-hydroxylated and 7-hydroxylated, N-dealkylated metabolites from human plasma, and utilizes ultraviolet (UV) detection of quetiapine and electrochemical detection of the metabolites. The method provides a linear response from a quantitation limit of 2.50 to 500 ng ml(-1) for each analyte using 0.4 ml plasma. The assay is applicable from 500 to 5000 ng ml(-1) by sample dilution with de-ionized water. The inter-assay precision of quetiapine in plasma calibration standards across 4 validation days averaged 11.9% relative standard deviation (RSD) over the range 2.50 to 500 ng ml(-1), with intra-assay precision averaging 16.0% RSD and mean accuracy of 98.6% of theory. Similarly, the inter-assay precision of the 7-hydroxylated metabolite in plasma calibration standards across 4 validation days averaged 13.7% RSD over the range 2.50 to 500 ng ml(-1), with intra-assay precision averaging 17.6% RSD and mean accuracy of 109% of theory. The 7-hydroxylated, N-dealkylated metabolite demonstrated inter-assay precision of 16.2% RSD, intra-assay precision of 19.9% RSD, and mean accuracy of 104% of theory over the range 2.50 to 500 ng ml(-1). The present assay method was used to support a study comparing the pharmacokinetic profile of quetiapine with the time course of dopamine D2 and serotonin 5-HT2 receptor occupancy in the brain using positron emission tomography

(PET). We describe in this paper the bioanalytical method and the plasma concentrations of quetiapine and its metabolites resulting from this study [12].

2.8 Zhiling Zhou ; Xin Li ; Kunyan Li ; Zhihong Xie ; Zeneng Cheng ; Wenxin Peng ; Feng Wang ; Ronghua Zhu ; Huande Li have developed method for simultaneous determination of clozapine, olanzapine, risperidone and quetiapine in plasma by high-performance liquid chromatography-electrospray ionization mass spectrometry. Clozapine (CLZ), olanzapine (OLZ), risperidone (RIP) and quetiapine (QTP) have been widely used in the treatment of schizophrenia. However, no study (or little study) has been conducted to determine the four drugs simultaneously by the use of high-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-MS/ESI). Objective: To develop a sensitive method for simultaneous determination of CLZ, OLZ, RIP and QTP in human plasma by HPLC-MS/ESI. Methods: The analytes were extracted twice by ether after samples had been alkalinized. The HPLC separation of the analytes was performed on a MACHEREY-NAGEL C_{18} (2.0 mm x 125 mm, 3 μm, Germany) column, using water (formic acid: 2.70 mmol/l, ammonium acetate: 10 mmol/l)-acetonitrile (53:47) as mobile phase, with a flow-rate of 0.16 ml/min. The compounds were ionized in the electrospray ionization (ESI) ion source of the mass spectrometer and were detected in the selected ion recording (SIR) mode. Results: The calibration curves were linear in the ranges of 20-1000 ng/ml for CLZ and QTP, 1-50 ng/ml for OLZ and RIP, respectively. The average extraction recoveries for all the four analysts were at least above 80%. The methodology recoveries were higher than 91% for the analysts. The intra- and inter-day R.S.D. were less than 15%. Conclusion: The method is accurate, sensitive and simple for routine therapeutic drug monitoring (TDM) and for the study of the pharmacokinetics of the four drugs [13].

3. Aim of present work

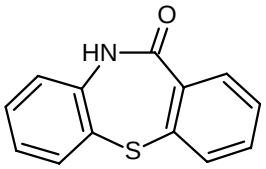
Literature reveals that the drug is not official in any pharmacopeia. An HPLC method for quetiapine assay using UV detector for drug substance and formulation is available [6]. A method with identification, isolation, synthesis and characterization of ***six***

impurities for quetiapine fumarate was reported [7]. A method for quetiapine and it's metabolite in serum is reported [8]. Many methods are available for quetiapine drug substance and it's metabolite in plasma or urine [9-13]. But here ***seven*** known impurities are reported and for that method needs to developed and validated as per current regulated requirement with establishment of RRF and RRT for all known impurities (Fig. 1C).

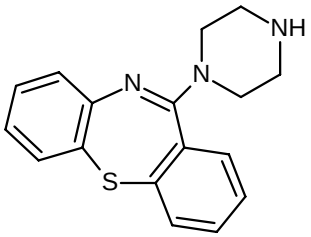
The aim and scope of the proposed work are as under

- HPLC method development for determination of related impurities in Quetiapine fumarate drug substance.
- Forced degradation study under stress condition
- To resolve all known impurities and generated during the force degradation studies
- Perform analytical method validation for the proposed method as per ICH guideline.

Fig.1C: Quetiapine fumarate impurity A to G



Impurity-A



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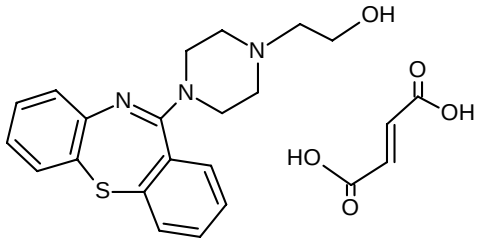
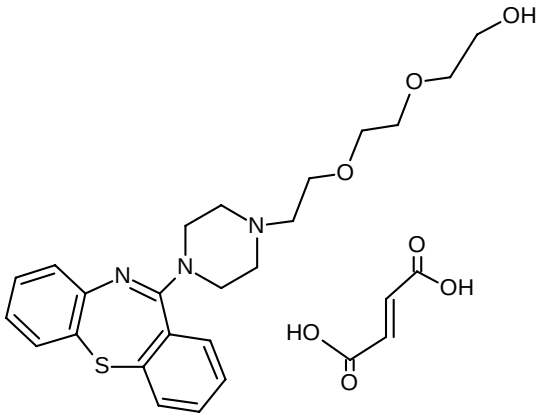


Impurity-C



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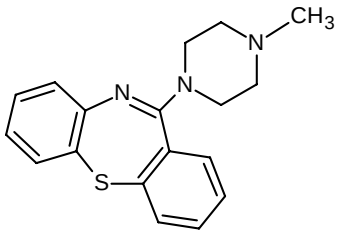

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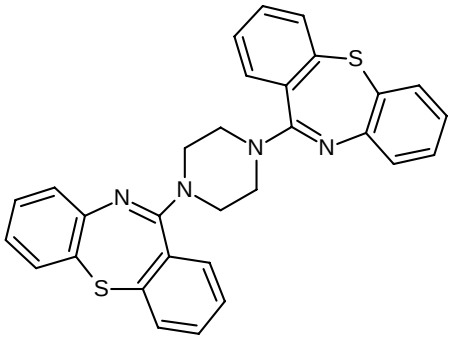

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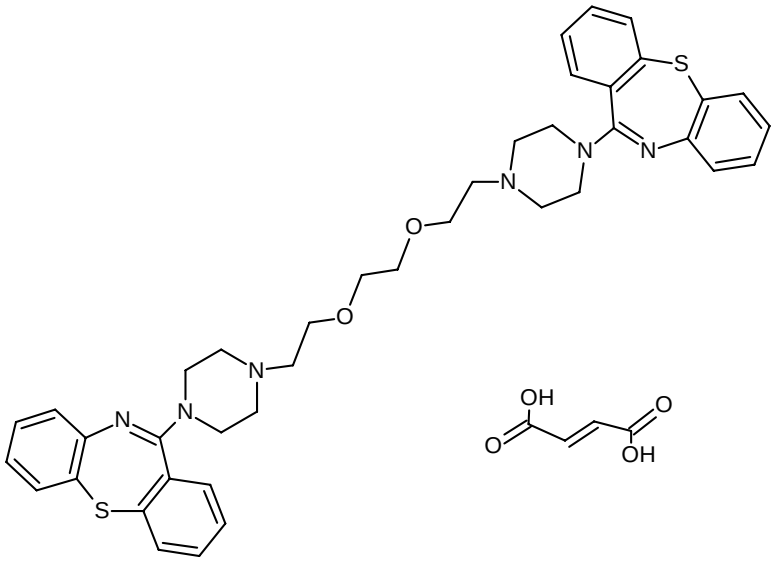


Impurity-G

4. Experimental

4.1 Materials, chemicals and reagents

Materials, chemicals and reagents were used as mentioned in Table 1 and Table 2.

Table 1: Materials

Sr.No	Material Name	Lot. No. / Batch no./Adl Cert. No.	Potency / Purity
1	Quetiapine fumarate standard	OTV/2008/0203	99.60%
2	Quetiapine fumarate sample	QTP3/XIV/005	--
3	Impurity-A = Dibenzo [b,f] [1,4] thiazepine-11-(10H)-one	OTV/2007/758/01	99.53%
4	Impurity-B = 11-Piperazinyl dibenzo[b,f] [1,4] thiazepine	OTV/2007/1502	82.79%

5	Impurity-C = 2-(4-dibenzo[b,f][1,4,]thiazepin-11-yl-1-piperazinyl) ethanol)	OTV/2007/1313	98.20%
6	Impurity-D = 2-[2-(4-dibenzo[b,f][1,4,]thiazepin-11-yl-1-piperazinyl)ethoxy]ethoxy]ethanol)	OTV/2007/1314	88.95%
7	Impurity-E = 11-(4-methylpiperazin-1-yl)dibenzo[b,f][1,4]thiazapine)	OTV/2007/893	87.72%
8	Impurity-F = (1,4 Bis(dibenzo[b,f][1,4]thiazapine-11-yl piperazine)	OTV/2007/1503	98.35%
9	Impurity-G = 1,2-Bis[2-(4-dibenzo[b,f][1,4]thiazepin-11-yl-1-piperazinyl)ethoxy]ethane	QTP/XII/022	83.59%

Table 2: Chemicals and reagents

Sr. No.	Chemical/Reagent	Grade
1	Methanol	HPLC grade (Merck, India)
2	Acetonitrile	HPLC grade (Merck, India)
3	Ammonia solution (25%v/v)	AR grade (Rankem, India)
4	Fumaric acid	GR grade (Merck, India)
5	Hydrochloric acid	AR grade (Rankem, India)
6	Sodium hydroxide pellets	GR grade (Merck, India)
7	Hydrogen peroxide solution (30%)	Purified (Merck, India)
8	Mill-Q-water	By Milli-Q system (Millipore, USA)

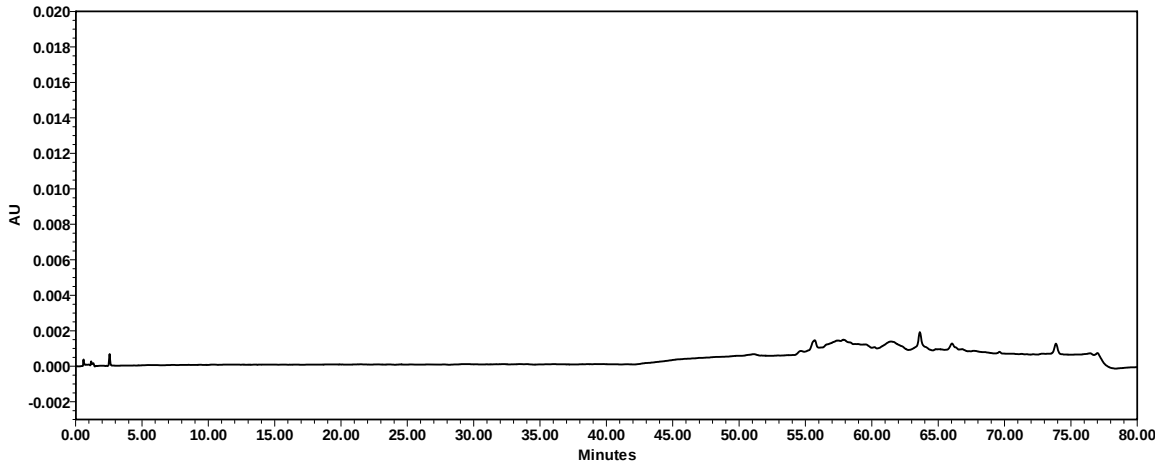
4.2 Instrumentation

The HPLC system of alliance with Waters 2695, waters 2487 and waters 2996 with Empower software was used for this entire study. Chromatographic separation was achieved on X terra RP -8 ,150 mm X 4.6mm 5µm (150 mm X 4.6mm 5µm) column as stationary phase with binary gradient mode.

4.3 Blank preparation

Diluent was used as a blank Fig. 2.

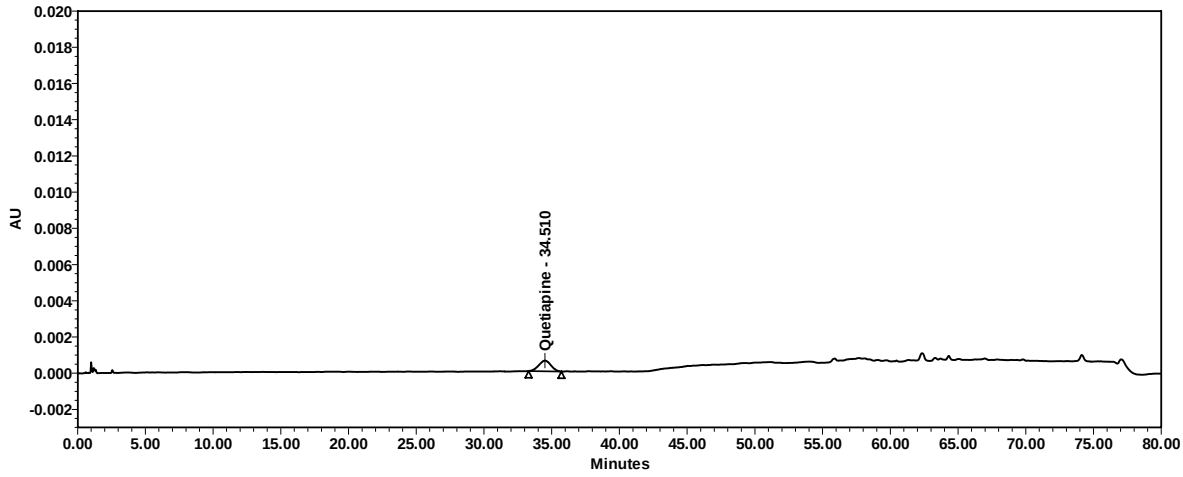
Fig.2: Chromatogram of blank



4.4 Standard solution

Transferred about 15 mg of each of quetiapine fumarate standard into 100ml volumetric flask and 75ml diluent was added to dissolve with aid of sonication and diluted to volume with diluent. In a 200ml volumetric flask, 2 ml of this solution was diluted to volume with diluent (1.0 µg/ml of quetiapine fumarate) Fig [3].

Fig.3: Chromatogram of standard solution



4.5 Preparation of impurity –A stock solution

Transferred about 2.5 mg of impurity –A into 10ml volumetric flask and 7ml diluent was added to dissolve with aid of sonication and diluted to volume with diluent.

4.6 Preparation of impurity –B stock solution

Transferred about 2.5 mg of impurity –B into 10ml volumetric flask and 7ml diluent was added to dissolve with aid of sonication and diluted to volume with diluent.

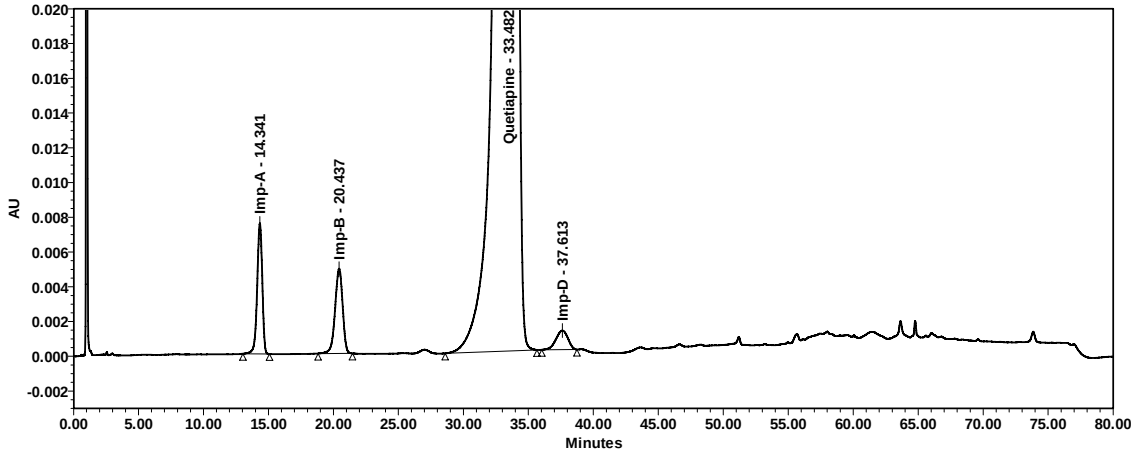
4.7 Preparation of impurity –D stock solution

Transferred about 25 mg of impurity –D into 100ml volumetric flask and 70ml diluent was added to dissolve with aid of sonication and diluted to volume with diluent.

4.8 Resolution solution

Transferred about 50 mg of quetiapine fumarate into 50ml volumetric flask and 35ml diluent was added to dissolve with aid of sonication and 1.0ml of each of impurity stock solution were added and diluted to volume with diluent Fig [4].

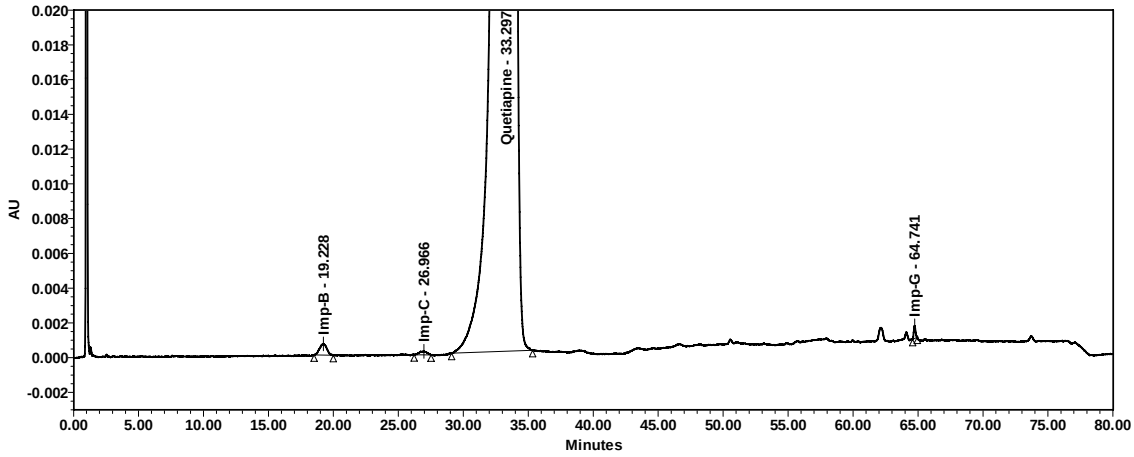
Fig.4: Chromatogram of resolution solution



4.9 Sample solution

Transferred about 50 mg of sample into 50ml volumetric flask and 35ml diluent was added to dissolve with aid of sonication and diluted to volume with diluent (1000 µg/ml of quetiapine fumarate) Fig[5].

Fig.5: Chromatogram of sample solution (as such preparation)



5. Result and discussion

5.1 Development and optimization of the LC method

Method development was based on several considerations like diluent selection, column selection, and detection.

5.1.1 Selection of diluent

Initially method development was started with quetiapine fumarate standard preparation in methanol. But, it was observed that known impurities were not easily soluble in methanol. So, a mixture of acetonitrile and water in the ratio of 80:20 was chosen to achieve solubility of impurity-A to impurity-E. During the course of development an unknown impurity was observed in late eluting region of chromatogram and it was identified and made it known as an impurity-F. But, during the recovery stock solution preparation it was observed that impurity-F was also very less soluble in proposed diluent. So, again different pH diluents were employed in same composition of acetonitrile and water and finally 0.1 ml of perchloric acid in a mixture of 1000 ml of acetonitrile: water in ratio of 80:20 was optimized to achieve desired solubility and solution stability of QF and its known impurities.

5.1.2 Selection of column

A mobile phase consisting of buffer of pH 9.2 was explored to achieve adequate separation between impurity-D and QF peak fig [2]. So for such a higher pH X-terra RP-8 column (150 mm x 4.6 mm, 5 µm) was used for chromatographic separation [14-15]

Table 3: Chromatographic condition

Mobile phase-A	Buffer : Acetonitrile : Methanol 70: 13: 17 (v/v/v) (Buffer, 40 mM ammoniaum acetate, pH was adjusted with ammonia solution to 9.2, filtered through 0.45µm PVDF membrane)		
Mobile phase-B	Acetonitrile		
Column	X terra RP -8 ,150 mm X 4.6mm 5µm		
Column oven temp	45°C		
Detection	UV at 250 nm		
Flow rate	1.5ml/min		
Injection volume	20µl		
Run time	80 min		
Gradient program	**Time (min)**	**Mobile phase-A (%)**	**Mobile phase-B (%)**
	0.0	100	0
	40	100	0
	65	50	50
	75	50	50
	76	100	0
	80	100	0
Diluent	0.1ml of perchloric acid (70%) in 1000 ml of mixture of water and Acetonitrile in ratio of 20:80		

5.1.3 Selection of detection

A UV spectrum of QF and its impurities were recorded with the help of photo diode array detector, spectrum pattern was found similar for all components of interest with maxima at about 208 nm, 250 nm and 290 nm, but detection at 208 nm was not selected with consideration of UV cut-off of ammonium acetate buffer [16] and 250 nm was selected for adequate response at working concentration for intended application to reproduce the results of an impurity at reporting threshold [17-18] or at LOQ.

Impurities spiked sample solution was used for gradient optimization. Once desired separation for known impurities and main component was achieved forced degradation was performed. Finally proposed method with chromatographic condition as per table 3 was subjected to method validation as per ICH guideline.

5.2 Forced degradation study

Forced degradation was performed on Quetiapine fumarate drug substance to achieve desired degradation. Based on development trials optimized forced degradation conditions were established as mentioned below, Final sample concentration was achieved 1000µg/ml of Quetiapine fumarate with diluent as proposed sample concentration.

5.2.1 Oxidative degradation

Drug substance was subjected to oxidative stress condition by 5ml of 3% v/v H_2O_2 solution on water bath at 50°C for 5 min and sample solution was prepared as per procedure.

5.2.2 Acidic degradation

Drug substance was subjected to acid stress condition by 5ml of 1N HCl on water bath at 70°C for 8 hours in 50ml volumetric flask and sample was neutralized with 1N NaOH and sample solution was prepared as per procedure

5.2.3 Base degradation

Drug substance was subjected to base stress condition by 5ml of 5N NaOH on water bath at 70°C for 12 hours in 50ml volumetric flask and sample was neutralized with 5N HCl and sample solution was prepared as per procedure

5.2.3 Thermal degradation

Drug substance was kept in temperature controlled oven at 105 °C for 72 hours and sample solution was prepared as per procedure.

5.3 Method validation

Method validation approach was to establish performance characteristic of method for the requirements of intended analytical application.

5.4.1 Specificity

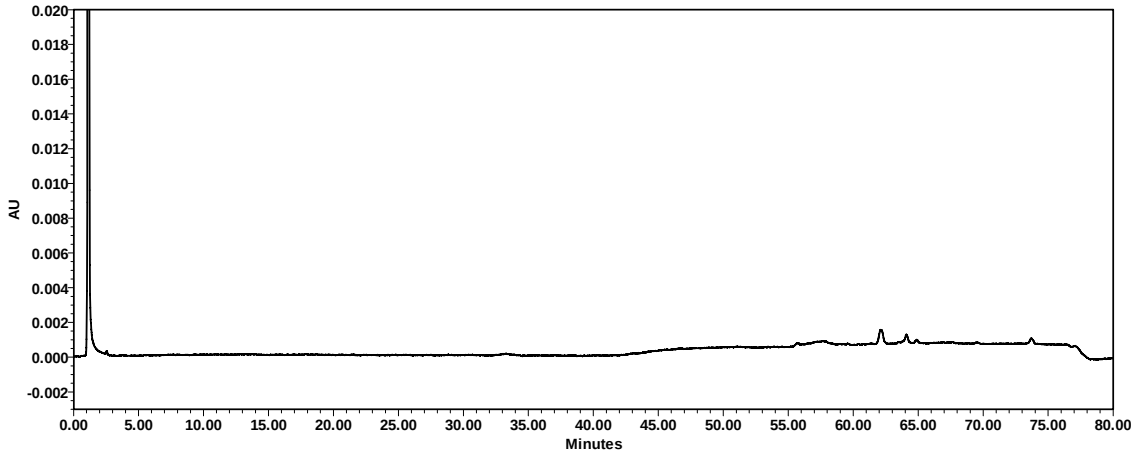
Interference from blank was not observed at any peak of interest and peak purity of all known impurities in spiked sample (as per method precision) observed within the acceptance criteria and forced degradation data as discussed in section 5.3 summarized in table 5 and chromatograms are as shown in fig.7,(A) to 7(L).

Table 5: Forced degradation data

Stress condition	% of degradation
Oxidative degradation	17.1
Acid degradation	22.4
Base degradation	5.0
Photo degradation	0.2
Thermal degradation	3.9

Fig 7: Forced degradation chromatograms

Fig.7 (A): Peroxide degradation



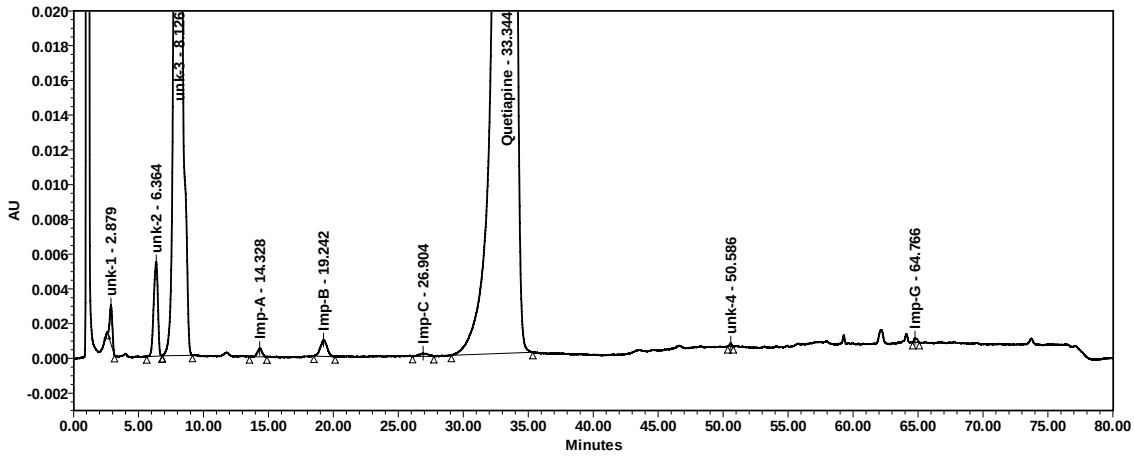
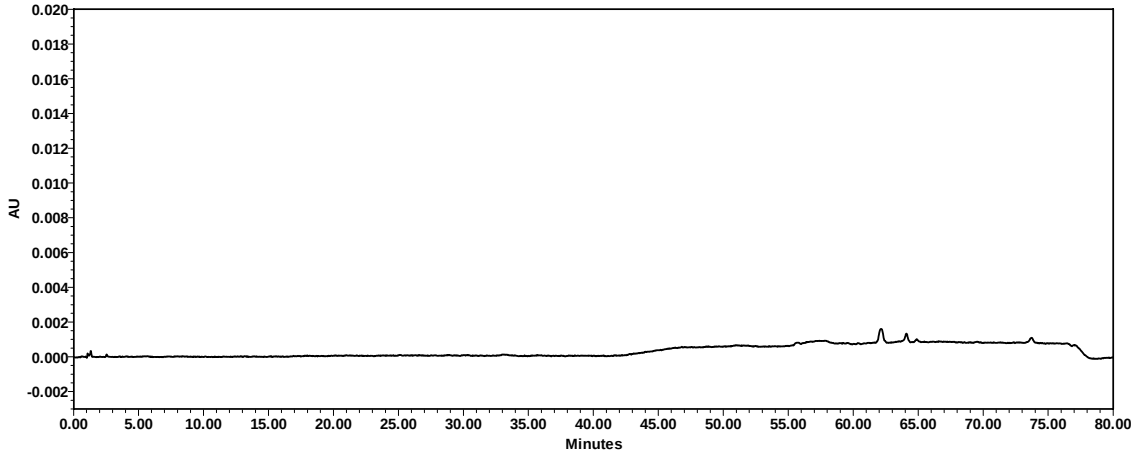


Fig.7 (B): Acid degradation



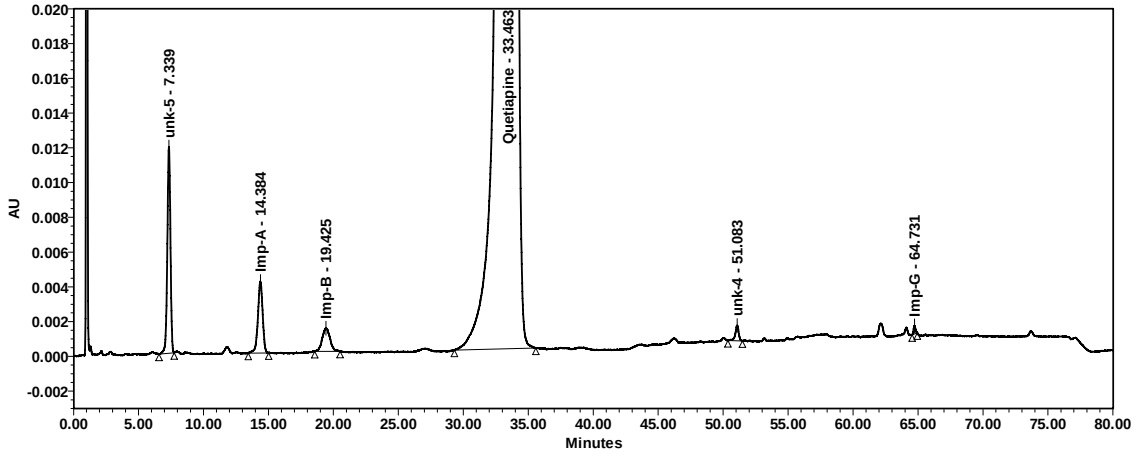


Fig.7 (C): Base degradation

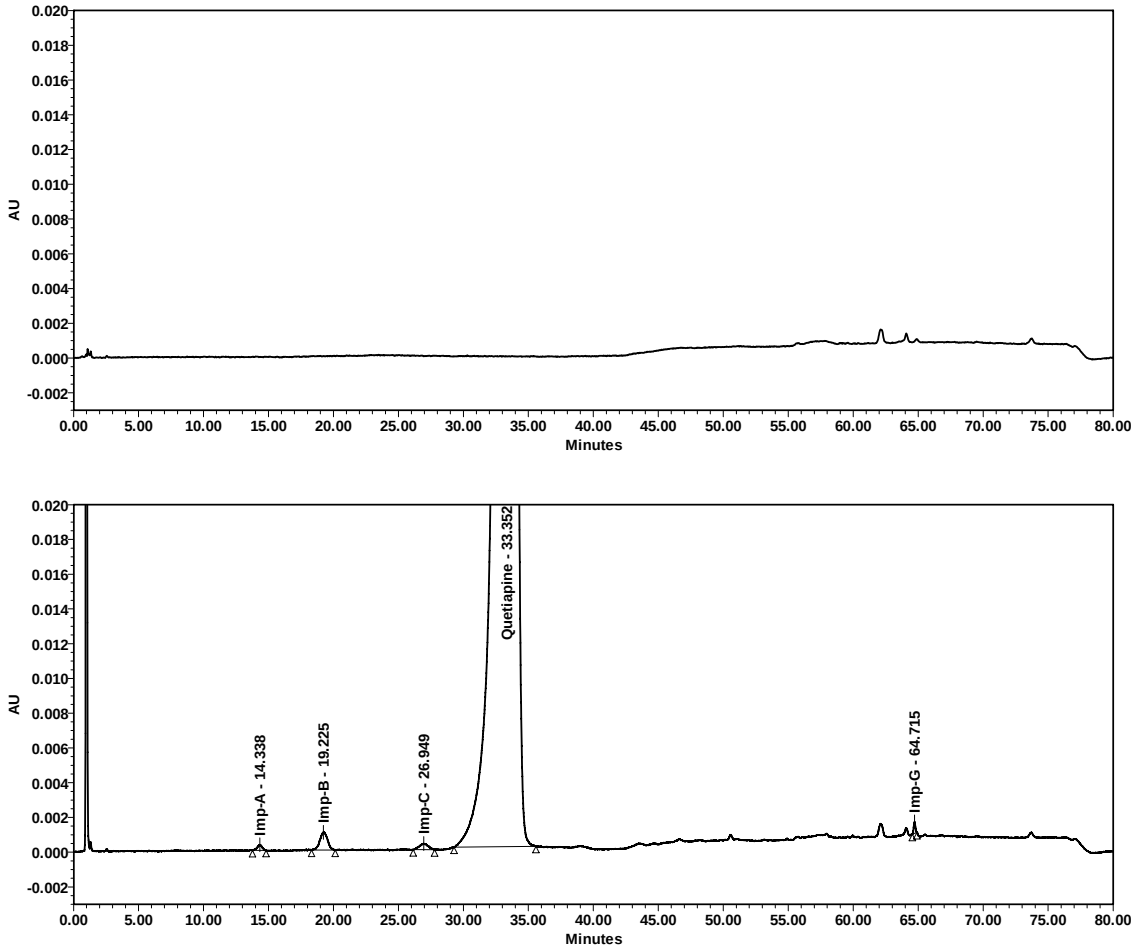


Fig.7 (D): Thermal degradation

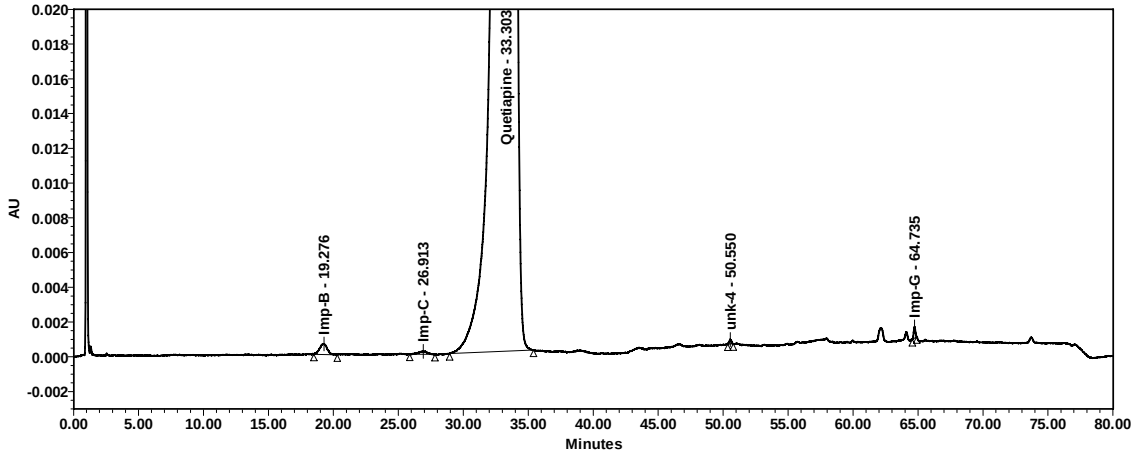


Fig.7 (E): Chromatogram of impurity-A

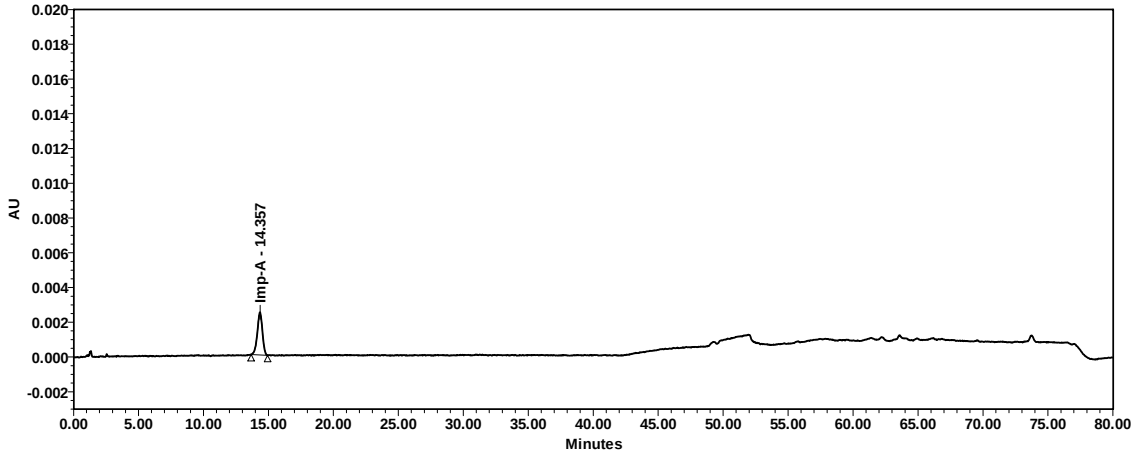


Fig.7 (F): Chromatogram of impurity-B

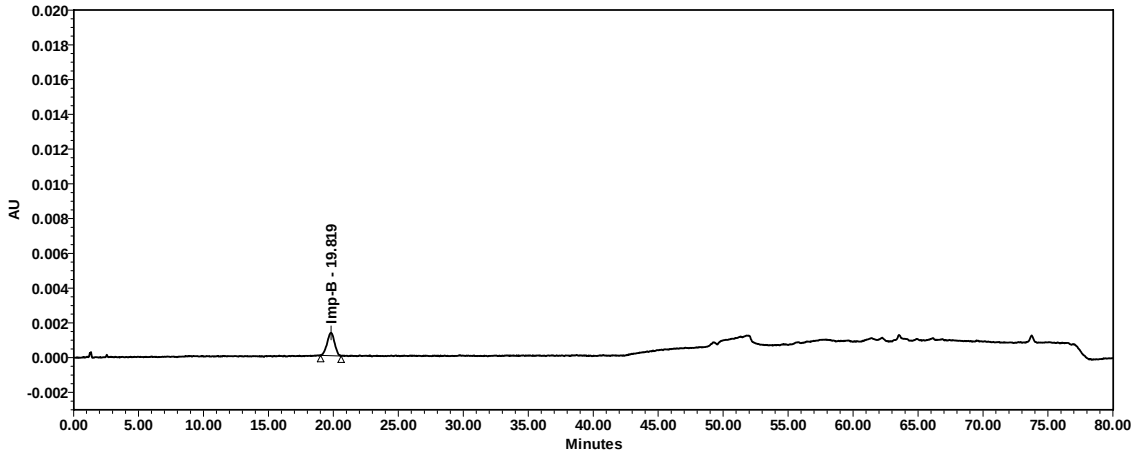


Fig.7 (G): Chromatogram of impurity-C

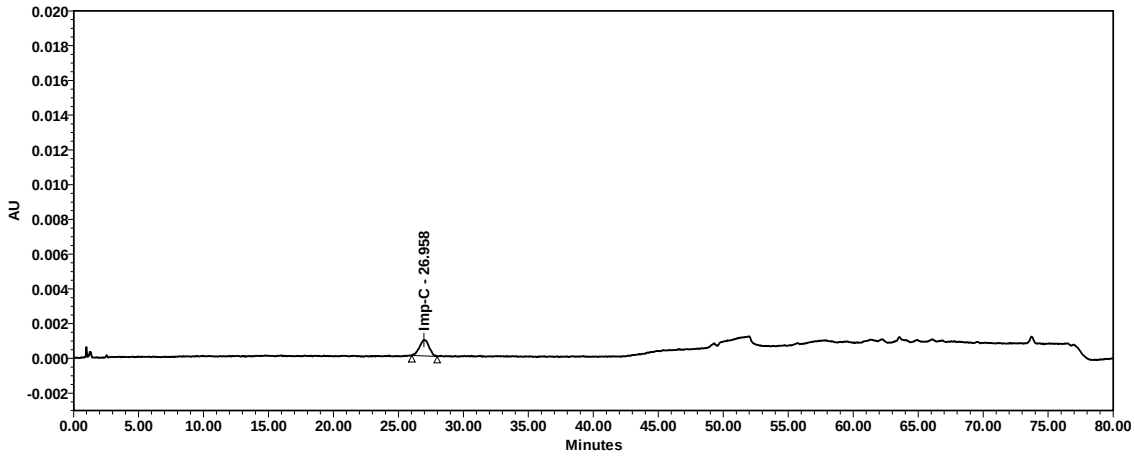


Fig.7 (H): Chromatogram of impurity-D

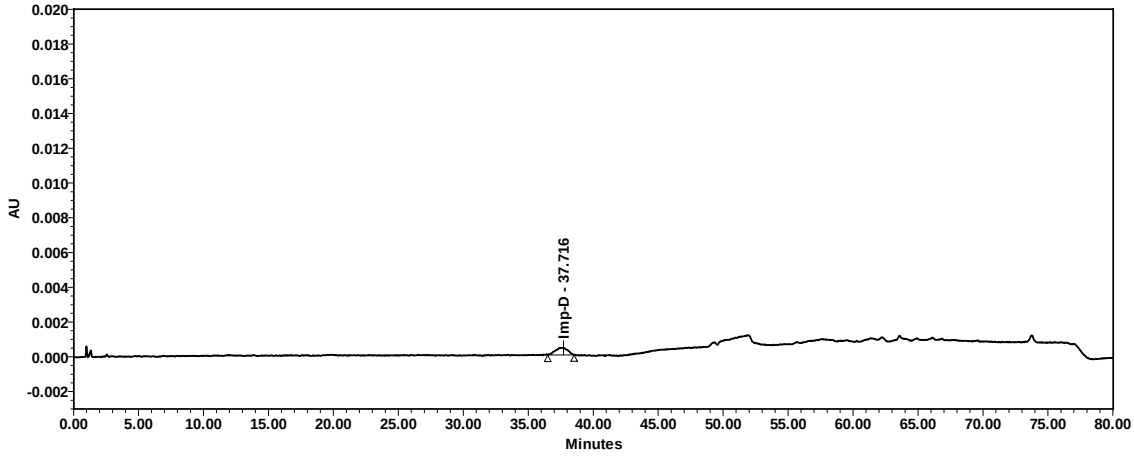


Fig.7 (I): Chromatogram of impurity-E

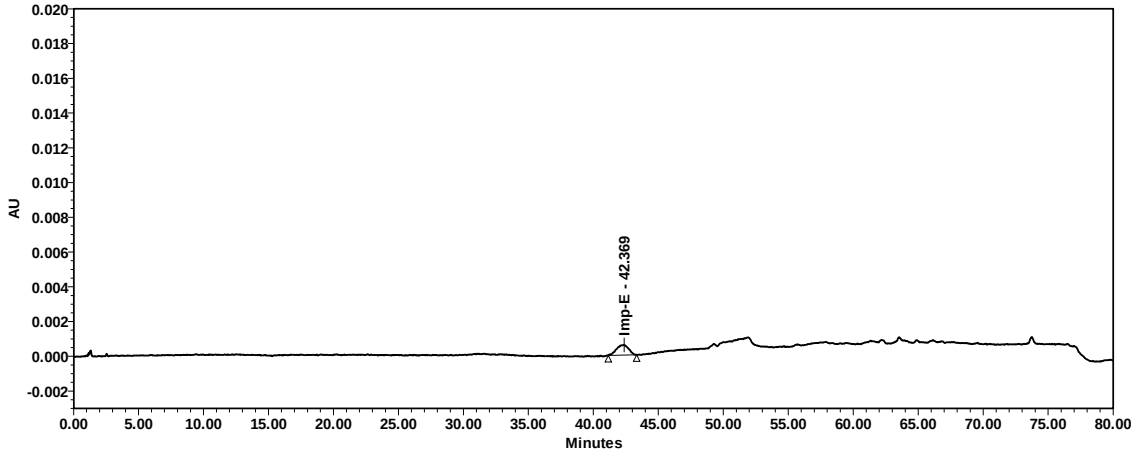


Fig.7 (J): Chromatogram of impurity-F

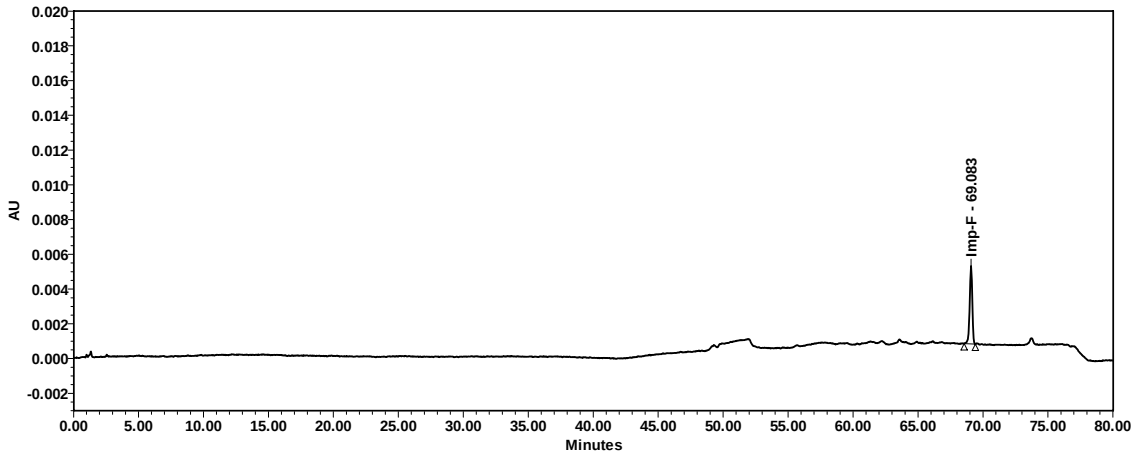


Fig.7 (K): Chromatogram of impurity-G

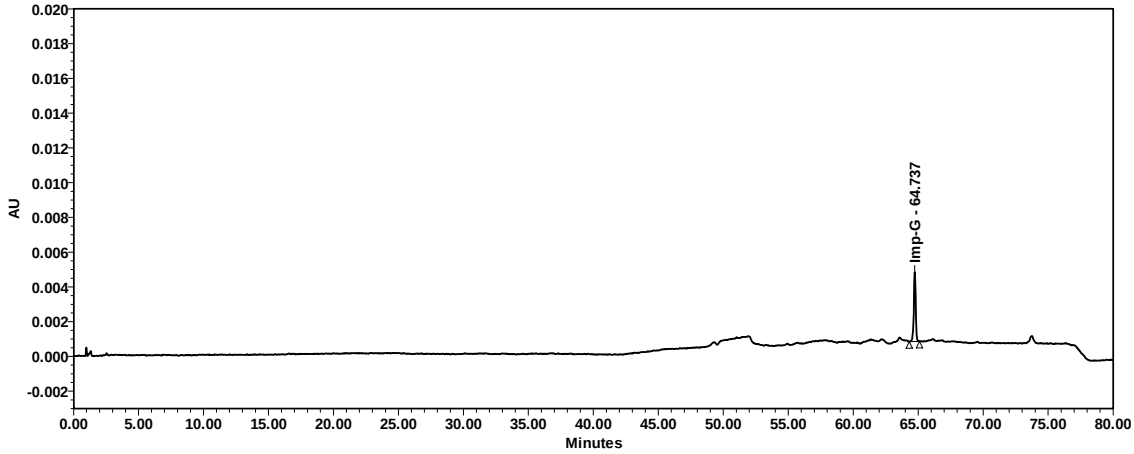
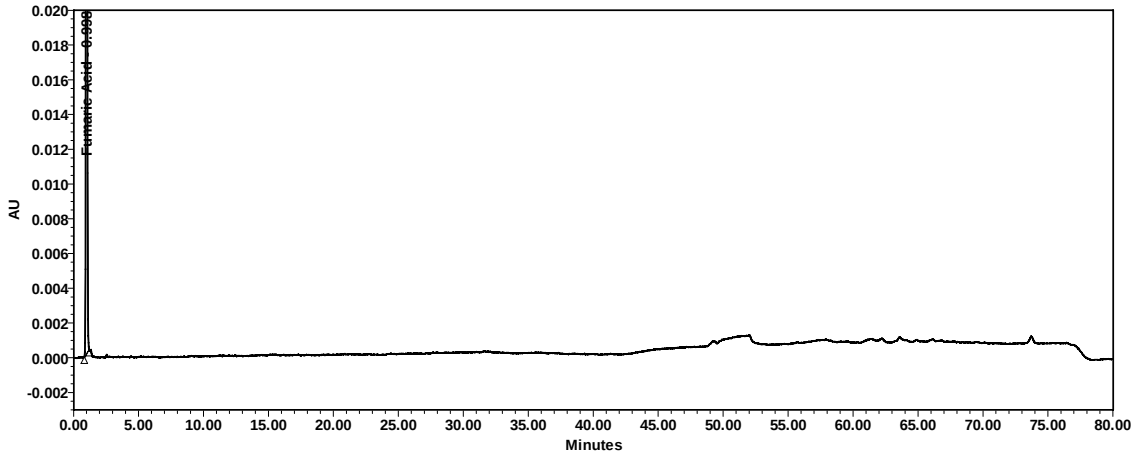


Fig.7 (L): Chromatogram of fumaric acid



5.5.2 Linearity

Linearity and relative response factor determination were established by constructing the calibration curve for QF and its impurities over the concentration range of 0.5-2.25 µg/ml (LOQ to 150% of maximum allowable % known impurities Fig [8]. Peak area of each impurity and quetiapine fumarate versus concentration were plotted and linear regression analysis performed on resultant curves. Relative Response Factor (RRF) was calculated for each known impurities by taking the ratio of impurity slope to QF slope. % impurity was calculated based on RRF value [19]. Statistical data summarized in table 6.

Table 6(A): Linearity and RRT and RRF

Sr. No.	Linearity Level	Impurity-A		Impurity –B		Impurity -C		QF	
		Conc. (%)	Area	Conc. (%)	Area	Conc. (%)	Area	Conc. (%)	Area
1.	LOQ (Level-1)	0.05	22693	0.05	20338	0.05	15316	0.05	11488
2.	60% (Level-2)	0.08	40516	0.09	34872	0.09	27976	0.06	14253
3.	80% (Level-3)	0.11	53447	0.12	46942	0.11	38004	0.08	18598
4.	100% (Level-4)	0.14	66147	0.15	58360	0.14	47941	0.10	25416
5.	150% (Level-5)	0.21	100264	0.22	87225	0.22	70490	0.15	39229
Correlation coefficient (r)		0.99888		0.99968		0.99597		0.99879	
Slope of regression line		475412		394495		326593		279105	
Y-intercept		514.78		-158.97		101.11		-2764.4	
Residual sum of squares		7679461		1660951		14076936		1184090	
RRF		1.70		1.41		1.17		1.00	

Table 6(B): Linearity and RRT and RRF

Sr. No.	Linearity level	Impurity-D		Impurity –E		Impurity -F		Impurity-G	
		Conc. (%)	Area	Conc. (%)	Area	Conc. (%)	Area	Conc. (%)	Area
1.	**LOQ (Level-1)**	0.05	10748	0.05	14984	0.05	20030	0.05	12494
2.	**60% (Level-2)**	0.09	15757	0.09	26815	0.08	36065	0.09	22729
3.	**80% (Level-3)**	0.12	23599	0.12	35425	0.11	48050	0.12	32250
4.	**100% (Level-4)**	0.15	28593	0.15	43742	0.14	59613	0.15	40037
5.	**150% (Level-5)**	0.22	45870	0.22	65841	0.20	89500	0.22	60784
Correlation coefficient (r)		0.99382		0.99968		0.99858		0.99959	
Slope of regression line		210189		297953		453460		285308	
Y-intercept		-1570.4		-180.71		-1949.8		-2290.1	
Residual sum of squares		9102257		941897		7782186		1108623	
RRF		0.75		1.07		1.62		1.02	

RRF = Relative response factor

Fig 8: Linearity chromatograms

Fig.8 (A): Linearity level-1

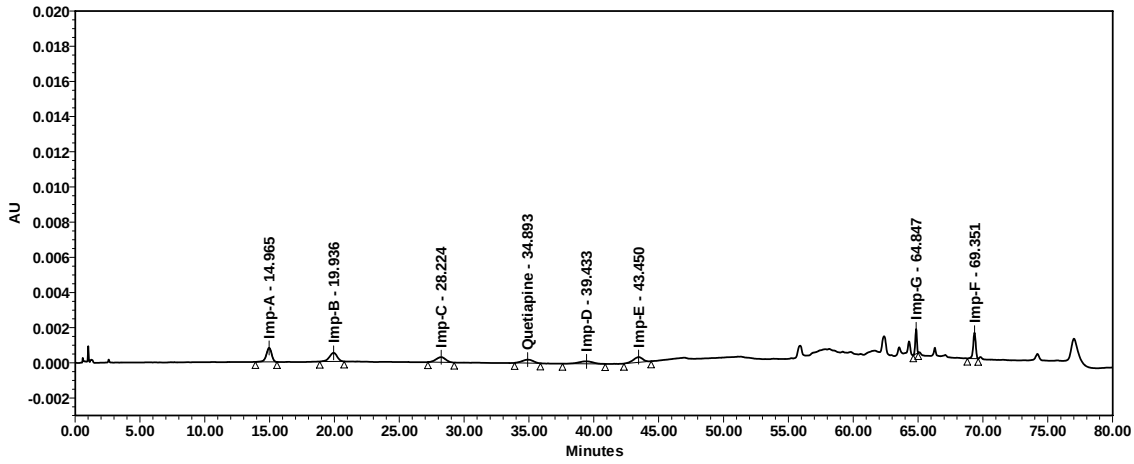


Fig.8 (B): Linearity level-2

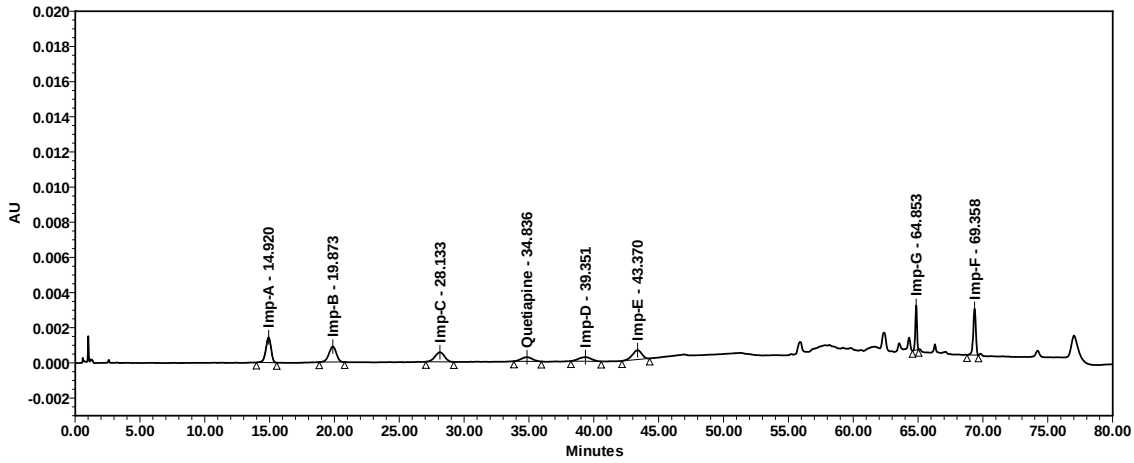


Fig.8 (C): Linearity level-3

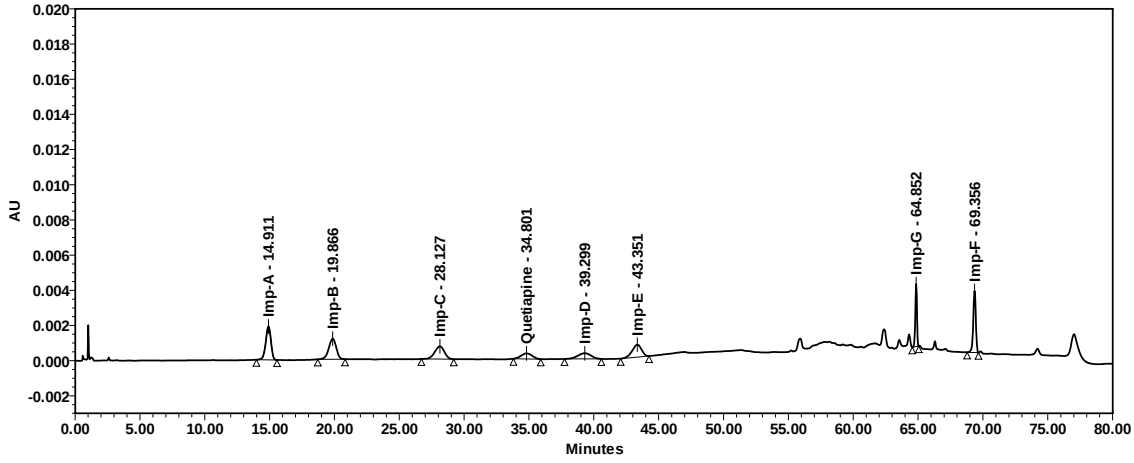


Fig.8 (D): Linearity level-4

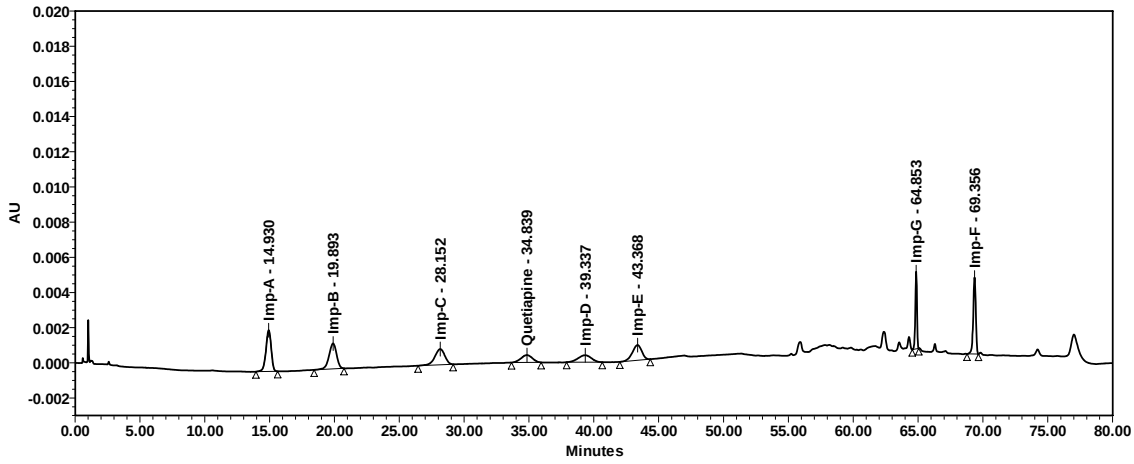
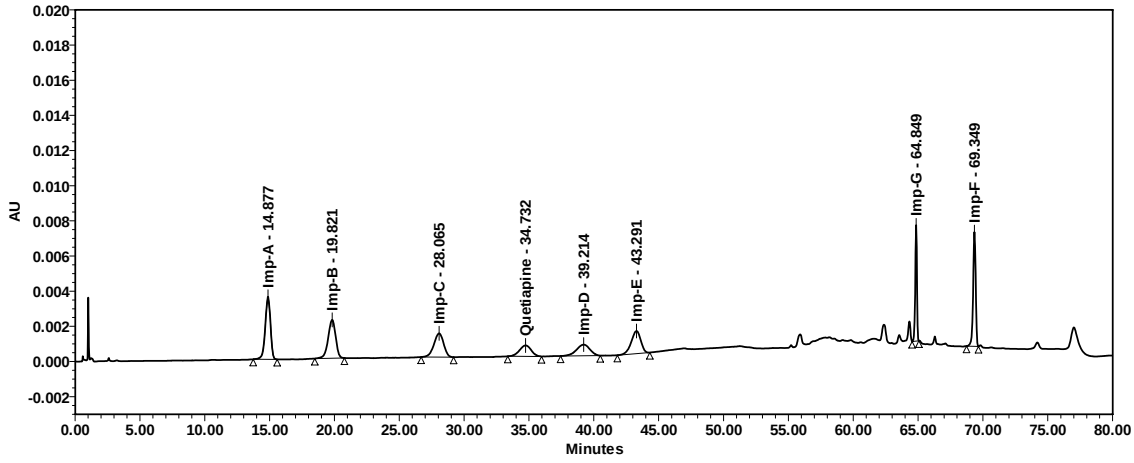


Fig.8 (E): Linearity level-5



5.5.3 Precision at LOQ

The LOQ concentrations for impurities were determined 0.5 µg/ml as 0.05 % with respect to sample concentration of QF. The reproducibility at LOQ was determined by six injections of composite mixture of impurities and quetiapine solution. % RSD of area for each impurities and quetiapine were determined as mentioned in table 7 and Fig.9.

Fig 9: Chromatogram of precision at LOQ

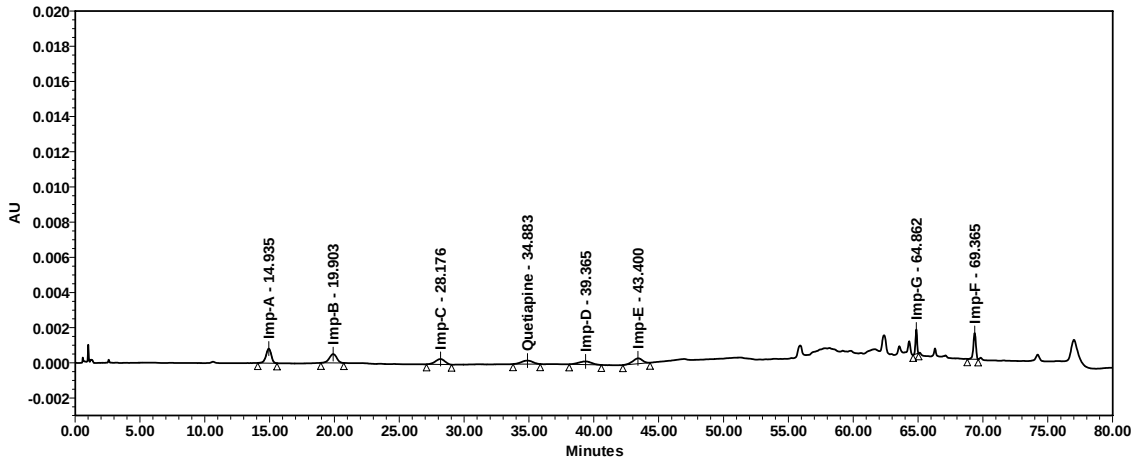


Table 7: Precision at LOQ

	Peak Area							
Injection	**Imp A**	**Imp B**	**Imp C**	**QF**	**Imp D**	**Imp E**	**Imp F**	**Imp G**
1	23275	19910	15773	11870	12209	15712	20223	12188
2	23631	20121	15906	12571	12069	15192	20139	12005
3	22990	20579	14778	12383	12442	15298	19984	11946
4	23469	20410	14954	12118	12856	16039	20146	12062
5	22531	20380	15439	12639	10758	16096	20321	12115
6	22493	20287	15932	12168	11759	15996	20123	12099
Mean Area	23064.8	20281.2	15463.7	12291.5	12015.5	15722.2	20156.0	12069.2
SD	478.7	236.0	498.2	293.3	717.7	394.0	112.0	85.4
%RSD	2.08	1.16	3.22	2.39	5.97	2.51	0.56	0.71

5.5.4 Precision

Method precision was established by calculating impurities in six sample preparations. To demonstrate the method precision all known impurities were spiked at 0.15 % level to all six sample preparation fig 4. All impurities were calculated by formula fig 10 using the RRF value as established in linearity study. Method precision data are summarized in table 8.

Table 8: Summary of precision

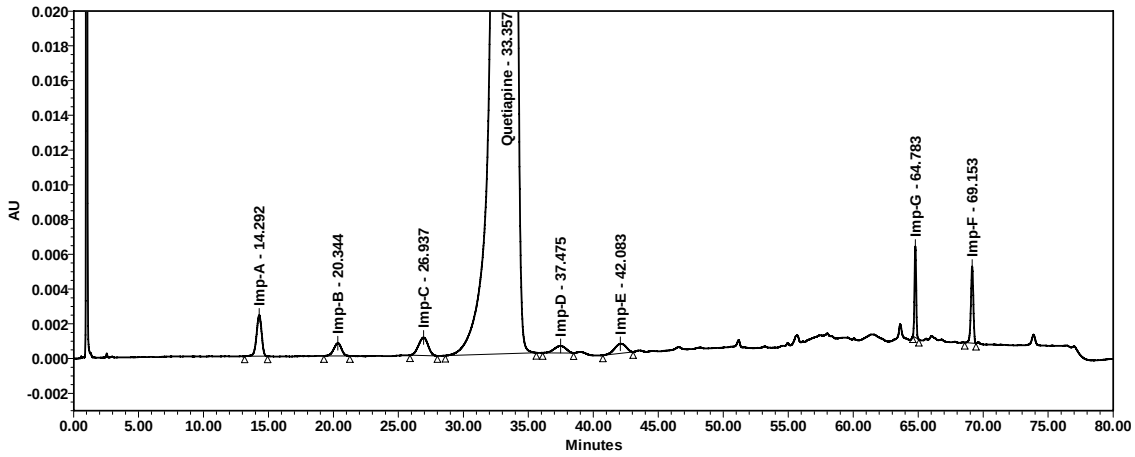
Day-1									
Preparation	**Imp-A (%)**	**Imp-B (%)**	**Imp-C (%)**	**Imp-D (%)**	**Imp-E (%)**	**Imp-F (%)**	**Imp-G (%)**	**Unk max imp (%)**	**Total imps**

									(%)
1	0.16	0.09	0.19	0.14	0.13	0.15	0.20	BQL	1.06
2	0.16	0.09	0.19	0.14	0.13	0.15	0.19	BQL	1.05
3	0.16	0.09	0.20	0.14	0.13	0.15	0.19	BQL	1.06
4	0.16	0.09	0.19	0.14	0.13	0.15	0.19	BQL	1.05
5	0.16	0.09	0.19	0.14	0.13	0.15	0.19	BQL	1.05
6	0.16	0.09	0.20	0.14	0.13	0.15	0.19	BQL	1.06
Mean	0.16	0.09	0.19	0.14	0.13	0.15	0.19	BQL	1.06
SD	0.000	0.000	0.005	0.000	0.000	0.000	0.004	BQL	0.005
%RSD	0.00	0.00	2.72	0.00	0.00	0.00	2.15	BQL	0.52
95% confidence interval	0.000	0.000	±0.005	0.000	0.000	0.000	±0.004	BQL	±0.006
Day-2									
Preparation	**Imp-A (%)**	**Imp-B (%)**	**Imp-C (%)**	**Imp-D (%)**	**Imp-E (%)**	**Imp-F (%)**	**Imp-G (%)**	**Unk max imp (%)**	**Total imps (%)**
1	0.15	0.09	0.19	0.14	0.13	0.15	0.19	BQL	1.04
2	0.15	0.09	0.19	0.13	0.13	0.15	0.19	BQL	1.03
3	0.15	0.09	0.19	0.14	0.13	0.15	0.18	BQL	1.03
4	0.15	0.09	0.19	0.13	0.13	0.15	0.18	BQL	1.02
5	0.15	0.09	0.19	0.14	0.13	0.15	0.18	BQL	1.03
6	0.15	0.09	0.19	0.13	0.13	0.15	0.18	BQL	1.02
Mean	0.15	0.09	0.19	0.14	0.13	0.15	0.18	BQL	1.03
SD	0.000	0.000	0.000	0.005	0.00	0.00	0.005	BQL	0.008
%RSD	0.00	0.00	0.00	3.91	0.000	0.000	2.87	BQL	0.73
95% confidence interval	0.000	0.000	0.000	±0.006	0.000	0.000	±0.005	NA	±0.008

SD = Standard Deviation, %RSD = Relative Standard Deviation

Unk imp = Unknown impurity, CI = confidence interval

Fig. 10: Chromatogram of method precision



5.5.5 Accuracy (Recovery)

The accuracy of the method was established at three levels in the range of LOQ-150% of specification limit. Calculated amount of known impurities were added in form of solution to the sample preparation to attain LOQ, 100% and 150% of specification limit as shown in fig 11(A) to 11(C). Sample preparations were prepared in triplicate for each level. Data summarized in table 9(A) to 9(G)

Fig. 11(A): Chromatogram of accuracy level-1

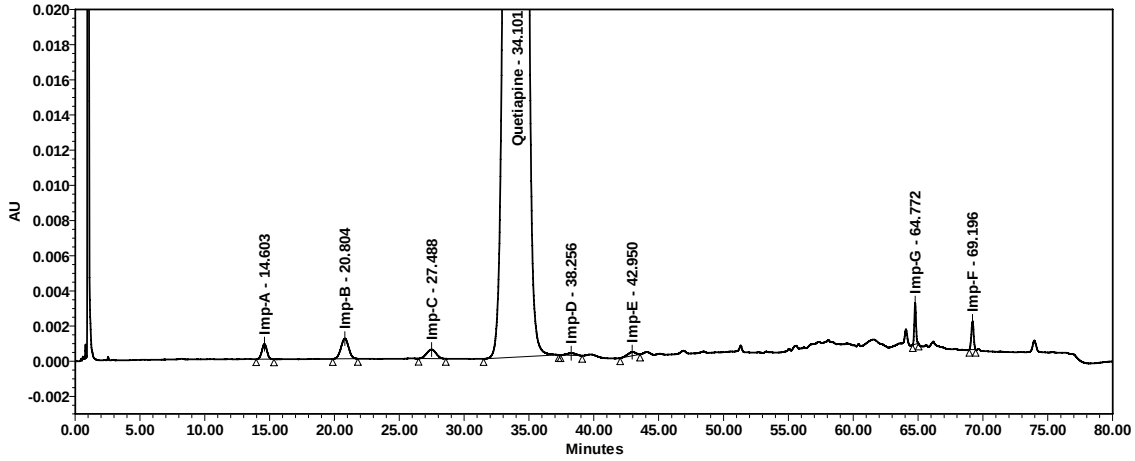


Fig. 11(B): Chromatogram of accuracy level-2

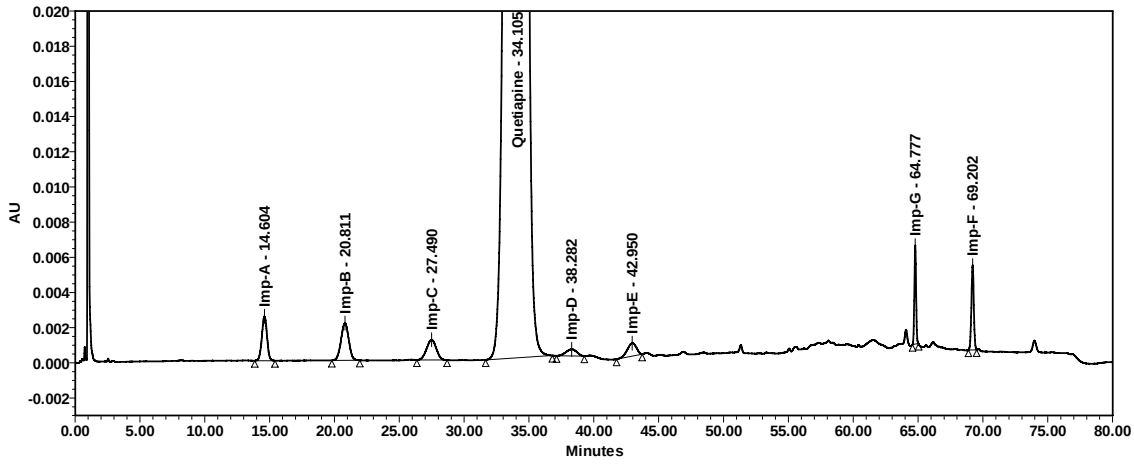


Fig. 11(C): Chromatogram of accuracy level-3

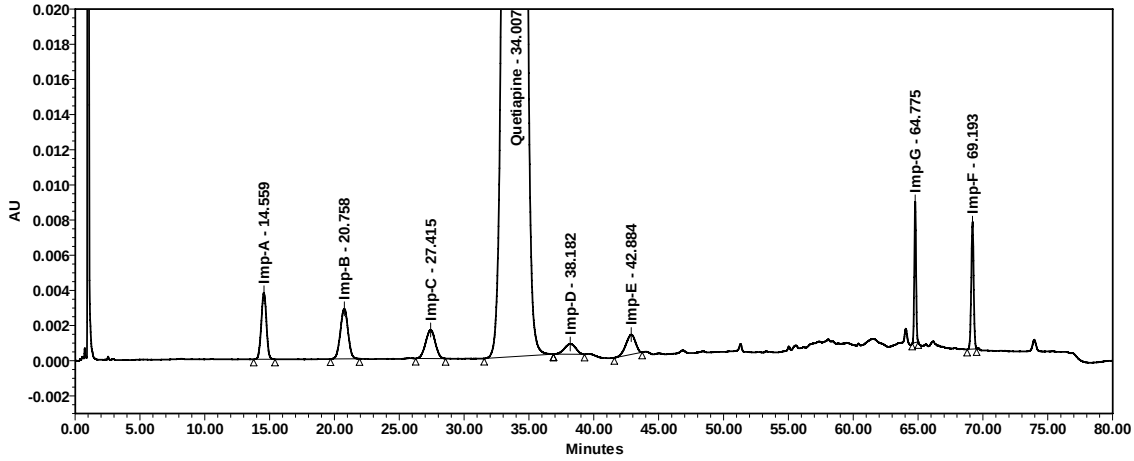


Table 9(A): Summary of recovery data for impurity-A

Level	Amount added (ppm)	Amount found (ppm)	Recovery (%)	Mean (%)
Level-1	0.5176	0.5572	107.7	108.7

(LOQ)	0.5176	0.5576	107.7	
	0.5176	0.5736	110.8	
Level-2 (100%)	1.5527	1.6588	106.8	106.4
	1.5527	1.6505	106.3	
	1.5527	1.6466	106.0	
Level-3 (150%)	2.3290	2.4806	106.5	106.3
	2.3290	2.4821	106.6	
	2.3290	2.4653	105.9	

Table 9(B): Summary of recovery data for impurity-B

Level	**Amount added (ppm)**	**Amount found (ppm)**	**Recovery (%)**	**Mean (%)**
Level-1 (LOQ)	0.5315	0.5827	109.6	109.8
	0.5315	0.5791	109.0	
	0.5315	0.5882	110.7	
Level-2 (100%)	1.5945	1.7289	108.4	108.1
	1.5945	1.7196	107.8	
	1.5945	1.7246	108.2	
Level-3 (150%)	2.3918	2.5759	107.7	107.4
	2.3918	2.5952	108.5	
	2.3918	2.5362	106.0	

Table 9(C): Summary of recovery data for impurity-C

Level	Amount added (ppm)	Amount found (ppm)	Recovery (%)	Mean (%)
Level-1 (LOQ)	0.5284	0.5969	113.0	113.3
	0.5284	0.5843	110.6	
	0.5284	0.6151	116.4	
Level-2 (100%)	1.5851	1.7348	109.4	109.7
	1.5851	1.7564	110.8	
	1.5851	1.7247	108.8	
Level-3 (150%)	2.3777	2.5698	108.1	108.2
	2.3777	2.5961	109.2	
	2.3777	2.5520	107.3	

Table 9(D): Summary of recovery data for impurity-D

Level	Amount added (ppm)	Amount found (ppm)	Recovery (%)	Mean (%)
Level-1 (LOQ)	0.4543	0.3188	70.2	72.5
	0.4543	0.3275	72.1	
	0.4543	0.3416	75.2	
Level-2 (100%)	1.3628	1.1901	87.3	86.0
	1.3628	1.1670	85.6	
	1.3628	1.1613	85.2	
Level-3 (150%)	2.0442	1.9008	93.0	93.5
	2.0442	1.9575	95.8	
	2.0442	1.8747	91.7	

Table 9(E): Summary of recovery data for impurity-E

Level	Amount added (ppm)	Amount found (ppm)	Recovery (%)	Mean (%)
Level-1 (LOQ)	0.4865	0.3468	71.3	73.3
	0.4865	0.3563	73.2	
	0.4865	0.3673	75.5	
Level-2 (100%)	1.4594	1.3581	93.1	92.9
	1.4594	1.3504	92.5	
	1.4594	1.3590	93.1	
Level-3 (150%)	2.1891	2.1795	99.6	99.8
	2.1891	2.1706	99.2	
	2.1891	2.2050	100.7	

Table 9(F): Summary of recovery data for impurity-F

Level	Amount added (ppm)	Amount found (ppm)	Recovery (%)	Mean (%)
Level-1 (LOQ)	0.5075	0.4909	96.7	95.1
	0.5075	0.4701	92.6	
	0.5075	0.4870	96.0	
Level-2 (100%)	1.5225	1.5378	101.0	101.2
	1.5225	1.5485	101.7	
	1.5225	1.5357	100.9	
Level-3 (150%)	2.2837	2.3408	102.5	102.4
	2.2837	2.3496	102.9	
	2.2837	2.3274	101.9	

Table 9(G): Summary of recovery data for impurity-G

Level	Amount added (ppm)	Amount found (ppm)	Recovery (%)	Mean (%)
Level-1 (LOQ)	0.5255	0.5986	113.9	115.4
	0.5255	0.6025	114.7	
	0.5255	0.6180	117.6	
Level-2 (100%)	1.5765	1.8060	114.6	114.4
	1.5765	1.7650	112.0	
	1.5765	1.8361	116.5	
Level-3 (150%)	2.3648	2.6920	113.8	115.0
	2.3648	2.7917	118.1	
	2.3648	2.6713	113.0	

5.5.6 Solution stability

The sample preparation of Quetiapine fumarate API was prepared and % related impurities were determined as per method. The diluted standard solution and sample preparation were stored upto 52 hours and 47 hours respectively at room temperature. The diluted standard solution was reanalyzed after 29 hours and 52 hours against freshly prepared diluted standard solution and results are shown in Table-10(A). The sample preparation was re-analyzed for impurities after 27 hours and 47 hours against freshly prepared diluted standard preparation. The impurity results obtained at different time intervals were compared with the initial value and shown in Table-10(B).

Table 10(A): Standard solution stability

For diluted standard preparation			
Time	**Condition**	**% Assay**	**Absolute difference (%)**
Initial	Room temperature	100.0	Not applicable
After 29 hours	Room temperature	101.1	1.1
After 52 hours	Room temperature	98.2	1.8

Table 10(B): Sample solution stability

For sample preparation				
Time	**Condition**	**% Impurity**	**Absolute diff.**	**% Difference**
A) For impurity-A (%)				
Initial	Room temperature	0.16	Not applicable	Not applicable
After 27 hours	Room temperature	0.16	Not applicable	0.0
After 47 hours	Room temperature	0.16	Not applicable	0.0
B) For impurity -B (%)				
Initial	Room temperature	0.09	Not applicable	Not applicable
After 27 hours	Room temperature	0.09	0.00	Not applicable
After 47 hours	Room temperature	0.09	0.00	Not applicable
C) For impurity -C (%)				
Initial	Room temperature	0.19	Not applicable	Not applicable
After 27 hours	Room temperature	0.19	Not applicable	0.0
After 47 hours	Room temperature	0.19	Not applicable	0.0
D) For impurity-D (%)				
Initial	Room temperature	0.14	Not applicable	Not applicable
After 27 hours	Room temperature	0.14	Not applicable	0.0
After 47 hours	Room temperature	0.15	Not applicable	7.1
E) For impurity-E (%)				
Initial	Room temperature	0.13	Not applicable	Not applicable
After 27 hours	Room temperature	0.13	Not applicable	0.0
After 47 hours	Room temperature	0.13	Not applicable	0.0
F) For impurity-F (%)				
Initial	Room temperature	0.15	Not applicable	Not applicable
After 27 hours	Room temperature	0.15	Not applicable	0.0
After 47 hours	Room temperature	0.15	Not applicable	0.0
G) For impurity-G (%)				
Initial	Room temperature	0.20	Not applicable	Not applicable
After 27 hours	Room temperature	0.19	Not applicable	5.0
After 47 hours	Room temperature	0.19	Not applicable	5.0
H) For any single maximum unknown impurity (%)				
Initial	Room temperature	BQL	Not applicable	Not applicable
After 27 hours	Room temperature	BQL	Not applicable	Not applicable
After 47 hours	Room temperature	BQL	Not applicable	Not applicable
I) For total impurities (%)				
Initial	Room temperature	1.06	Not applicable	Not applicable
After 27 hours	Room temperature	1.05	Not applicable	0.9
After 47 hours	Room temperature	1.06	Not applicable	0.0

5.3.6 Robustness:

Procedure:

The robustness of the method was established by making deliberate minor variations in the following method parameters.

1. Change flow rate by ± 10% [i.e. flow rate 1.35mL/min and 1.65mL/min]
2. Change the minor component in the mobile phase by ± 2 % absolute (i.e. Buffer: Acetonitrile: Methanol:: 68:15:17 and Buffer: Acetonitrile: Methanol:: 72:11:17)
3. Change column oven temperature by ± 5°C. [i.e. column oven temperature 40°C and 50°C]
4. Change in buffer pH by ±0.2 unit (Use buffer pH 9.0 and 9.4)
5. Change in column lot.

Blank, resolution solution, diluted standard preparation and the sample preparation were prepared and injected. The effect of changes was observed on system suitability parameters and on impurities results and shown in table-11(A) & 11(B) respectively.

Table 11(A): System suitability summary of robustness study

Condition	System Suitability parameters		
	Resolution between imp-A and imp-B (NLT 2.5)	**Resolution between quetiapine and Imp-D (NLT 1.5)**	**% RSD of six replicate injections of diluted standard preparation (NMT 5.0)**
A) Change in flow rate			
Normal Condition* (1.5 ml per minute)	7.22	2.57	3.82
1.35 ml per minute	5.76	2.42	2.30
1.65 ml per minute	5.50	2.29	1.52
B) Change in column oven temperature			
Normal condition**(45°C)	5.43	2.33	1.11
oven temperature 40°C	5.50	2.28	1.03
oven temperature 50°C	5.13	2.28	0.71
C) Change in buffer pH			
Normal condition (pH-9.2)*	7.22	2.57	3.82

Buffer pH 9.0	4.38	2.40	1.44
Buffer pH 9.4	6.51	2.51	3.05
D) Change in column lot.			
Normal*Sr. No. 0126373161	7.22	2.57	3.82
Changed** Sr. No. 0126380041	5.43	2.33	1.11
E) Change in mobile phase composition			
Normal: Buffer: Acetonitrile: Methanol :: 70:13:17**	5.43	2.33	1.11
Buffer: Acetonitrile: Methanol :: 72:11:17	7.03	2.47	3.59
Buffer: Acetonitrile: Methanol :: 68:15:17	4.30	1.89	1.20

* Data taken from the method precision.

** Data taken from the intermediate precision.

Table 11(B): System suitability summary of robustness study

Condition	% Impurities								
	Imp-A (%)	**Imp-B (%)**	**Imp-C (%)**	**Imp-D (%)**	**Imp-E (%)**	**Imp-F (%)**	**Imp-G (%)**	**Max Unk Imp (%)**	**Total Imps (%)**
A) Change in flow rate									
Normal condition (1.50 ml per minute)*	0.16	0.09	0.19	0.14	0.13	0.15	0.20	BQL	1.06
(1.35 ml /minute)	0.15	0.08	0.18	0.15	0.13	0.15	0.20	BQL	1.04
Absolute difference from normal condition	NA	0.01	NA	NA	NA	NA	NA	NA	NA
% Difference from normal condition	6.3	NA	5.3	7.1	0.0	0.0	0.0	NA	1.9
(1.65 ml / minute)	0.16	0.08	0.19	0.13	0.13	0.15	0.19	BQL	1.03
Absolute difference from normal condition	NA	0.01	NA	NA	NA	NA	NA	NA	NA
% difference from normal condition	0.0	NA	0.0	7.1	0.0	0.0	5.0	NA	2.8
B) Change in column oven temperature									
Normal condition (45.0°C)**	0.15	0.09	0.19	0.14	0.13	0.15	0.19	BQL	1.04

Condition	% Impurities								
	Imp-A (%)	**Imp-B (%)**	**Imp-C (%)**	**Imp-D (%)**	**Imp-E (%)**	**Imp-F (%)**	**Imp-G (%)**	**Max Unk Imp (%)**	**Total Imps (%)**
Change in oven temperature by + 5°C(50.0°C)	0.15	0.08	0.19	0.13	0.13	0.15	0.19	BQL	1.02
Absolute difference from normal condition	NA	0.01	NA	NA	NA	NA	NA	NA	NA
% difference from normal condition	0.0	NA	0.0	7.1	0.0	0.0	0.0	NA	1.9
Change in oven temperature by - 5°C(40.0°C)	0.15	0.08	0.18	0.13	0.22	0.15	0.18	BQL	1.09
Absolute difference from normal condition	NA	0.01	NA	NA	NA	NA	NA	NA	NA
% difference from normal condition	0.0	NA	5.3	7.1	69.2	0.0	5.3	NA	4.8
C) Change in buffer pH									
Normal condition (pH-9.2)*	0.16	0.09	0.19	0.14	0.13	0.15	0.20	BQL	1.06
Change in buffer pH by -0.2 unit (pH-9.0)	0.15	0.08	0.18	0.13	0.14	0.15	0.19	BQL	1.02
Absolute difference from normal condition	NA	0.01	NA	NA	NA	NA	NA	NA	NA
% Difference from normal condition	6.3	NA	5.3	7.1	7.7	0.0	5.0	NA	3.8
Change in buffer pH by +0.2 unit (pH-9.4)	0.15	0.08	0.18	0.13	0.14	0.16	0.19	BQL	1.03
Absolute difference from normal condition	NA	0.01	NA	NA	NA	NA	NA	NA	NA
% Difference from normal condition	6.3	NA	5.3	7.1	7.7	6.7	5.0	NA	2.8
D) Change in mobile phase composition									
(Buffer: Acetonitrile: Methanol :: 70:13:17)**	0.15	0.09	0.19	0.14	0.13	0.15	0.19	BQL	1.04

Condition	% Impurities								
	Imp-A (%)	**Imp-B (%)**	**Imp-C (%)**	**Imp-D (%)**	**Imp-E (%)**	**Imp-F (%)**	**Imp-G (%)**	**Max Unk Imp (%)**	**Total Imps (%)**
Changed in minor component by -2% (Buffer: Acetonitrile: Methanol :: 72:11:17)	0.14	0.07	0.17	0.23	0.13	0.14	0.20	BQL	1.08
Absolute difference from normal condition	NA	0.02	NA	NA	NA	NA	NA	NA	NA
% Difference from normal condition	6.7	NA	10.5	64.3	0.0	6.7	5.3	NA	3.8
Changed in minor component by +2% (Buffer: Acetonitrile: Methanol :: 68:15:17)	0.15	0.08	0.19	0.11	0.18	0.14	0.18	BQL	1.03
Absolute difference from normal condition	NA	0.01	NA	NA	NA	NA	NA	NA	NA
% Difference from normal condition	0.0	NA	0.0	21.4	38.5	6.7	5.3	NA	1.0
E) Change in column lot									
Normal: XT/RP8/150-5/19 Sr. No. 0126373161*	0.16	0.09	0.19	0.14	0.13	0.15	0.20	BQL	1.06
Changed: XT/RP8/150-5/23 Sr. No. 0126380041**	0.15	0.09	0.19	0.14	0.13	0.15	0.19	BQL	1.04
Absolute difference	NA	0.00	NA	NA	NA	NA	NA	NA	NA
% Difference from normal condition	6.3	NA	0.0	0.0	0.0	0.0	5.0	NA	1.9

6. Quantitation and Calculation formulae

6.1 Relative Standard Deviation (%)

$$\text{Relative Standard Deviation (\%)} = \frac{SD \times 100}{\overline{X}}$$

By Microsoft excel

Average = AVERAGE (Number 1)

Standard Deviation = STDEV (Number 1)

6.2 Calculation formulae in Linearity and RRF

Correlation coefficient (r) = $\frac{n\ (\sum xy)\ -\ (\sum x)(\sum y)}{\sqrt{\{[n\sum x^2-(\sum x)^2]\ [n\sum y^2-(\sum y)^2]\}}}$

Correlation coefficient (r) = CORREL (Array 1, Array 2)

Slope of regression line = $\frac{n\ (\sum xy)\ -\ (\sum x)(\sum y)}{n\sum x^2-(\sum x)^2}$

Slope of regression line = SLOPE (Array 1, Array 2)

y- intercept = $A = \bar{y} - B\bar{x}$

y- intercept = INTERCEPT (Array 1, Array 2)

Residual sum of squares = $\sum (Y_{original} - Y_{calculated})^2$

Where,

$Y_{calculated}$ = A + Bx

n = Number of measurements

x = Individual concentration

y = Individual area

$\bar{x}$ = Average concentration

$\bar{y}$ = Average area

Residual sum of squares = SUMXMY2 (Array 1, TREND (Array 1, Array 2, Array2))

$$\text{Relative Response Factor} = \frac{\text{Slope of regression line for impurity}}{\text{Slope of regression line for standard}}$$

6.3 Accuracy (% Recovery)

$$\% \text{ Recovery} = \frac{\text{Ammount of sbustance (mg) found}}{\text{Ammount of substance (mg) added}} \text{ x } 100$$

6.4 % Impurity in Quetiapine fumarate drug substance

$$\% \text{ Impurity} = \frac{\text{Asp}}{\text{Astd}} \times \frac{\text{Std. wt}}{\text{Dilution}} \times \frac{\text{Dilution}}{\text{Spl Wt}} \times \frac{1}{\text{RRF}} \times \text{Std. assay \% (as such)}$$

e.g.

$$\% \text{ Impurity-A} = \frac{66288}{39225} \times \frac{15.6}{100} \times \frac{2}{200} \times \frac{50}{50.07} \times \frac{1}{1.70} \times 99.6$$

$$\% \text{ Impurity-A} = 0.15$$

$$\% \text{ Impurity-B} = \frac{30952}{39225} \times \frac{15.6}{100} \times \frac{2}{200} \times \frac{50}{50.07} \times \frac{1}{1.41} \times 99.6$$

$$\% \text{ Impurity-B} = 0.09$$

$$\% \text{ Impurity-C} = \frac{56934}{39225} \times \frac{15.6}{100} \times \frac{2}{200} \times \frac{50}{50.07} \times \frac{1}{1.17} \times 99.6$$

$$\% \text{ Impurity-C} = 0.19$$

$$\% \text{ Impurity-D} = \frac{26564}{39225} \times \frac{15.6}{100} \times \frac{2}{200} \times \frac{50}{50.07} \times \frac{1}{0.75} \times 99.6$$

$$\% \text{ Impurity-D} = 0.14$$

$$\% \text{ Impurity-E} = \frac{35062}{39225} \times \frac{15.6}{100} \times \frac{2}{200} \times \frac{50}{50.07} \times \frac{1}{1.07} \times 99.6$$

$$\% \text{ Impurity-E} = 0.13$$

$$\% \text{ Impurity-F} = \frac{61764}{39225} \times \frac{15.6}{100} \times \frac{2}{200} \times \frac{50}{50.07} \times \frac{1}{1.62} \times 99.6$$

% Impurity-F = 0.15

$$\% \text{ Impurity-G} = \frac{48586}{39225} \times \frac{15.6}{100} \times \frac{2}{200} \times \frac{50}{50.07} \times \frac{1}{1.02} \times 99.6$$

% Impurity-G = 0.19

% Total impurities = Total known impurities + Total unknown impurities

% Total impurities = (0.15 + 0.09 + 0.19 + 0.14 + 0.13 +0.15 + 0.19) + (0.00)

% Total impurities = 1.04

Asp : Area of impurity in sample

Astd : Mean area of diluted standard

Std. wt : Std weight in mg

Spl wt : Sample weight in mg

6.5 95% Confidence Interval (CI)

$$95\% \text{ Confidence Interval (CI)} = \pm \frac{t\alpha \times SD}{\sqrt{n}}$$

Where,

SD = Standard Deviation

$\overline{X}$ = Average concentration

n = Number of measurements

t_α = t value at n-1 from t table (two tail)

95% Confidence Interval (CI) = SD (2.57058)/SQRT (6)

References

[1] Physicians' Desk Reference, Thomson Healthcare INC., PDR 62 ed 2008 p.919-920

[2] http://www.freshpatents.com/Novel-polymorph-of-quetiapine-fumarate-and-a-process-for-its-preparation-dt20061005ptan20060223994.php

[3] http://img.orion.fi/liitteet/200869.pdf

[4] http://www.pbs.gov.au/pi/appseroq10807.pdf

[5] 2(17) Seroquel® Product Information CNS.000-149-416.5.0

[6] Vijaya Kumar M., Muley, Indian Drugs 41-5(2004) 272-275

[7] Bharathi C.H., Prabahar K. J., Prasad C. H. S., Srinivasa Rao M. Trinadhachary G. N., Handa V. K., Dandala R., Naidu A Pharmazie 63(2008) pp. 14-19

[8] Julia Sachse, Johannes Köller Sebastian Härtter and Christoph Hiemke, Journal of Chromatogr.B, 830 (2006) 342-348

[9] B. Barrett , M. Hol¡capek , J. Huclov´a ,, V. Bo¡rek-Dohalsk´y , P. Fejt, N¡emeca, I. Jel´ýnek Journal of Pharmaceutical and Biomedical Analysis, 44(2007)498–505

[10] Mandrioli R.; Fanali S.; Ferranti A.; Raggi M, Journal of Pharmaceutical and Biomedical Analysis, 30(2002) 969-977

[11] Vasil N, Atanasov, Kamen P. Kanev and Mariana Io. Mitewa, Central European journal of medicine 3(2008) 327-331

[12] Davis PC, Wong J Gefvert O, Journal of Pharmaceutical and Biomedical Analysis, 20(1999)271-82

[13] Zhiling Zhou ; Xin Li ; Kunyan Li ; Zhihong Xie ; Zeneng Cheng ; Wenxin Peng ; Feng Wang ; Ronghua Zhu ; Huande Li ; J Chromatgraphy B 802(2004) 257-262

[14] Johan W. Dolan, Retaining Polar Compounds, 1132 LCGC Volume 19 Number 11 November 2001

[15] Craig S. Young & Raymond J. Weigand, An Efficient Approach to Column Selection in HPLC Method Development, 464 LCGC North America Volume 20 Number5 May 2002

[16] Lloyed R. Snyder, Joseph J. Krikland, Joseph L. Glajch, 2nd ed., John wiley & Sons, INC. 1997 p.299

[17] ICH guideline, Q2 (R1) step 4, Validation of Analytical Procedures: Text and Methodology (2005)

[18] ICH guideline, Q3B (R2) step 4, Impurities in new drug products (2006)

[19] Behnam Davani, Karen A. Russo, Andrzej Wilk, Lokesh Bhattacharya USP PF 31(2) (2005) 626-636.

Section-III: HPLC Method development and validation for assay of Pramipexole in Pramipexole tablets

1. Introduction to pramipexole

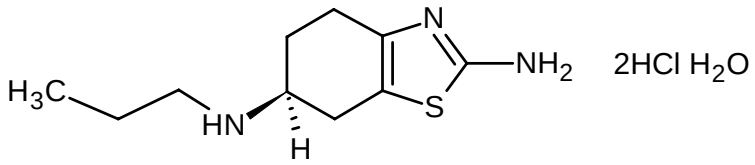
Parkinson's disease (PD) is a neurodegenerative disorder characterized by bradykinesia, rigidity, postural imbalance and tremor. The incidence of PD increases with age and on average, 2 to 3% of the population in western world will develop PD. The cause of the disease is still unknown. PD develops due to loss of neuronal functions within the basal ganglia and the substania nigra of the brain. More specifically, there is marked deficiency in the nigrostrital dopamine (DA) system due to degeneration of nigral DA neurons. Thus, restoration of the dopaminergic transmission forms the central strategy for the treatment of PD.

Pramipexole is a synthetic amino-benzothiazole derivative. It has been shown to be a selective and specific full DA receptor agonist with high affinity and selectivity for the DA D2 receptor subfamily, and particularly the D3 receptor subtype [1].

Innovator: Boehringer Ingelheim, Germany.

1.1 Physico-chemical properties

Pramipexole is chemically (S)-2-amino-4,5,6,7-tetrahydro-6-(propylamino) benzothiazole. Commercially available tablets conations Pramipexole dihydrochloride monohydrate salt (Fig.1) in formulation (Sifrol®, Mirapex®, and Mirapexin®) [3]. Molecular formula of Pramipexole dihydrochloride monohydrate is $C_{10}H_{17}N_3S$ 2HCl H_2O having molecular weight 302.28 g/mole. Its dissociation constants are pKa_1 = 5.0 and pKa_2 = 9.6, partition coefficient Log *P* (octanol water) is 0.87. Melting point is 296°C to 301°C with decomposition [2].



S-isomer

Fig.1: Chemical structure of pramipexole dihydrochloride monohydrate

1.2 Pharmaceutical and biological aspects

Pramipexole tablets containing Pramipexole dihydrochloride monohydrate available in 5 strengths as Pramipexole base they are 0.088 mg, 0.18 mg, 0.35 mg, 0.7 mg, 1.1 mg as an active ingredient.

Active substance:

Pramipexole is white to off-white crystalline powder, which is freely soluble in water in a pH independent way, soluble in methanol, slightly soluble in ethanol and insoluble in dichloromethane. At a relative humidity above 92% it liquefies but at lower relative humidity no absorption of water occurs. The active substance is not light sensitive itself, but has been found to degrade in the tablets matrix of binary mixtures with each excipient. A verity of degradation products have been identified but the mechanism of degradation is not known. By appropriate light protection during manufacture and by using a light protecting aluminium blister for packing, photo degradation is avoided.

Stability:

In the solid state, the active substance has shown good stability characteristics when tested under a number of different conditions. A 24-months shelf life is accepted when stored below 30°C and protected from light.

1.3 Toxico - pharmacological aspects

Pharmacodynamics:

The pharmacodynamic action of pramipexole has been studied *in vitro* and *in vivo*. In brain homogenates or cell lines expressing cloned human receptors, pramipexole effectively bound to receptor of the DA D2 subfamily (i.e.D_2, D_3 and D_4), with the highest affinity for D_3 binding sited (Ki 0.5 nM). Binding to adrenergic alpha-2, $5HT_{1A}$ and histamine-2 sited was weak to moderate (Ki > 240 nM). It lacked affinity for binding sites within the DA D1 subfamily (D_1, D_5) and for a large number of centrally acting neurotransmitters. Further *in vitro* studies (e.g. proton generation in a microphysometer assay, stimulation of CHO cell mitogenesis) showed that pramipexole was a full agonist for receptors within the DA D2 subfamily. *In vivo* studies, e.g. electrophysiology,

inhibition of prolactin release, DA turnover in specific brain region, indicated that pramipexole possessed full agonist activity.

Pharmacokinetics:

The pharmacokinetic profile of pramipexole was studied in mouse, rat, rabbit, minipig and monkey, the main species used in the preclinical program. Protein binding was low, <20% in all species including humans. Distribution study was conducted in rats with radiolablled compound. A wide tissue distribution was observed, with up to 9-fold higher tissue exposure to parent compound observed in brain compared to plasma. In pregnant rates, extensive placental transfer to fetuses was observed and in lacting females, levels of drug-related radioactivity in milk were up to 6 times higher than plasma. After oral administration, absorption was rapid and the bioavailability high (70-90%).

1.4 Disposition in the body

Pramipexole is readily, rapidly and completely absorbed from the gastro-intestinal tract. It is widely and extensively distributed throughout the body. Metabolism is minimal. More than 90% of the dose is excreted unchanged in urine via renal tubular secretion. Steady state is achieved by 2 days. No metabolites have been identified in plasma or urine.

Absorption:

- Rapid and complete. Absolute bioavailability is > 90 %
- Linear Pharmacokinetics over the clinical dosage range
- Cmax – 0.85 ng/ml (0.304 mg/ day), Tmax – 1-3 hrs
- AUC – 10.43 ng.ml/hr (0.304 mg/ day)
- Food dose not reduce the extent of pramipexole absorption, but the rate of absorption was reduced by around 1 hr.

Half life: Men 11.6 hour; women, 14.1 hour; also reported as 8 to 12hours

Volume of distribution: Men 7.34 L/kg; women 7.01 L/kg; also reported as 400L

Clearance:

Total 500mL/min. Renal clearance is approx.400mL/min (3 times higher than the glomerular filtration rate) or 5.5 mL/min/kg. Clearance is about 30% lower in women as compared to men, elderly compared to young people and those with Parkinson's disease compared to healthy people. It is about 75% lower for people with renal treatment.

Distribution in Blood: The erythrocyte: plasma ratio is 2.

Protein binding: 15%

Dose: 125 to 500µg; three times daily. Maximum daily dose is 4.5 mg.

1.5 Contraindications

- Vascular disorders: Hypotension (dose limiting adverse effect). Beyond 0.25 mg not tolerated in healthy subjects. (RLD – 0.25 mg)
- Psychiatric disorders: Insomnia, hallucinations, confusion
- Nervous system disorders: Dizziness, dyskinesia, somnolence
- Gastrointestinal disorders: Nausea, constipation
- General disorders: Oedema peripheral

2. Literature Review

The literature reviews regarding pramipexole suggest that various analytical methods were reported for drug substance as well as in pharmaceutical formulation and in various biological fluids. The literature reviews for analysis of pramipexole are as under [3].

2.1 Srinubabu, K. Jaganbabu, B. Sudharani1, K. Venugopal, G. Girizasankar and J. V. L. N. S. Rao have developed method for determination of pramipexole in tablets. Chromatography was performed with mobile phase containing a mixture of acetonitrile/phosphate buffer (60/40; v/v) with a flow rate of 0.8 ml/min. Quantitation was accomplished with the internal standard method; the procedure was validated by linearity (correlation coefficient = 0.99892), accuracy, robustness and intermediate precision [4].

2.2 AU YI LAU, SELENKA J. M., ANSON G. D., TALAAT R., ICHHPURANI N have developed a highly sensitive and selective HPLC-MS-MS method for the determination of pramipexole in human plasma. The analytes, pramipexole and BHT-920 (internal standard), were extracted from plasma at basic pH with methyl tert.-butyl ether (MTBE). MTBE was evaporated to dryness and reconstituted in 100 µl of (95:5) methanol-water. Chromatographic separation was achieved on a Zorbax SB-CN column with a mobile phase of (15:5:80) water-0.1 M ammonium acetate-methanol. The analytes were detected utilizing HPLC in conjunction with atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS-MS). The assay was linear in the concentration ranges from 50 to 5000 pg/ml [5].

2.3 Ramakrishna V. S. Nirogi, Vishwottam Kandikere, Wishu Shrivastava, Koteshwara Mudigonda, Santosh Maurya, Devender Ajjala have developed a high-performance liquid chromatography/electrospray ionization tandem mass spectrometry method and validated for the quantification of pramipexole in human plasma. Following liquid-liquid extraction, the analytes were separated using an isocratic mobile phase on a reverse-phase column and analyzed by MS/MS in the multiple reaction monitoring mode using the respective $[M + H]^+$ ions, *m*/*z* 212/152 for pramipexole and *m*/*z* 409/228 for the IS. The method exhibited a linear dynamic range of 200-8000 pg/ml for pramipexole in human plasma. The lower limit of quantification was 200 pg/ml with the RSD value less than 8% [6].

2.4 Biljana Janci, Mirjana Medenica, Darko Ivanovic, Anddelija Malenovic have developed method for its impurities as 2-amino benzothiazole and BI-II 546 CL and BI-II 751 xx using Zorbax Extended-C18 150 mm x 4.6 mm, 5 µ column with UV detection at 262 nm for pramipexole, BI-II 751 xx and 2-amino benzothiazole and at 326 nm for BI-II 546 CL. The flow rate was 1.0 ml/min. Column temperature 25°C and 20 µl was injection volume. Mobile phase was water, TEA pH adjusted to 7.0 with orthophosphoric acid [7].

2.5 A Pathare DB, Jadhav AS, Shingare MS have developed a chiral liquid chromatographic method for the enantiomeric resolution of pramipexole

dihydrochloride monohydrate, (S)-2-amino-4,5,6,7-tetra-hydro-6-(propylamino) benzothiazole dihydrochloride monohydrate, a dopamine agonist in bulk drugs. The enantiomers of pramipexole dihydrochloride monohydrate were resolved on a Chiralpak AD (250 mm x 4.6 mm, 10 microm) column using a mobile phase system containing n-hexane:ethanol:diethylamine (70:30:0.1, v/v/v). The resolution between the enantiomers was found not less than eight. The presence of diethylamine in the mobile phase has played an important role in enhancing chromatographic efficiency and resolution between the enantiomers. The developed method was extensively validated and proved to be robust. The limit of detection and limit of quantification of (R)-enantiomer were found to be 300 and 900 ng/ml. ng/ml. respectively for 20 microl injection volumes. The percentage recovery of (R)-enantiomer was ranged from 97.3 to 102.0 in bulk drug samples of Pramipexole dihydrochloride monohydrate. Pramipexole dihydrochloride monohydrate sample solution and mobile phase were found to be stable for at least 48 h. The proposed method was found to be suitable and accurate for the quantitative determination of (R)-enantiomer in bulk drugs [8].

3. Aim of present work

As per discussion in the literature review it is not official in pharmacopeia [9-14], method for assay by UV was reported with internal standard preparation. Methods for pramipexole in human plasma, chiral purity by normal phase and related impurities method were reported. So, a simple assay method without any internal standard preparation is the aim of present work. Hence, the method is recommended for routine quality control analysis and also stability sample analysis.

The aim and scope of the proposed work are as under

- To developed suitable HPLC assay method for Pramipexole in formulation
- Forced degradation study of Pramipexole tablets under stress condition
- To resolve all major impurities generated during the force degradation studies of Pramipexole tablets
- Perform analytical method validation for the proposed method as per ICH guideline.

4. Experimental

4.1 Materials, chemicals and reagents

A) Materials required:

Sr. No.	Material Name	Lot. No./ Batch no.	Mfg.by	Potency/ Purity
1	Pramipexole dihydrochloride monohydrate reference standard	OTV/2007/112	TRC,Bhat	99.8%
2	Pramipexole dihydrochloride monohydrate API	PRA/015/005	TRC,Bhat	--
3	Pramipexole impurity A	OTV/2007/123	TRC,Bhat	98.99%
4	Pramipexole impurity B	OTV/2007/114	TRC,Bhat	98.53%
5	Pramipexole impurity C	OTV/2007/113	TRC,Bhat	99.56%

B) Chemicals and Reagents:

Sr. No.	Chemical/Reagent	Grade
1	Acetonitrile	HPLC
2	Ammonia solution (25% v/v)	AR
3	Methanol	HPLC
6	Water	Milli-Q-water
7	Hydrogen peroxide	AR
8	Hydrochloric acid	AR
9	Sodium hydroxide	AR

4.2 Instrumentation

Shimadzu LC 2010C with Class VP software and Waters alliance 2996 and 2487 with empower software HPLC systems were used.

4.3 Mobile phase preparation

The mobile phase consisted of 0.1 % v/v ammonia solution: acetonitrile: methanol (70: 15: 15, v/v/v). To prepare the 0.1 % v/v ammonia solution 1ml concentrated ammonia solution (25% v/v) was diluted to 1000 ml with HPLC grade water. Mobile phase was degassed by sonication with an ultrasonic bath.

4.4 Diluent preparation

Mixed both the solvents Methanol : water, 50:50.

4.5 Standard preparation

A pramipexole standard solution containing 0.1 mg/ml, was prepared in a 250 ml volumetric flask by dissolving 25 mg of Pramipexole dihydrochloride monohydrate in 50 ml of diluent by sonication and then diluted to volume with diluent.

4.6 Test preparation

Sample tablets were weighed and transferred to the volumetric flask as per below the table, after addition of about 70 % of total volume of volumetric flask sonication was done with intermittent swirling, content was brought back to room temperature and diluted to volume to achieve about 0.1 mg/ml Pramipexole concentration as of standard preparation. The preparation was filtered through 0.45µm PVDF membrane disk filter.

4.7 Chromatographic conditions

Chromatographic analysis was performed on X-TERRA RP-8 (150mm × 4.6mm , 5 µm) column with oven temperature of 25˚C. The flow rate of the mobile phase was adjusted to 1.0 ml/min and the injection volume was 10 µl. Detection was performed at 260nm.

5. Result and Discussion

5.1 Development and optimization of the HPLC method

Proper selection of the methods depends upon the nature of the sample (ionic or ionisable or neutral molecule) its molecular weight and solubility. Pramipexole dihydrochloride monohydrate is dissolved in polar solvent hence RP-HPLC was selected for it’s estimation in formulation. To develop a rugged and suitable HPLC method for the

quantitative determination of pramipexole, the analytical condition were selected after the consideration of different parameters such as diluent, buffer, buffer concentration, organic solvent for mobile phase and mobile phase composition and other chromatographic conditions. Preliminary trials were taken with different composition of buffer and organic phase of mobile phases with pH range of 2-7 but pramipexole peak was appear in early eluting region even at certain composition there was double peak phenomena.

Pramipexole is basic compound with amine groups in its structure so, peak retention can be achieved above it's pKa in alkaline pH and further optimized with organic phase. Based on this fact just 0.1 % ammonia solution as aqueous part with different composition with acetonitrile as well as with methanol were tried to achieve desired chromatography. Finally 0.1 % ammonia solution-acetonitrile-methanol (70:15:15, v/v/v) was optimized with consideration of peak shape and pump pressure. Here, X-terra RP-8 column was used due to its higher pH stability of mobile phase (pH=10 to 11). Detection at 260 nm was selected based on its UV spectrum. For sample preparation water-methanol (50:50, v/v) was chosen based on solubility of drug and to get easy dispersion of tablet formulation. Moreover this diluent gave good solution stability for sample as well as standard preparation. Here, acetonitrile was also tried along with water as a diluent but somewhat that diluent is not able to give reproducible result for assay value even peak area of standard preparation, Hence, selected diluent was best among the all trials by many aspects. Fig.1 to Fig.3 represents the chromatogram of standard preparation, sample preparation and blank respectively.

Fig.1: Chromatogram of standard preparation

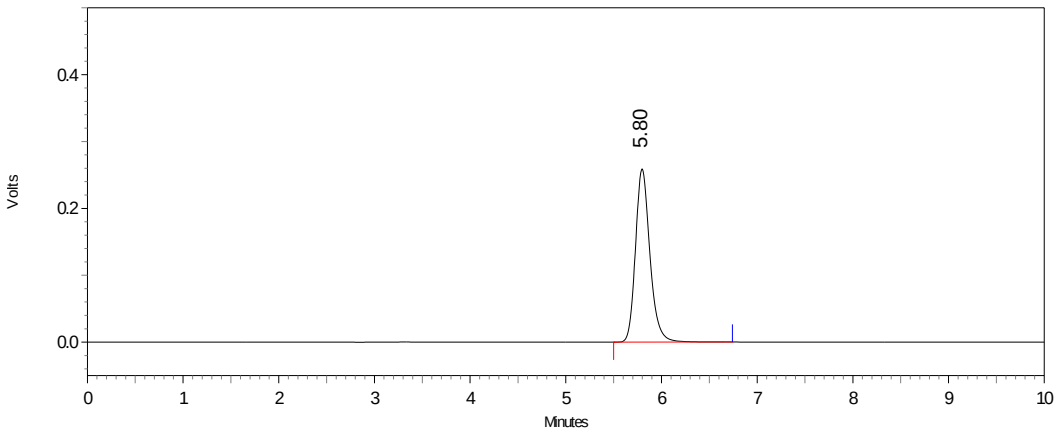


Fig.2: Chromatogram of sample preparation

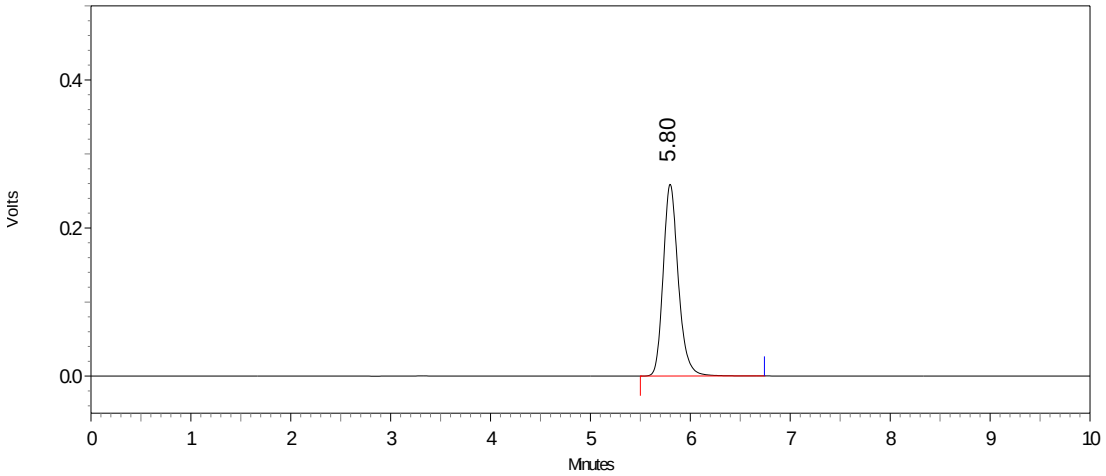
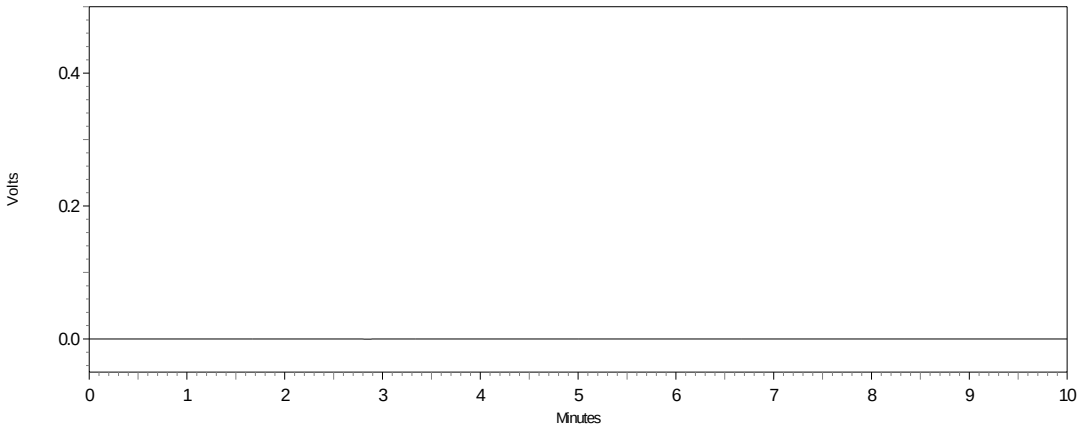


Fig.3: Chromatogram of blank preparation



5.2 Degradation study

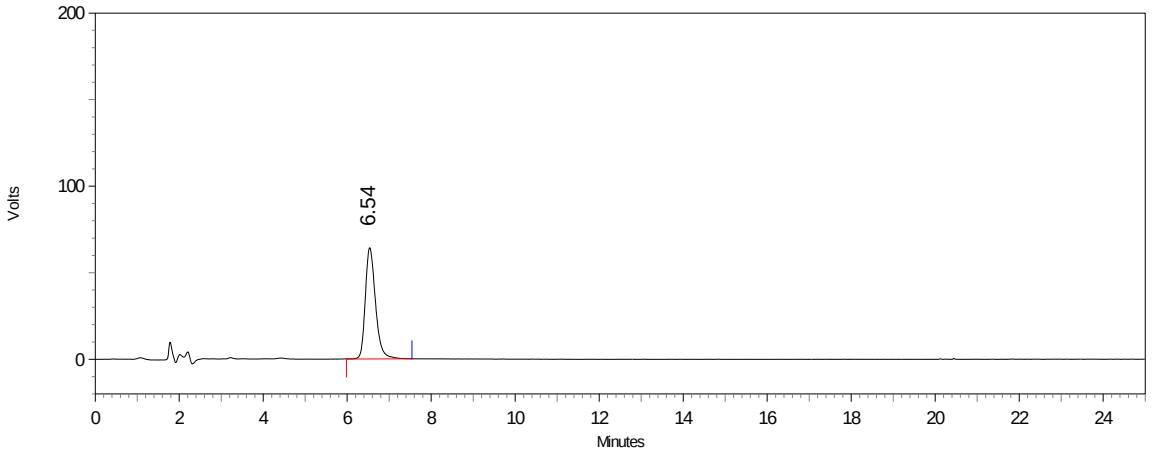
The degradation samples were prepared by transferring intact tablets, samples were employed for acidic, alkaline and oxidant media and also for thermal and photolytic conditions. After the degradation treatments were completed, the stress content solutions were diluted with diluent to attain about 0.1 mg/ml concentration. Specific conditions were described as follows.

5.2.1 Acidic degradation

Acidic degradation study was performed by refluxing the content in 30 ml of 5 N HCl at about 100˚ C for 12 hrs and after cooling to room temperature it was neutralized

with 5 N NaOH solution. Pramipexole degradation was found by 1 % in this acidic condition (Fig.4).

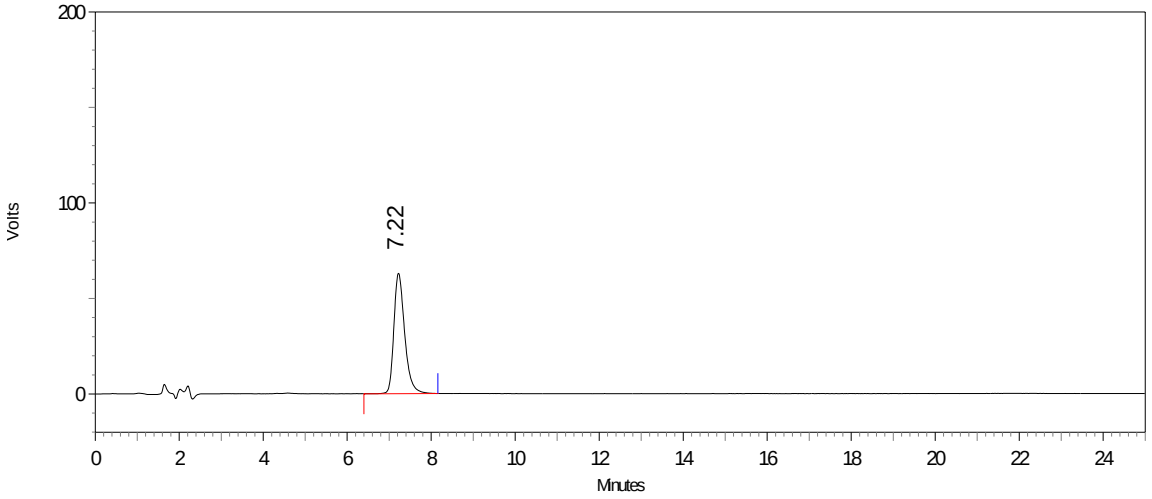
Fig.4: Chromatogram of acidic forced degradation study



5.2.2 Base degradation

Alkali degradation study was performed by heating the content in 30 ml of 5 N NaOH at about 80˚ C for 45 min and after cooling to room temperature it was neutralized with 5 N HCl solution. Pramipexole degradation was found by 23.4 % in this alkaline condition (Fig.5).

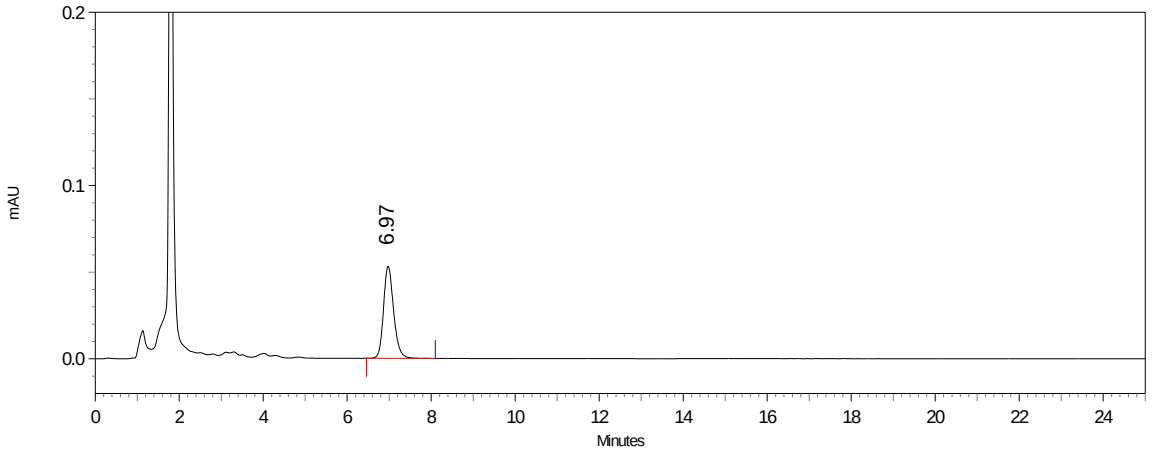
Fig.5: Chromatogram of alkali forced degradation study



5.2.3 Oxidative degradation

Oxidation degradation study was performed by heating the drug product in 30ml of 30% v/v H_2O_2 at 80˚ C for 15 minutes. Pramipexole degradation was found by 27.9 % in this oxidative condition (Fig.6).

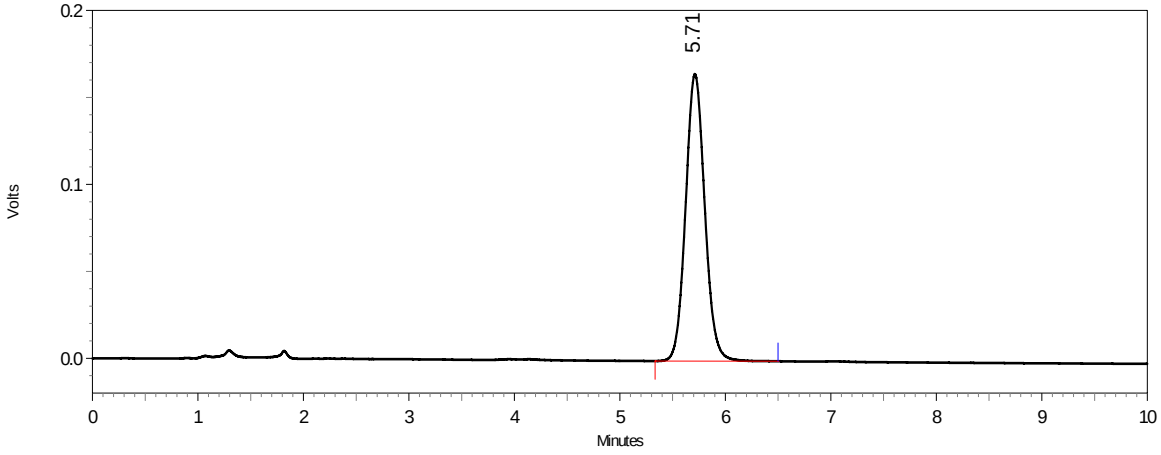
Fig.6: Chromatogram of oxidative forced degradation study



5.2.4 Thermal degradation

Thermal degradation was performed by exposing solid drug product at 105˚ C for 72 hrs. Pramipexole degradation was found by 14.5 % in this thermal condition (Fig.7).

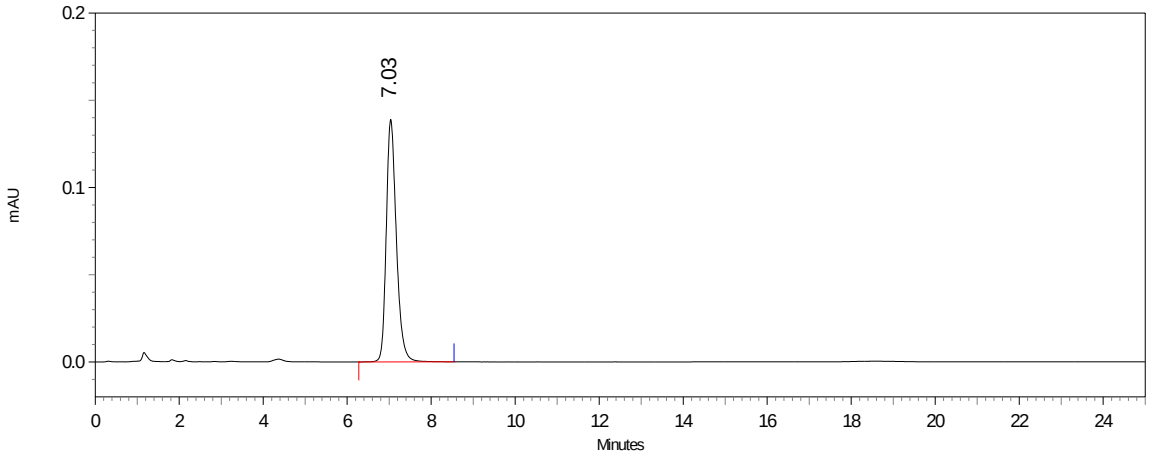
Fig.7: Chromatogram of thermal degradation study



5.2.5 Photolytic degradation

Photolytic degradation study was performed by exposing the drug product to UV light for 1.2 million lux hours. Pramipexole degradation was found by 7.7 % in this photolytic condition (Fig.8).

Fig.8: Chromatogram of UV-light degradation study



5.3 Method Validation

Method validation approach

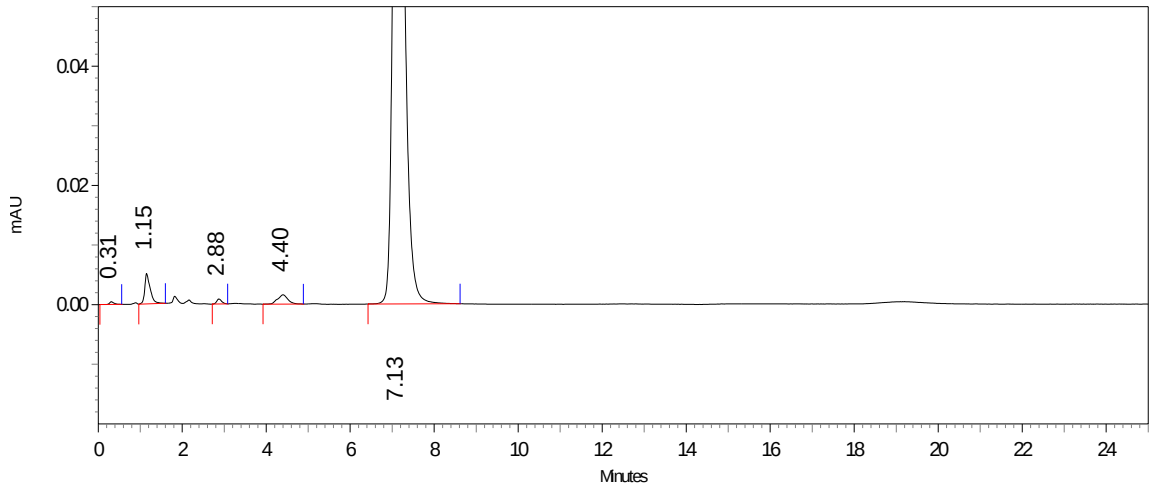
The assay method is same for all the strength. The standard concentration for 0.125mg, 0.25mg, 0.5mg, 1mg, 1.5mg are 100 ppm and sample concentration for 0.125mg, 0.25mg, 0.5mg, 1mg are 100 ppm and for 1.5mg is 90 ppm. Pramipexole dihydrochloride tablets 0.125 and 0.25mg are weight proportional formulations (scale up-down) and pramipexole dihydrochloride tablets 0.5, 1.0 and 1.5mg are also weight proportional formulations (scale up-down). Complete validation will be performed on higher strength (1.5 mg) and specificity (Part-A) and accuracy also performed on 0.125 mg strength by 3 replicate x 3 concentration and Linearity with 5 levels of concentration range [15]. Filter media interference study was covered in accuracy study.

5.3.1 Specificity

The specificity of the method was determined by checking the interference of placebo with analyte and the proposed method was evaluated by checking the peak purity

of pramipexole during the force degradation study. The peak purity was found satisfactory (>0.9999) under different stress condition. There was no interference of any peak of degradation product with drug peak and peak purity was also found satisfactory in impurity spiked sample (Fig.9).

Fig.9: Chromatogram of impurity spiked sample



Here, known pramipexole impurity-A was eluted before the pramipexole which can process cum degradation product while late eluting impurity was not eluted but as it is process impurity and controlled at API level it self and at proposed concentration of pramipexole for assay that impurity can be at below quantitation limit for proposed chromatography.

5.3.2 Linearity

Seven points calibration curve were obtained in a concentration range from 0.045-0.15 mg/ml for pramipexole. The response of the drug was found linear for entire concentration range and the linear regression equation was y = 23524x + 21527 with correlation coefficient 0.99998. (Fig.10) Chromatograms obtain during linearity study is shown in fig.11-15.

Fig.10: Linearity curve for pramipexole

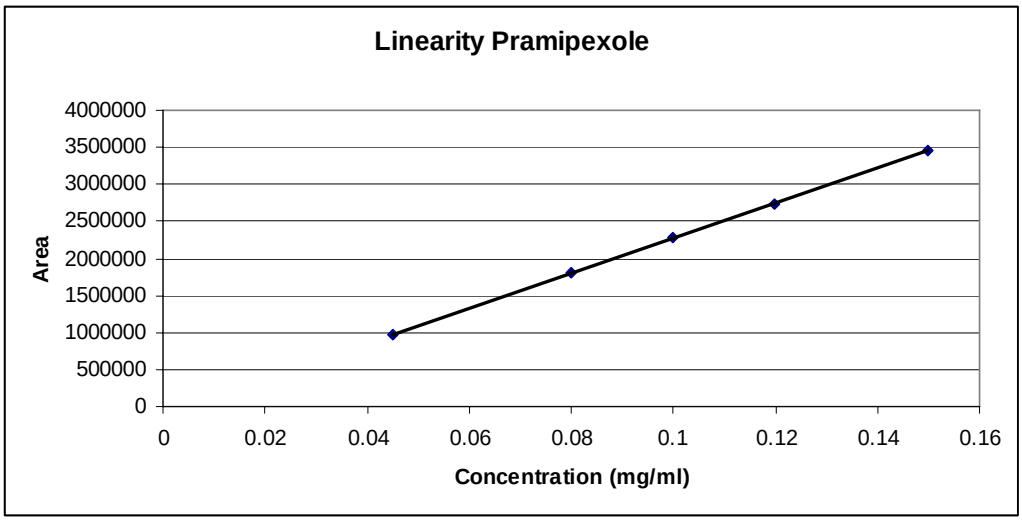


Fig.11: Linearity study chromatogram of level-1 (45%)

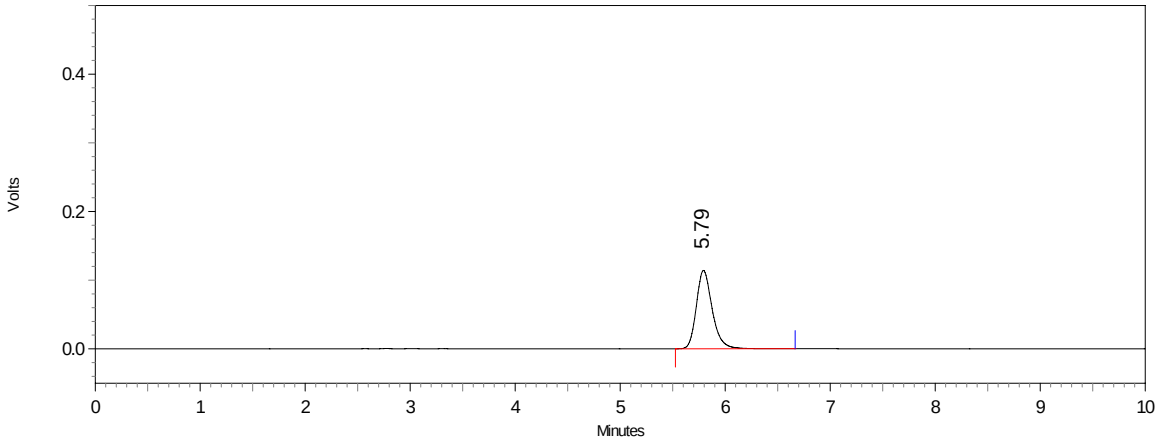


Fig.12: Linearity study chromatogram of level-2 (80%)

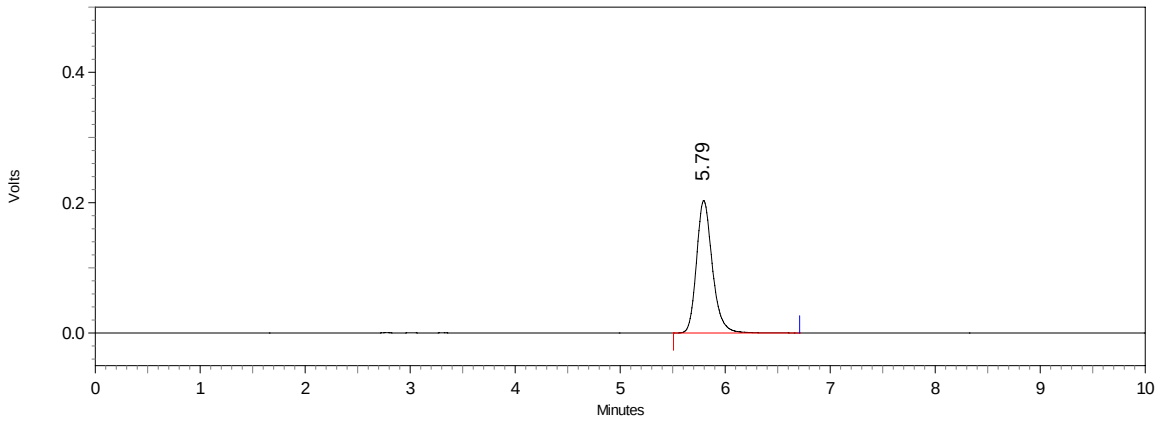


Fig.13: Linearity study chromatogram of level-3 (100%)

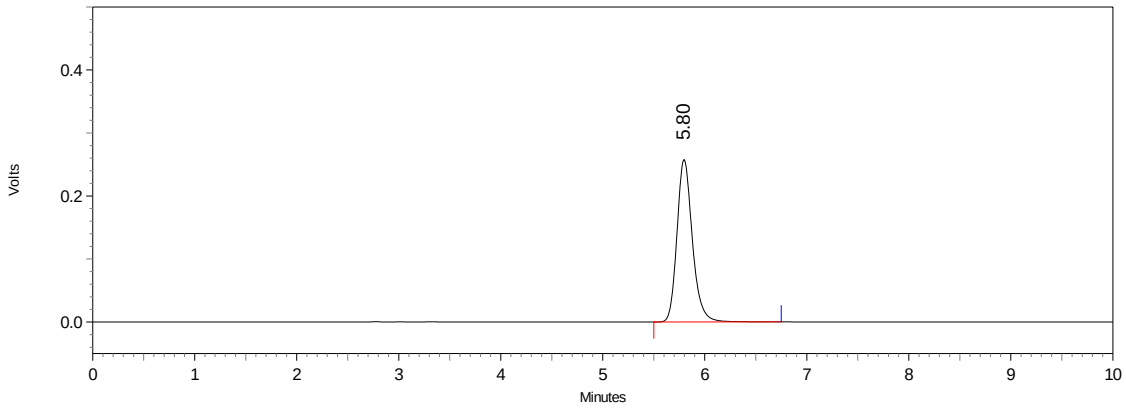


Fig.14: Linearity study chromatogram of level-4 (120%)

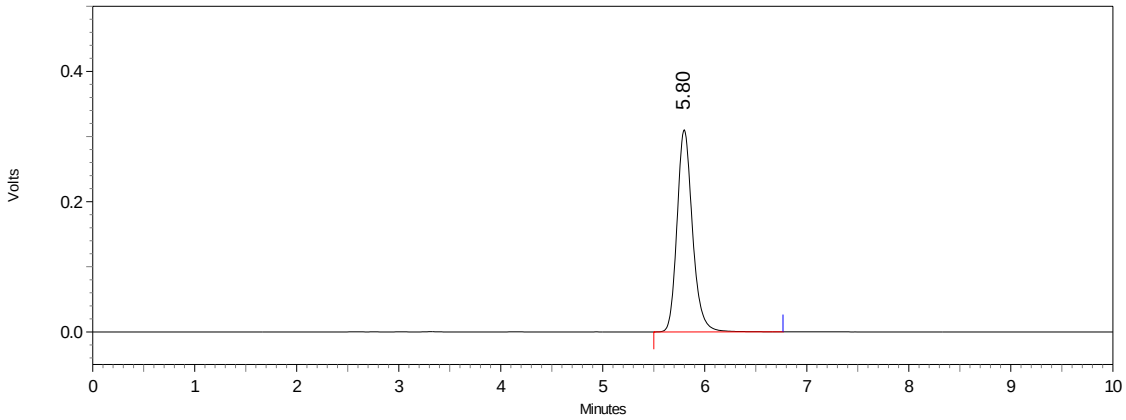
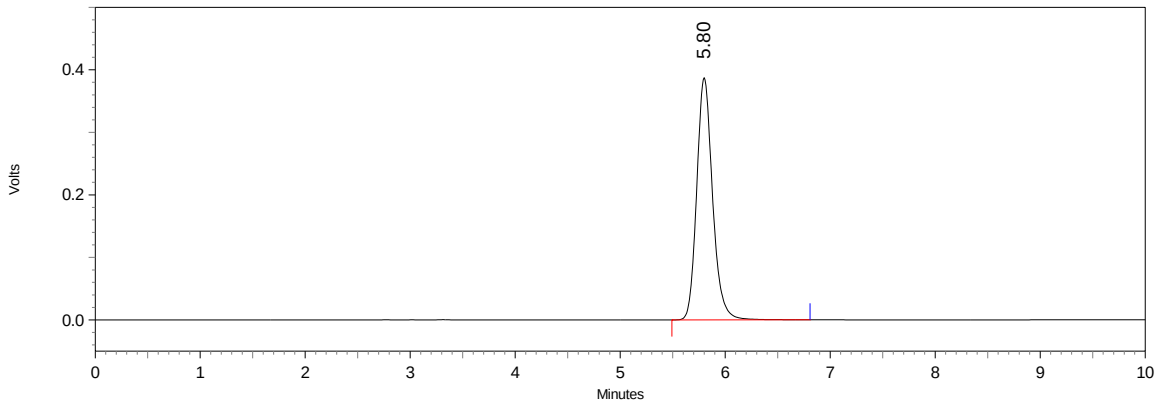


Fig.15: Linearity study chromatogram of level-5 (150%)



5.3.3 Precision

The result of repeatability and intermediate precision study are shown in Table 1. The developed method was found precise as the %RSD values for the method precision and intermediate precision studies were < 0.81 % and < 1.23 %, respectively.

Table 1: Evaluation data of precision study

Set	Day-1 (n = 6)	Day-2 (n = 6)
1	102.5	101.6
2	103.8	102.1
3	101.3	103.1
4	102.3	103.3
5	102.5	104.8
6	101.9	104.5
Mean	102.4	103.2
Standard deviation	0.83	1.27
% RSD	0.81	1.23
95% Confidence Interval (CI)	0.66	1.02

5.3.4 Accuracy

The accuracy of the method was established at three levels in the range of 50% to 150% of specification limit for 0.125 mg and 1.5 mg strengths. Above levels were prepared in triplicate. Each solution was injected in singlet. % Recovery was calculated at each level and recorded in Table-2 and 3 respectively for 0.125 mg and 1.5 mg strengths. The supporting chromatograms shown in fig.16-21.

Table 2: Evaluation data of accuracy study Part-A (with placebo of 0.125 mg)

Level	Replicate	Amount			Recovery	%RSD
		Added (ppm)	Found (ppm)	Recovery %	Mean %	
Level-1 (50%)	1	52.5946	52.9263	100.6	101.2	0.5
	2	52.4748	53.2075	101.4		
	3	52.5547	53.3170	101.5		
Level-2 (100%)	1	104.7900	106.7151	101.8	101.7	0.1
	2	105.0894	106.8262	101.7		
	3	104.8698	106.6460	101.7		
Level-3 (150%)	1	157.9235	159.3557	100.9	100.9	0.1
	2	158.1830	159.5583	100.9		
	3	157.6640	159.2088	101.0		

Table 3: Evaluation data of accuracy study Part-B (with placebo of 1.5 mg)

Level	Replicate	Amount			Recovery	%RSD
		Added (ppm)	Found (ppm)	Recovery %	Mean %	
Level-1 (50%)	1	51.8002	52.4149	101.2	101.1	0.6
	2	50.9140	51.7041	101.6		
	3	50.9200	51.1906	100.5		
Level-2 (100%)	1	101.7880	103.6629	101.8	100.9	0.8
	2	100.7980	101.2128	100.4		
	3	100.8060	101.2919	100.5		
Level-3 (150%)	1	150.3088	151.3185	100.7	100.2	0.5
	2	150.3228	150.0967	99.8		
	3	150.5024	150.7100	100.1		

Fig.16: Chromatogram of accuracy study Part-A level-1 (50%)

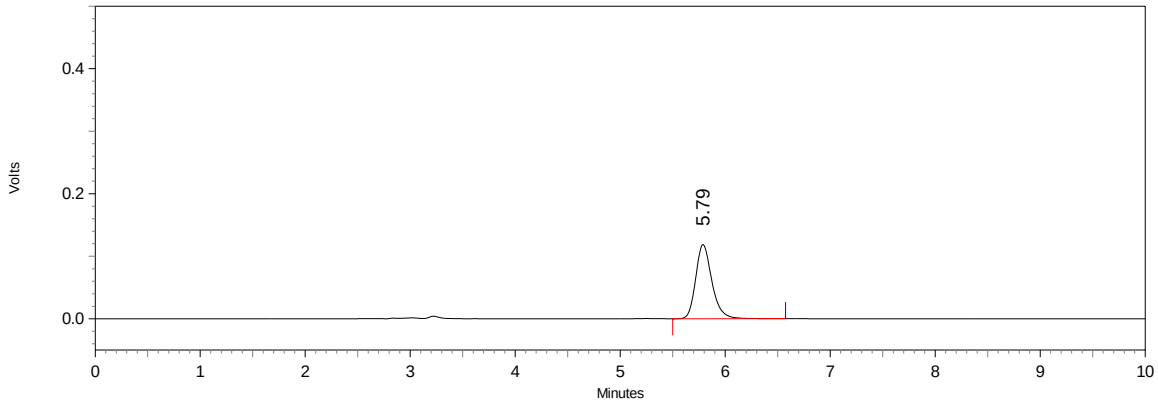


Fig.17: Chromatogram of accuracy study Part-A level-2 (100%)

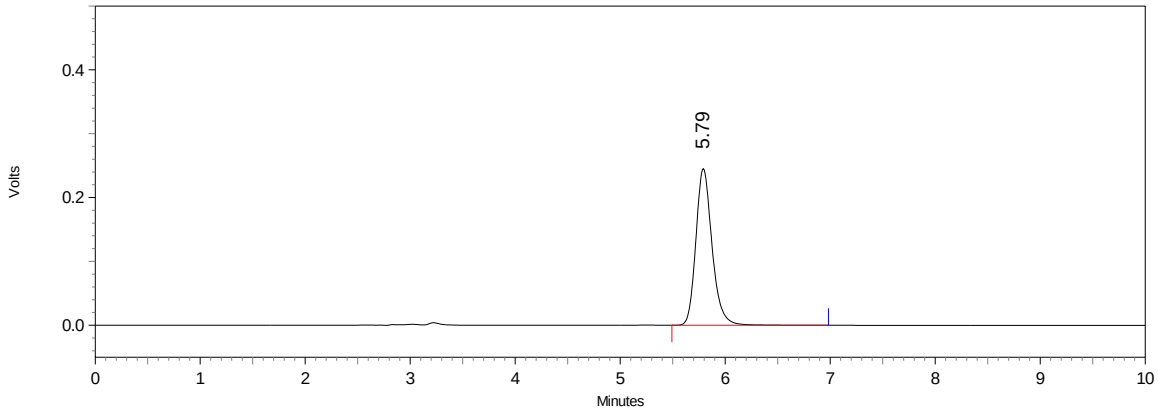


Fig.18: Chromatogram of accuracy study Part-A level-3 (150%)

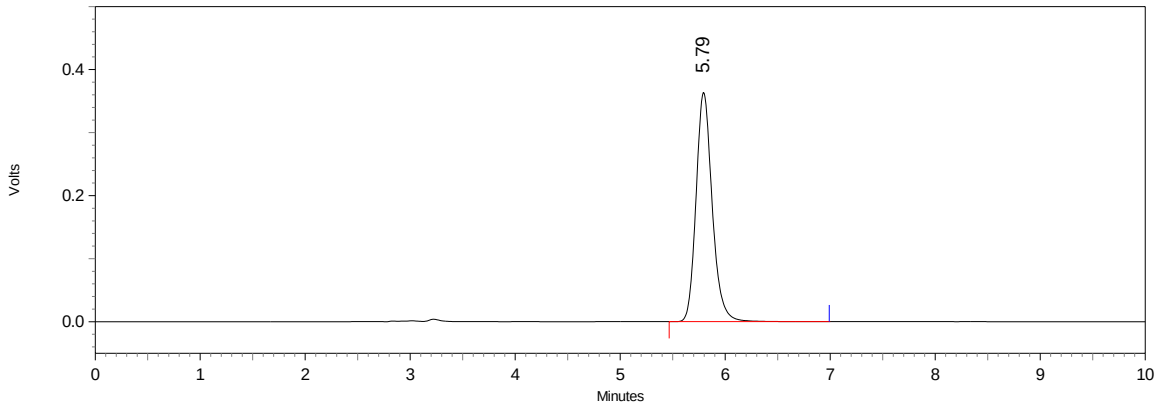


Fig.19: Chromatogram of accuracy study Part-B level-1 (50%)

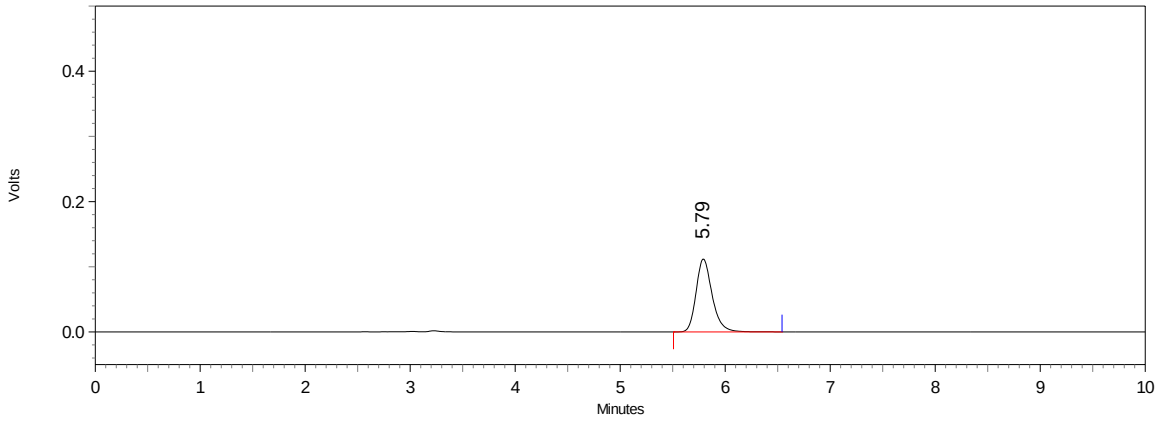


Fig.20: Chromatogram of accuracy study Part-B level-2 (100%)

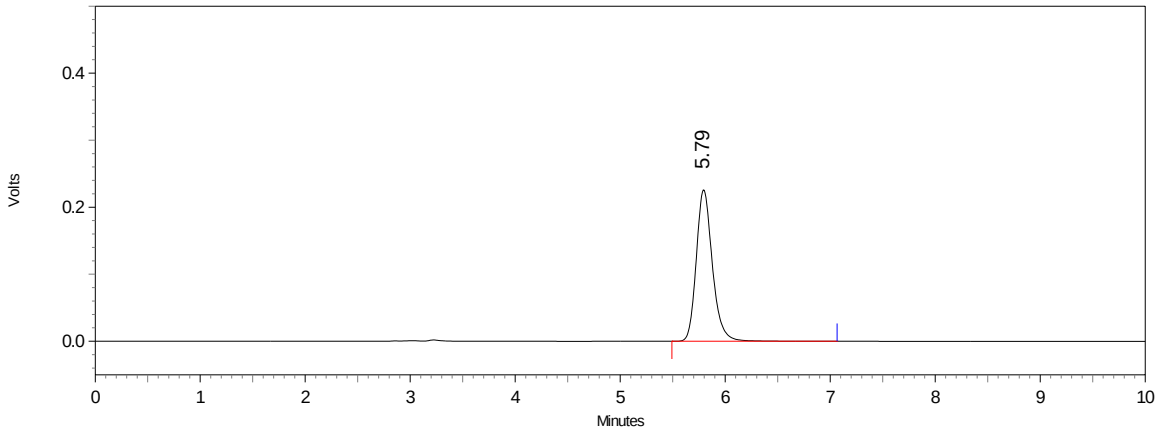
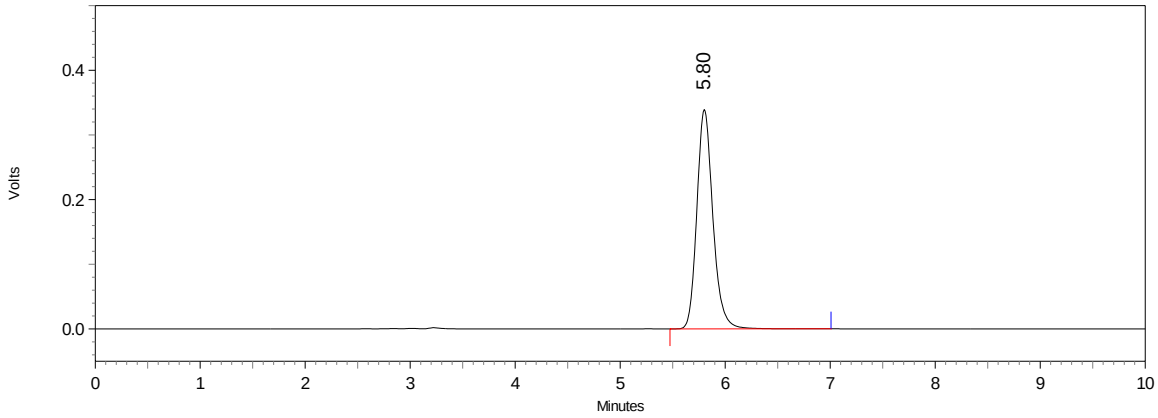


Fig.21: Chromatogram of accuracy study Part-B level-3 (150%)



5.3.5 Solution stability study

Table 4 and 5 shows the results obtain in the solution stability study at different time intervals for standard and sample preparation. It was concluded that the standard and sample preparation solution were found stable up to 60 hours at 25 ˚C.

Table 4: Solution stability for standard preparation

Standard Preparation		
Time	**% Assay**	**Absolute Difference (%)**
Initial*	100.0	**N/A**
After 25 hours	100.1	0.1
After 60 hours	100.5	0.5

Table 5: Solution stability for sample preparation

Sample Preparation		
Time	**% Assay**	**Absolute Difference (%)**
Initial*	101.6	**N/A**
After 25 hours	100.8	0.8
After 60 hours	100.2	1.4

***** Data taken from system suitability and Test preparation-1 injected for Intermediate precision respectively.

5.3.6 Robustness

The result of robustness study of the developed assay method was shown in Table 6 and 7. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of accepted true value from method precision study. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

Table 6: System suitability summary of robustness study

Condition	System suitability parameters		
	Theoretical plates	Asymmetry	% RSD
A) Change in flow rate			
Normal condition (1.0 ml per minute)	4480	0.98	0.46
(0.9 ml per minute)	4589	1.03	0.20
(1.1 ml per minute)	4111	1.02	0.13
B) Change in minor component in the mobile phase			
Normal condition Buffer : Methanol : Acetonitrile(70:15:15)	4480	0.98	0.46
Buffer : Methanol : Acetonitrile(72:14:14	4360	1.03	0.23
Buffer : Methanol : Acetonitrile(68:16:16)	4261	1.03	0.11
C) Change in column temperature			
Normal condition 25°C	4480	0.98	0.46
Column temperature 30°C	4516	1.04	0.12
D) Change in column Lot*			
Normal condition Column S/N 012437289133 24	4279	1.00	0.03
Changed condition Column S/N 012337101120 02	4139	1.13	0.14

***** This parameter was studied as part of precision (method precision and intermediate precision).

Note:

i) Theoretical plates, asymmetry and retention time values are from the first injection of the system suitability set.

ii) RSD was calculated for the five replicate injections of standard preparation.

iii) Normal condition data taken from the method precision (Repeatability).

Table 7: % Assay summary of robustness study

Condition	% Assay
A) Change in flow rate	
Normal condition (1.0 ml per minute)	100.7
Change in flow rate (0.9 ml per minute)	99.7
Absolute difference from normal condition = 1.0%	
Change in flow rate (1.1 ml per minute)	98.9
Absolute difference from normal condition = 1.8%	
B) Change in minor component in the mobile phase	
Normal condition Buffer : Methanol : Acetonitrile (70:15:15)	100.7
Change in methanol composition by – 2% absolute Buffer : Methanol : Acetonitrile(72:14:14)	98.8
Absolute difference from normal condition = 1.9%	
Change in methanol composition by + 2% absolute Buffer : Methanol : Acetonitrile(68:16:16)	98.8
Absolute difference from normal condition = 1.9%	
D) Change in column oven temperature	
Normal condition: (25°C)	100.7
Change in oven temperature by + 5°C: (30°C)	99.1
Absolute difference from normal condition = 1.6%	
C) Change in column lot*	
Normal column S/N 012437289133 24	102.4
Changed column S/N 012337101120 02	103.2
Absolute difference from normal condition = 0.8%	

5.3.7 System suitability

A system suitability test of the chromatographic system was performed before each validation run. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same. Acceptance criteria for system suitability, asymmetry not more than 2.0, theoretical plate not less then 2000 and % RSD of peak area not more then 2.0, were full fill during each validation parameter as shown in table 8.

Table 8: System suitability summary

Parameter	Theoretical plates	Asymmetry	%RSD
Limits:	Not less than 2000	Not more than 2.0	Not more than 2.0%
Specificity-Part A	4530	1.15	0.12
Accuracy	5293	1.19	0.19
Method precision	4279	1.00	0.03
Specificity –Part B	3187	1.11	0.25
Intermediate precision and Solution stability-initial	4139	1.13	0.14
Robustness	4480	0.98	0.46
Linearity	4084	1.13	0.10
Solution stability after 25 hours	4288	1.05	0.08
Solution stability after 60 hours	4199	1.02	0.09
Specificity (Photo degradation)	3756	1.30	1.74

6. Quantitation and Calculation formulae

6.1 Relative Standard Deviation (%)

$$\text{Relative Standard Deviation (\%)} = \frac{SD \times 100}{\overline{X}}$$

By Microsoft excel

Average = AVERAGE (Number 1)

Standard Deviation = STDEV (Number 1)

6.2 Calculation formulae in Linearity

$$\text{Correlation coefficient (r)} = \frac{n\ (\sum xy)\ -\ (\sum x)(\sum y)}{\sqrt{\{[n\sum x^2 - (\sum x)^2]\ [n\sum y^2 - (\sum y)^2]\}}}$$

Correlation coefficient (r) = CORREL (Array 1, Array 2)

$$\text{Slope of regression line} = \frac{n\ (\sum xy)\ -\ (\sum x)(\sum y)}{n\sum x^2 - (\sum x)^2}$$

Slope of regression line = SLOPE (Array 1, Array 2)

$$\text{y- intercept} = A = \bar{y} - B\bar{x}$$

y- intercept = INTERCEPT (Array 1, Array 2)

$$\text{Residual sum of squares} = \sum (Y_{original} - Y_{calculated})^2$$

Where,

$Y_{calculated} = A + Bx$

n = Number of measurements

x = Individual concentration

y = Individual area

$\bar{x}$ = Average concentration

$\bar{y}$ = Average area

Residual sum of squares = SUMXMY2 (Array 1, TREND (Array 1, Array 2, Array2))

6.3 Accuracy (% Recovery)

$$\%\ \text{Recovery} = \frac{\text{Ammount of sbustance (mg) found}}{\text{Ammount of substance (mg) added}} \times 100$$

6.4 % Assay

$$\% \text{ Assay} = \frac{\text{Asp}}{\text{Astd}} \times \frac{\text{Std. wt}}{\text{Dilution}} \times \frac{\text{Dilution}}{\text{Spl Wt}} \times \frac{\text{Avg Wt.}}{\text{LC}} \times \text{Std. assay \% (as such)}$$

e.g. Method precision Set-1:

$$\% \text{ Assay of Pramipexole 2HCl } H_2O = \frac{2003071}{2236837} \times \frac{25.80}{250} \times \frac{500}{9025.67} \times \frac{300}{1.5} \times 99.8$$

% Assay of pramipexole 2HCl H_2O = 102.5

Asp	:	Peak area in sample
Astd	:	Mean peak area of standard
Std. wt	:	Std weight in mg
Spl wt	:	Sample weight in mg
Avg wt	:	Average weight in mg
LC	:	Label claim per tablet (mg)

6.5 95% Confidence Interval (CI)

$$95\% \text{ Confidence Interval (CI)} = \pm \frac{t\alpha \times SD}{\sqrt{n}}$$

Where,

SD = Standard Deviation

$\overline{X}$ = Average concentration

n = Number of measurements

t_α = t value at n-1 from t table (two tail)

or

95% Confidence Interval (CI) = SD (2.57058)/SQRT (6)

References

[1] Sifrol, INN-Pramipexole; http://www.emea.europa.eu/humandocs/PDFs/EPAR/Sifrol/059197EN6.pdf

[2] Clarke's analysis of drug and poison in pharmaceuticals, body fluids and postmortem material 3 rd edition 2004 p.1468

[3] http://www.Mirapex.com

[4] Srinubabu, K. Jaganbabu, B. Sudharani1, K. Venugopal, G. Girizasankar and J. V. L. N. S. Rao, chromatographia 64 (2006) 95-100

[5] YAU YI LAU, SELENKA J. M., ANSON G. D., TALAAT R., ICHHPURANI N, journal of chromatography biomedical applications 683(1996) 209-216

[6] Ramakrishna V. S. Nirogi, Vishwottam Kandikere, Wishu Shrivastava, Koteshwara Mudigonda, Santosh Maurya, Devender Ajjala, Biomedical chromatography 21(2007) 1151-1158

[7] Biljana Janci, Mirjana Medenica, Darko Ivanovic, Anddelija Malenovic Acta Chim.Slov.54(2007)49-54

[8] APathare DB, Jadhav AS, Shingare MS J. Pharm Biomed Anal.41-4(2006)1152-1156

[9] US Pharmacopeia, USP 31 NF 26, 2008

[10] European Pharmacopeia, 6th edition, 2008

[11] British Pharmacopeia, 2008

[12] Indian Pharmacopeia, 2007

[13] Japanese Pharmacopeia, 15th edition (2006)

[14] Pharmacopeia of the people's republic of china (2005)

[15] ICH guideline, Q2 (R1) step 4, Validation of Analytical Procedures: Text and Methodology (2005)

INTRODUCTION TO CHIRAL CHROMATOGRAPHY

INTRODUCTION

A chiral molecule is defined as a molecule that is not superimposible on its mirror image. The two mirror image forms of a chiral molecule are called enantiomers.A chiral molecule has one or more stereogenic centers, which are typically carbons. These steriogenic carbon atoms are attached to four different substituents [1], e.g. chlorobromomethanol fig.1.

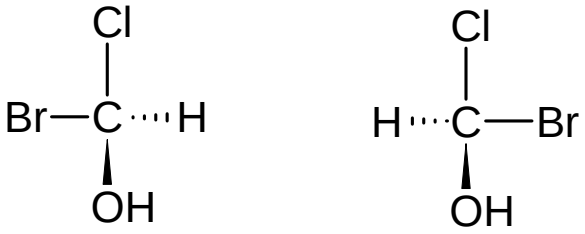


Fig.1: Chlorobromomethanol

The biological activity of chiral substances often depends upon their stereochemistry, since the living body is a highly chiral environment. A large percentage of commercial and investigational pharmaceutical compounds are enantiomers, and many of them show significant enantioselective differences in their pharmacokinetics and pharmacodynamics. The importance of chirality of drugs has been increasingly recognized, and the consequences of using them as racemates or as enantiomers has been frequently discussed in the pharmaceutical literature during recent years. With increasing evidence of problems related to stereoselectivity in drug action, enantioselective analysis by chromatographic methods has become the focus of intensive research of separation science. Most of the pharmaceutical and pharmacological studies of stereoselectivity of chiral drugs before the mid eighties involved pre-column derivatization of the enantiomers with chiral reagents, forming diastereomers. The diastereomers were subsequently separated in the normal or reversed phase mode of chromatography [2-5].

The majority of separation of enantiomers reported in old literature were based on an initial reaction of chiral molecule with an enantiomerically pure, chiral derivatizing agent. The reaction yields two diastereomers, one for each of the two enantiomeric

analytes in the starting sample. The resulting two diastereomers could than be separated by conventional HPLC. This overall procedure referred to as the ***indirect method*** of chiral analysis. The use of chiral stationary phases (CSPs) without derivatization is referred to as the ***direct method*** of chiral analysis. A method is to be considered to be direct if it involves the actual chromatographic separation of molecules that are enantiomerically related to each other (not converted to covalent diastereomerically related derivatives), and regardless of whether they are derivatized with an achiral reagent or not. Another type of direct method uses a chiral mobile phase additive (CMPA), which forms a transient diastereomeric complex with the analytes. Resolution of these diasereomeric complexes is then possible by HPLC [1].

Principle of chiral recognition

Chiral recognition referes to the ability of the CSP to interact differently with two enantiomers, leading to their HPLC separation. The nature of chiral recognition has been examines in several studies and depends on the different interactions as summarized in table 1. The ability of the analyte and CSP to form transient-diastereomeric complexes utilizing hydrogen bonding, π-π interactions, dipole stacking, inclusion complexing, and steric bulk is the driving force behind enantioseparation.

General considerations for chiral method development

Sample information:

-Solubility in different solvents

-π or hydrogen bonding capability

- pKa

-Functional groups

-UV spectrum

-Bulky substituents

-Inclusion-complexing capability

Table 1: Chiral stationary phases

Type of CSP	Chiral recognition mechanism	Analyte requirement	Mobile phase requirement
Protein based	Hydrophobic and electrostatic interactions	Ionizable groups (e.g. amide or acid); aromatic group helpful	Reversed phase only
Cyclodextrin	Inclusion complexation, H-bonding	Polar and aromatic group	Reversed and normal phase and polar organic mode
Polymer based carbohydrates	Inclusion complexation, attractive interactions	Ability to H bond; steric bulk near chiral center helps	Reversed and normal phase
Pirkle type	H-bonding, π-π interactions, dipole stacking	Ability to π or H bond, aromatic group helpful	Mostly normal phase; reversed phase usually less resolution
Ligand exchange	Coordination complexes to metals	α-amino and α-hydroxy amino acids	Reversed phase
Macrocyclic antibiotics	H-bonding, π-π interactions, dipole stacking; steric, hydrophobic pocket	Ability to π or H bond or dipole bond; steric bulk near chiral center assists	Reversed and normal phase

All of these characteristics may help a role in determining the ability of a CSP to resolve the sample enantiomers. The two most important considerations are the type of substituent groups present in the analyte molecule and their ability to either (1) π or H bond (attractive interaction) or (2) sterically interfere with bonding. The arrangement in space of these substituent groups (relative to the analyte chiral center) plays an important role in enantiomer separations. In most cases, the closer a group is to the chiral center, the more likely to chiral recognition and enantioselectivity. If there are no analyte groups that can bind to stationary support, derivatization of the analyte may be necessary.

Selecting chiral column

Over 100 CSP columns are commercially available, although many of these column are similar in structure and enantioselectivity. Some columns are better able to separate a wide range of sample types. A very rough order of CSP universality is;

Protein > carbohydrate > pirkle > cyclodextrin.

1. PROTEIN-BASED CHIRAL COLUMN

One of the most appealing types of chiral stationary phases for pharmaceutical analysis involves the use of protein immobilized to the surface of silica gel, or other support. Many protein based stationary phases have been proposed, six materials with somewhat different characteristics now have been commercialized in columns for chiral separations: bovine and human serum albumin, α_1-acid glycoprotein (orosomucoid), ovomucoid, cellobiohydrolase (cellulose) and pepsin. These proteins have been covalently bonded to wide pore silica and polymeric support for rapid HPLC separations that are suited for routine analytical methods. A special advantage of immobilized protein columns is that they are compatible with the aqueous buffered mobile phases widely used in many applications. Protein based chiral columns are less suited for preparative applications because of limited sample capacity and relative high column cost. Some commercially available protein based chiral columns are listed in table 2.

Table 2: Commercially available protein based chiral columns

Protein	Trade Name	Manufacturer	Support	Particle size (µm)	Pore size (A°)
AGP	Enantiopak	Pharmacia LKB	Silica	10	250
AGP	Chiral AGP	ChromTech AB	Silica	5	120
HSA	Chiral Protein-2	ASTEC	Silica	7	300
HSA	Chiral HSA	ChromTech AM	Silica	5	120
BSA	Resolvosil	Machery Nagel	Silica	7	100
BSA	BSA column	ASTEC	Silica	7	300
BSA	BA	Showa Denko KK	Polymer	15-20	500
OVM	Ultron ES-OVM	Shinwa chemicals	Silica	5	120
OVM	OV	Shinwa chemicals	Polymer	15-20	500
CELL	Chiral-CBH	ChromTech AB	Silica	5	120
Pepsin	Ultron ES-PEP	Shinwa chemicals	Silica	5	120

Human Serum Albumin (HSA):

Human Serum Albumin has about the same molecular weight as bovine serum albumin, but immobilized form of HSA exhibit some what different selectivity for many analytes. HSA covalently bonded to diol activated silica based support forms the basis for one commercial column. For many years, HSA has been used to study the binding of drugs to protein in solution, reflecting its utility in investigating drugs to be used in the human body.

α_1-Acid Glycoprotein (AGP)

α_1-Acid glycoprotein or orosomucoid is made up of a polypeptide containing 181 amino acid residues and sialic acid residues incorporated in the carbohydrate portion of the molecule. Salic acid has been suggested to be associated with binding of basic compounds at neutral pH. Two different commercial columns of immobilized AGP are available, and the variation in performance characteristics for these two products probably is the result of different immobilization chemistry.

Ovomucoid (OVM)

Ovomucoid is a trypsin inhibitor obtained from the egg white of chickens. This protein is known to undergo strong hydrophobic interactions with hydrophobic aromatic solutes. Ovomucoid protein is immobilized onto aminopropyl silica, and the amount of protein bound to the support apparently influences the degree of sample resolution. Recent studies have identified the active component of OVM column as an ovoglycoprotein.

Cellobiohydrolase (CBH; cellulose)

Cellobiohydrolase forms the basis for a diol silica-based commercial column that uses this stable covalently bound enzyme produced by the fungus *Trichoder ma reesei.* Pepsin is distinctive among enzymes for having a low isoelectric point (pI <1). The chromatographic packing is prepared by bonding this protein to aminopropyl silica via N,N'-disuccinimidyl carbonate [1].

2. POLYSACCHARIDE (CARBOHYDRATE) BASED CHIRAL COLUMNS

The naturally occurring polysaccharides form the basis for an important group of columns designed for chiral separations. Derivatives of these polymers, especially cellulose and amylose, exhibits excellent properties as stationary phases for HPLC. Commercially available columns of these materials now are used extensively for both analysis and preparative separations of a wide range of enantiomers. Although most columns used in normal phase mode, columns are also available for applications with aqueous reversed mobile phases. Some commercially available polysaccharide based chiral columns are shown in table 3.Non-ionic or moderately ionic samples generally are compatible with normal phase chromatography. Experience with many compound types shown that a decreasing order of successful separations with polysaccharide-based column is

OD > AD > OJ > OG > AS > OF > OB > OC > OK > OA. Statistically, the top four in this list are successful in separating more than 80% of unknown samples.

Table 3: Commercially available polysaccharide based chiral columns

Polysaccharide Derivative	**Trade Name**	**Distributor**
Microcrystalline cellulose triacetate*	Chiralcel CA-1	Daicel
	Cellolose triacetate	Merck
	Celolose Cel-AC-40XF	Macherey-Nagel
Cellolose triacetate	Chiralcel OA	Daicel
Cellolose tribenzoate	Chiralcel OB	Daicel
Cellolose trisphenylcarbamate	Chiralcel OC	Daicel
Cellolose tris(3,5-dimethylphenyl-carbamate)	Chiralcel OD Chiralcel OD-R	Daicel
Cellolose tris(4-chlorophenyl-carbamate)	Chiralcel OF	Daicel
Cellolose tris(4-methylphenyl-carbamate)	Chiralcel OG	Daicel
Cellolose tris(4-methylbenzoate)	Chiralcel OJ	Daicel
Cellolose tricinnamate	Chiralcel OK	Daicel
Amylose tris(3,5-dimethylphenyl-carbamate)	Chiralpak AD	Daicel
Amylose tris[(S)-1-phenylethyl-carbamate)	Chiralpak AS	Daicel

*other than this columns are coated on silica gel

3. DONOR- ACCEPTOR (PIRKLE) CHIRAL COLUMN

Separation of these CSPs is based on the three point rule. For chiral recognition to occur, a minimum of three simultaneous points of interactions is necessary between the solute and the CSP, with at least one interaction being strereochemically dependent. These interactions can be either attractive or repulsive in nature. The pioneering work of Pirkle had such an impact on the field that the whole category of donor-acceptor type stationary phases was named after him. The structure of these types of stationary phases is based on single strands of chiral selectors, connected via amidic linkage onto aminopropyl silica as of napthylaniline CSP, a π-electron donor CSP as shown in Fig.2 and Chemical structure of 3,5-dinitrophenyl phenylglycine (3,5-DNBPG) CSP, a π-electron acceptor CSP in fig.3.

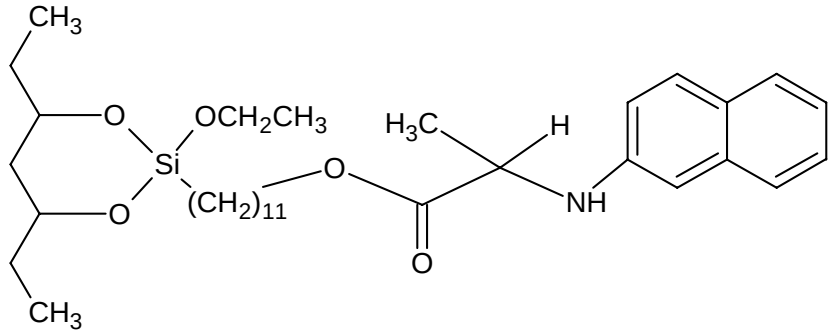


Fig.2: Napthylaniline CSP, a π-electron donor CSP

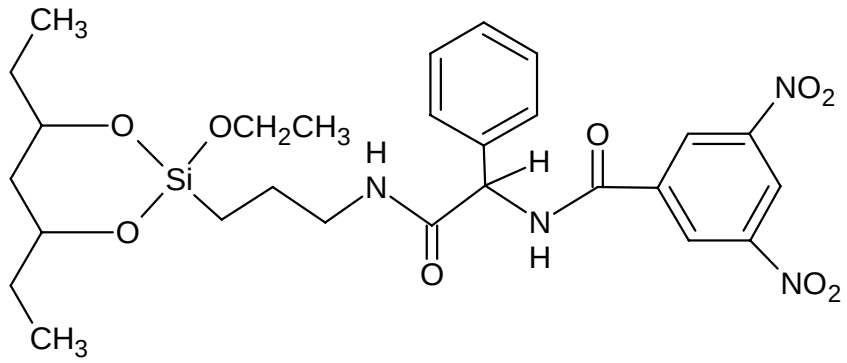


Fig.3: Dinitrophenyl phenylglycine (3,5-DNBPG) CSP, a π-electron acceptor CSP

Table 4: Commercially available Pirkle-type chiral columns

π-electron donor or acceptor CSP	Trade Name	Distributor
D or L Phenylglycine	DNBPG	Regis, J.T.Baker, ES Industries, E. Merck, Serva
D or L Leucine	Leucine	Regis, J.T.Baker, ES Industries, Serva
(R,R) or (S,S)-β-Gem 1	β-Gem 1	Regis
(R) or (S) α-Burke 1	α-Burke 1	Regis
D or L N-2-Naphthylamine	Naphthylamine	Regis
(S) N-1-Naphthylleucine	Naphthylleucine	Regis
Whelk-O 1	Whelk-O 1	Regis
Sumimoto OA Series	OA-2000, 2500, to 5000	Regis, HPLC technology, LTD, Sumimoto
Phenomenex-Chirex	Chirex CSPs	Phenomenex
DNB-phenylethylamine	Nucleosil chiral 2	Machery Nagel
Phenylmethyl urea	Spherisorb chiral 1	Phase-Sep
(R)-Naphthyl urea	Spherisorb chiral 2	Phase-Sep
(R)-Naphthylethylamine	YMC-Pack-K-Series	YMC, Inc.
α- Naphthyl urea	Bakerbond α- naphthyl urea	J. T. Baker
(R) Phenethyl urea	Chromegabond-(R)-phenethyl urea	E. S. Industries

4. CAVITY TYPE CHIRAL COLUMNS

Cavity type CSP allows inclusion of one or both enantiomers into a chiral cavity, thereby providing enhanced chiral discrimination between the two enantiomers. Cyclodextrin bonded phases (CD) comprise most cavity CSPs, but synthetic chiral polymers have also been used for inclusion complexation. Cyclodextrin CSPs make use of interactions such as H-bonding and inclusion complexation. These interactions require that the analyte contain at least a hydrophobic portion that fits into the CD cavity. If the

analyte also contains polar groups that can hydrogen bond to the CD hydroxyls, enantioselectivity can be enhanced. Cyclodextrin are available in α-, β-, γ-CD types that are formed from 6(α), 7(β), or 8(γ) glucopyranose units connected by α-(1,4)-glycosidic linkages as shown in fig.4 and its mechanism shown in fig.5. Some commercially available cyclodextrin columns are mentioned in table 5.

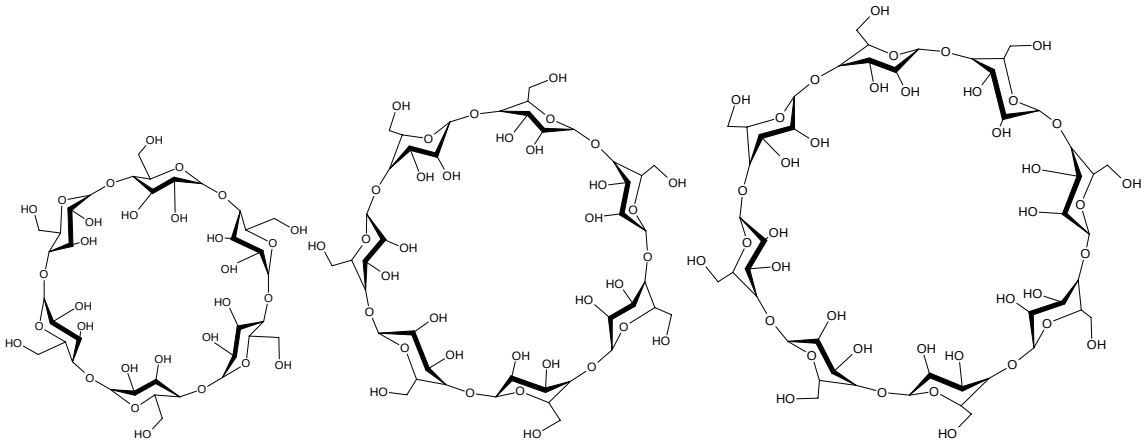


Fig.4: Alpha-Beta- Gamma Cyclodextrin

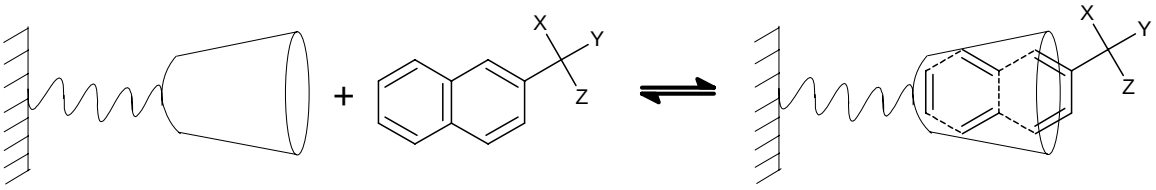


Fig.5: Cyclodextrin cavity and inclusion mechanism

Table 5: Commercially available cyclodextrin columns*

Trade Name	Support
Cyclobond I 2000	β - Cyclodextrin
Cyclobond II 2000	γ - Cyclodextrin
Cyclobond III 2000	α – Cyclodextrin
Cyclobond I Ac 2000	Acetylated β-CD
Cyclobond I SP 2000	Hydroxypropyl ether β-CD
Cyclobond I RSP 2000	Hydroxypropyl ether β-CD
Cyclobond I RN 2000	R-Naphthylmethyl carbamate β-CD
Cyclobond I SN 2000	S-Naphthylmethyl carbamate β-CD
Cyclobond I DMP 2000	3,5-Dimethylphenyl carbamate β-CD
Cyclobond I PT 2000	p-toluoyl ester β-CD

* Manufacturer and supplier : Astec , with 5µm particle size.

References

[1] Lloyed R. Snyder, Joseph J. Krikland, Joseph L. Glajch, 2nd ed., John wiley & Sons, INC. 1997 p.538-539

[2] http: // www.chromatography-online.org/topics/chiral/stationary/phase.html

[3] S. Levin and S. Abu-Lafi, "The Role of Enantioselective Liquid Chromatographic Separations Using Chiral Stationary Phases in Pharmaceutical Analysis", in Advances in Chromatography. E. Grushka and P. R. Brown, Eds., Marcel Dekker Inc.: NY, 33(1993) 233-266

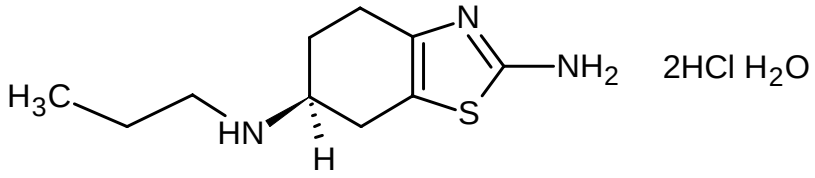
[4] http:/ /www. chromtech.se/pdf/handbook.pdf

[5] http:// www.esslab.com/daicel_chiral_columns.htm

Section-I: Chiral RP-HPLC method development and validation for determination of R-isomer in Pramipexole

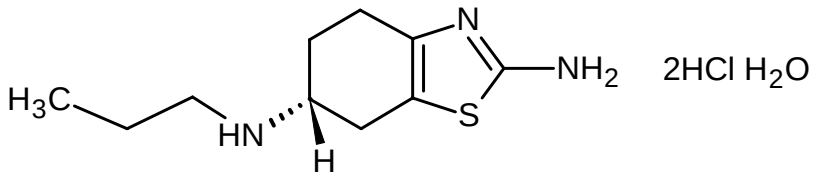
1. Introduction to pramipexole

Pramipexole is chemically (S)-2-amino-4,5,6,7-tetrahydro-6-benzothiazole. Commercially available tablets conations pramipexole dihydrochloride monohydrate salt (Fig.1A) in formulation (Sifrol®, Mirapex®, and Mirapexin®). Molecular formula of pramipexole dihydrochloride monohydrate is $C_{10}H_{17}N_3S$ 2HCl H_2O having molecular weight 302.28 g/mole. Its dissociation constants are pKa_1 = 5.0 and pKa_2 = 9.6, partition coefficient Log *P* (octanol water) is 0.87. Melting point is 296°C to 301°C with decomposition [1-3]. R-Isomer of pramipexole dihydrochloride monohydrate salt is an impurity of drug substance (Fig.1B). Detail information is described in section of pramipexole assay method development for formulation tablets.



S-isomer

Fig.1A: Pramipexole dihydrochloride monohydrate (S-isomer)



R-isomer

Fig.1B: Pramipexole dihydrochloride monohydrate (R-isomer)

2. Literature review

2.1 A Pathare DB, Jadhav AS, Shingare MS have developed a chiral liquid chromatographic method for the enantiomeric resolution of Pramipexole dihydrochloride monohydrate, (S)-2-amino-4,5,6,7-tetra-hydro-6-(propylamino) benzothiazole dihydrochloride monohydrate, a dopamine agonist in bulk drugs. The

enantiomers of pramipexole dihydrochloride monohydrate were resolved on a Chiralpak AD (250 mm x 4.6 mm, 10 microm) column using a mobile phase system containing n-hexane:ethanol:diethylamine (70:30:0.1, v/v/v). The resolution between the enantiomers was found not less than eight. The presence of diethylamine in the mobile phase has played an important role in enhancing chromatographic efficiency and resolution between the enantiomers. The developed method was extensively validated and proved to be robust. The limit of detection and limit of quantification of (R)-enantiomer were found to be 300 and 900 ng/ml. ng/ml. respectively for 20 microl injection volumes. The percentage recovery of (R)-enantiomer was ranged from 97.3 to 102.0 in bulk drug samples of Pramipexole dihydrochloride monohydrate. Pramipexole dihydrochloride monohydrate sample solution and mobile phase were found to be stable for at least 48 h. The proposed method was found to be suitable and accurate for the quantitative determination of (R)-enantiomer in bulk drugs [4].

3. Aim of present Work

Pramipexole dihydrochloride monohydrate is identical as S-isomer or basically it is the salt form of (S)-2-amino-4,5,6,7-tetrahydro-6-(propylamino) benzothiazole. From the structure at the position of 6^{th} carbon all attached groups and / or atom is different. So, there is one chiral center is present in the basic molecule part of the API. Hence, as per the chiral theory two isomers are possible for this structure. Out of them S-isomer is the identified and reported as an active pharmaceutical ingredient for the treatment of Parkinson's disease. Finally R-isomer needs to be control as per ICH guideline [5].

As per discussion in the literature review it is not official in pharmacopeia [6-11], method for Assay by UV was reported with internal standard preparation. Methods for pramipexole in human plasma and related impurities method were reported. A method for **chiral purity by *normal phase*** also reported [4] but **eco friendly and simple *reversed phase* method for chiral purity** is the aim of present work.

The aim and scope of the proposed work are as under

- Method development for chiral purity / R-isomer content for Pramipexole drug substance by HPLC.

- Resolution optimization for R-isomer and S-isomer (Drug substance)
- Perform analytical method validation for the proposed method as per ICH guideline [12].

4. Experimental

4.1 Materials, chemicals and reagents

A) Materials :

All the materials are manufactured and provided by Torrent Research Center

Sr. No.	Material Name	Lot No. / Batch no.	Potency /Purity
1	R-Isomer (+) 2,6-diamino -4,5,6,7-tetrahydro benzothiazole reference standard	OTV/2007/238	99.62
2	Pramipexole dihydrochloride monohydrate API	PRA/015/005	--
3	Pramipexole impurity A [2,6-Diamino-4,5,6,7-tetrahydro-benzothiazole]	OTV/2007/123	98.99%
4	Pramipexole impurity B [(6S)-N2,N6-dipropyl 4,5,6,7 tetrahydro 1,3 benzothiazole 2,6 diamino]	OTV/2007/114	98.53%
5	Pramipexole impurity C [(6S)-N6,N6-dipropyl 4,5,6,7 tetrahydro 1,3 benzothiazole 2,6 diamino]	OTV/2007/113	99.56%

B) Chemicals and reagents:

Sr. No.	Chemical/Reagent	Grade	Sr. No.	Chemical/Reagent	Grade
1	Acetonitrile	HPLC	6	Boric acid	AR
2	Ammonia solution	AR	7	Hydrogen peroxide	AR
3	Methanol	HPLC	8	Hydrochloric acid	AR
4	Water	Milli-Q	9	Sodium hydroxide	AR
5	Borax	AR			

4.2 Instrumentation

Shimadzu LC 2010C with Class VP software HPLC systems were used.

4.3 Mobile phase preparation

Buffer: Acetonitrile in ratio of 70: 30 (v/v) were mixed and sonicated to degas.

Buffer: pH of Buffer 1 adjusted to 8.5 with Buffer 2.

Buffer 1: 7.44 g of borax dissolved in 1000 ml of water.

Buffer 2: 13.66 g of boric acid dissolved in 1000 ml of water.

4.4 Diluent preparation

Methanol: 0.1% v/v ammonia solution, 40:60 mixed.

4.5 Resolution preparation

Accurately weighed and transferred each of about 2.5 mg of R-isomer of pramipexole working/reference standard and Pramipexole dihydrochloride monohydrate into 50 ml volumetric flask, 35 ml of diluent was added and dissolved it with aid of sonication, diluted to volume with diluent, mix. 5 ml of this solution was diluted to 50 ml with diluent.

4.6 Standard preparation

Accurately weighed and transferred about 2.5 mg of R-isomer of pramipexole working/reference standard into 50 ml volumetric flask, 35 ml diluent was added to dissolve with aid of sonication and diluted to volume with diluent. 5 ml of this solution was diluted to 50 ml with diluent. Further 5 ml of this solution was diluted to 50 ml with diluent.

4.7 Test preparation

Accurately weighed and transferred about 25 mg of test sample into 50 ml volumetric flask, 35 ml of diluent was added to dissolve with aid of sonication, diluted to volume with diluent.

4.8 Chromatographic conditions

Chromatographic separation of both the isomers achieved on CHIRAL PACK AD-RH 150 mm x 4.6 mm column with 10 x 4 mm guard cartridge as of same CSP in gourd holder [13], column oven temperature was 25˚C. The flow rate of the mobile phase was adjusted to 0.6 ml/min and the injection volume was 20 µl. Detection was performed at 260nm.

5. Result and discussion

5.1 Development and optimization of the HPLC method

Initially development was started with solution of mixture of R-isomer and S-isomer of Pramipexole. Mobile phase with buffer pH 4.5, 20mM potassium dihydrogen phosphate and methanol in binary isocratic mode at different compositions were tried but even at higher aqueous composition peaks were not separated or it gave ‘M’ shape peak. For the purpose of quantitation base line separation was the primary goal before method validation and a mobile phase with higher pH gave the base line resolution so, buffer with higher pH was explored but as a known fact that at higher pH phosphate buffers reduces the column life. Hence, borate buffer with higher pH was explored to increase the column life and buffer and acetonitrile composition was optimized to get desired separation for both the isomers. Before any sample analysis or study system was qualified with the resolution solution and standard solution as a part of system suitability.

5.2 Method validation

5.3.1 Specificity:

Interference from blank and impurities:

A blank, resolution solution, standard preparation and sample preparation were injected and peak purity index for analyte peak in each preparation were the observed within the acceptance criteria as mentioned in table 1 and chromatogram shown in fig.1 to fig.3. There was no peak at the retention time of any of isomer’s retention time of peak.

Table 1: Peak purity in specificity study

Solution	Peak purity index for R-Isomer
Standard preparation	0.99597
Resolution	0.99999
Sample preparation (as such)	Not detected
Sample preparation spiked with R-isomer	0.99227

Fig.1: Chromatogram of blank preparation

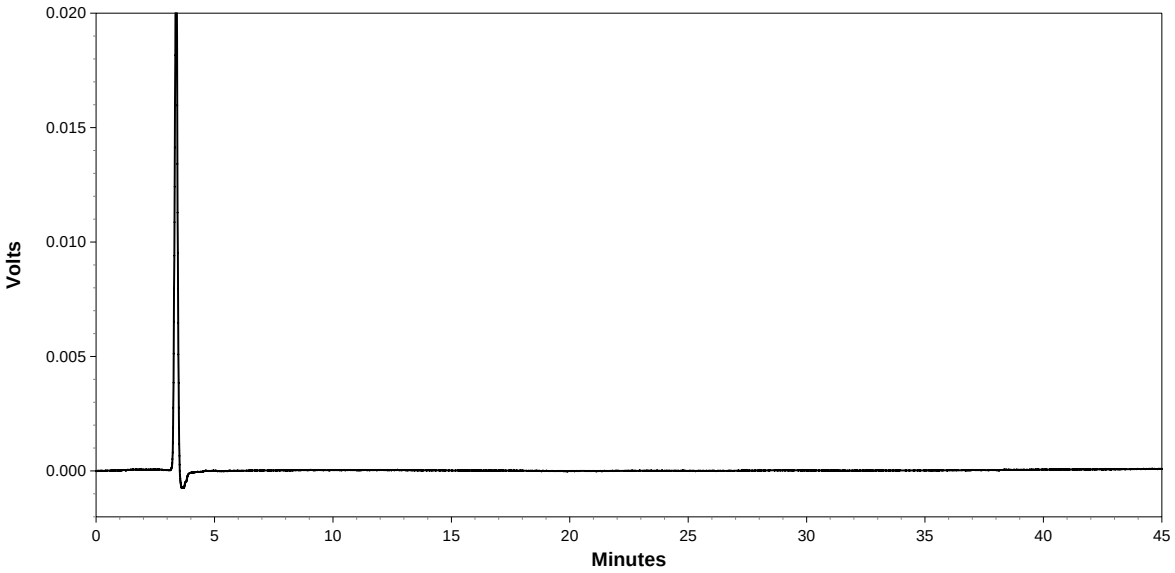


Fig.2: Chromatogram of resolution preparation

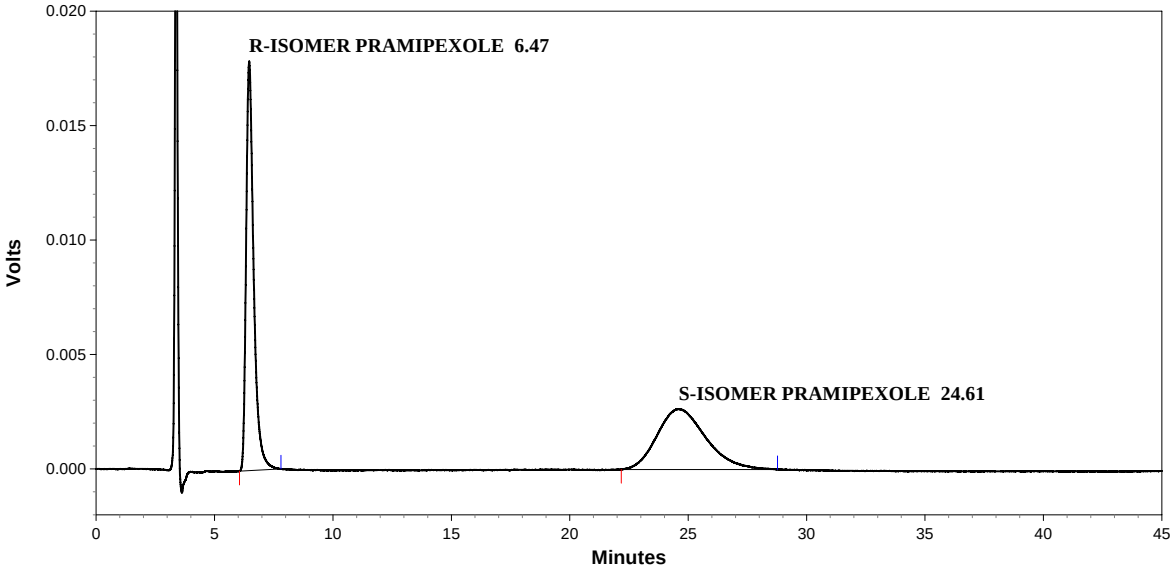
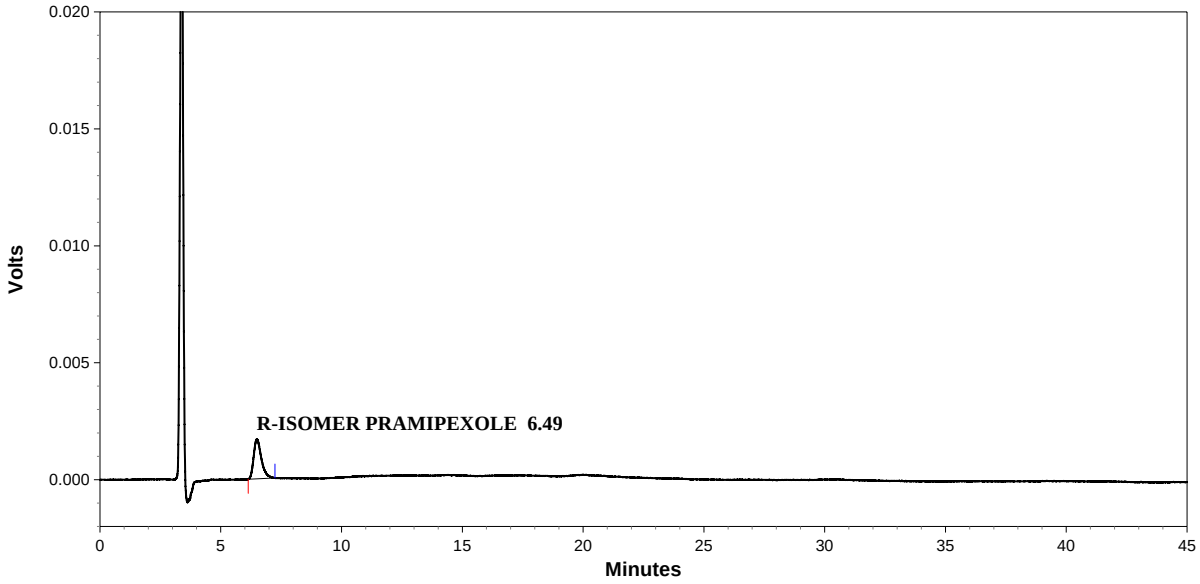


Fig.3: Chromatogram of R-isomer standard preparation



Interference from degradation products by stress study

The Pramipexole dihydrochloride monohydrate was subjected to thermal, water hydrolysis and photo degradation while other chemical stress conditions were not applied to avoid probable chiral column damage. For each stress conditions a corresponding blank was prepared.

A blank, unstressed and stressed pramipexole dihydrochloride monohydrate sample solutions were prepared and injected

5.3.2 Linearity:

Linearity was determined at five levels over the range of LOQ to 150% of specification limit. A standard stock solution was prepared and further diluted to attain concentration at LOQ (0.05%), 80%, 100%, 120% and 150% of working concentration. Each preparation was injected in singlet. A graph of area versus concentration was plotted. The correlation co-efficient (r), y- intercept, slope of regression line, residual sum of squares were calculated and mentioned in Table 2. Supporting plot and chromatograms shown in fig.3 to fig.5.

Table 2: Linearity data for pramipexole R-isomer

Sr.No.	Concentration (% of working concentration)	Area
1	49.80	18851
2	79.80	29169
3	99.60	37571
4	119.60	45874
5	149.40	58005
Correlation coefficient (r)		0.99917
Slope of regression line		396.76
Y-intercept		-1639
Residual sum of squares		1496806

Fig.3: Linearity curve for pramipexole R-isomer

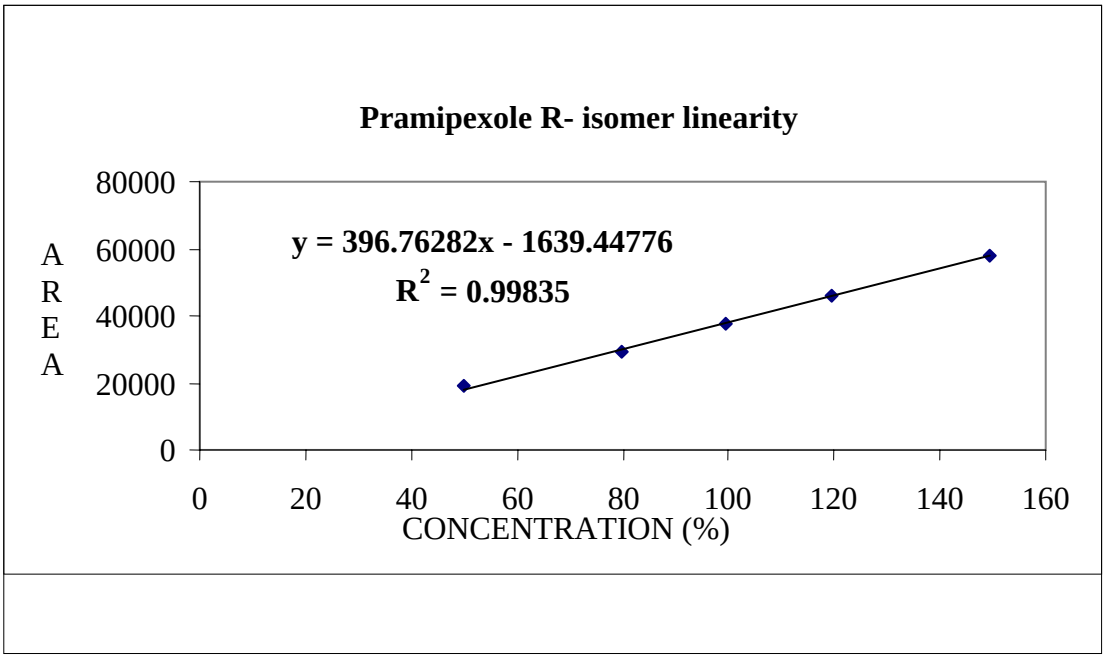


Fig.4: Linearity study chromatogram of level-1

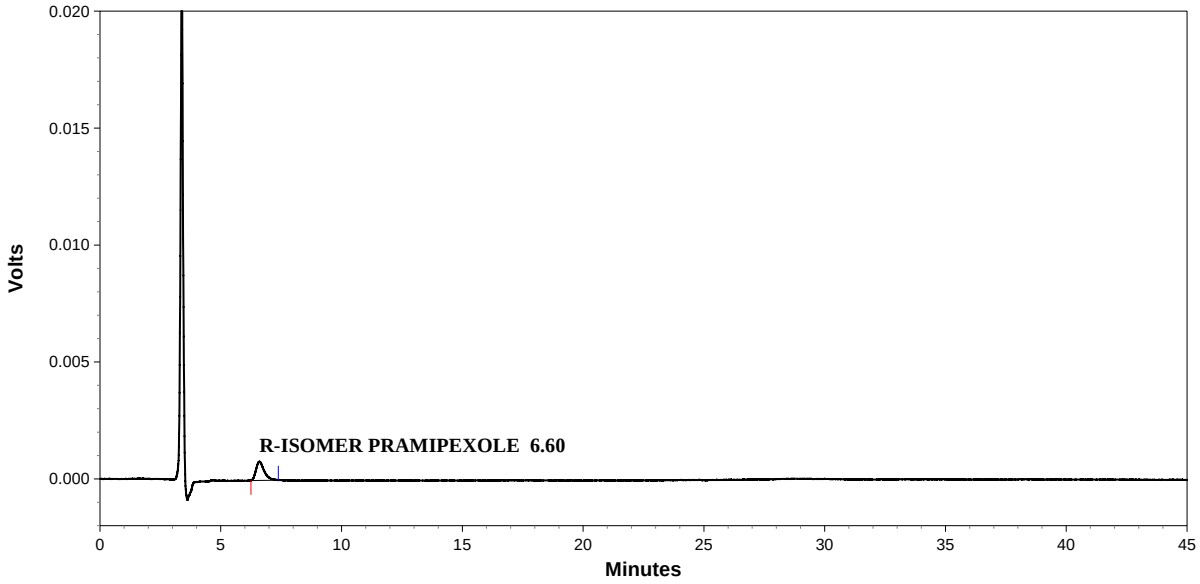


Fig.5: Linearity study chromatogram of level-2

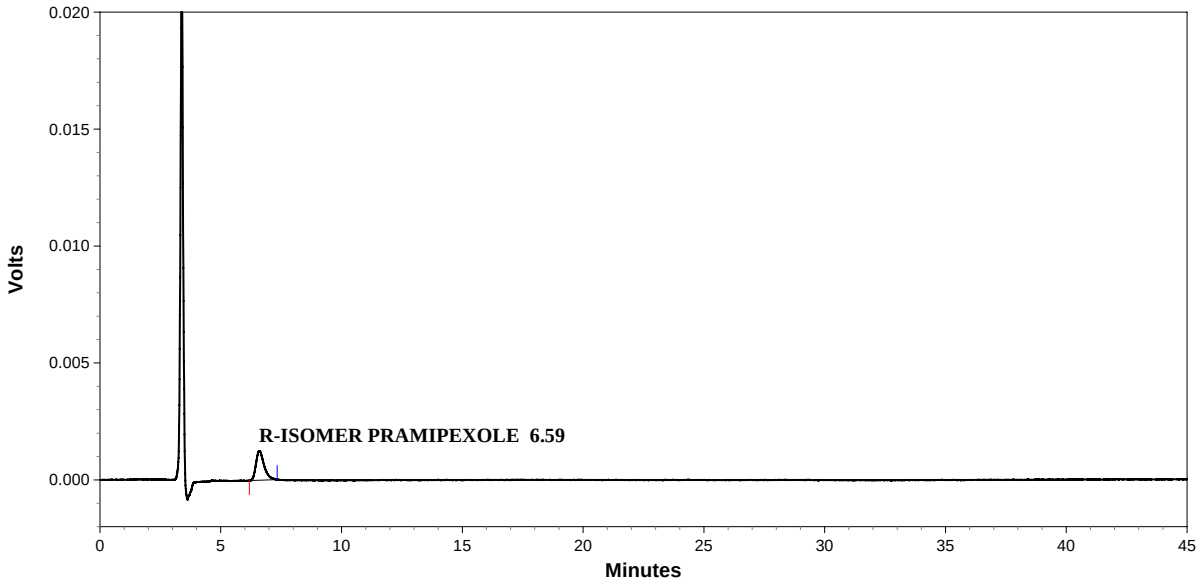


Fig.6: Linearity study chromatogram of level-3

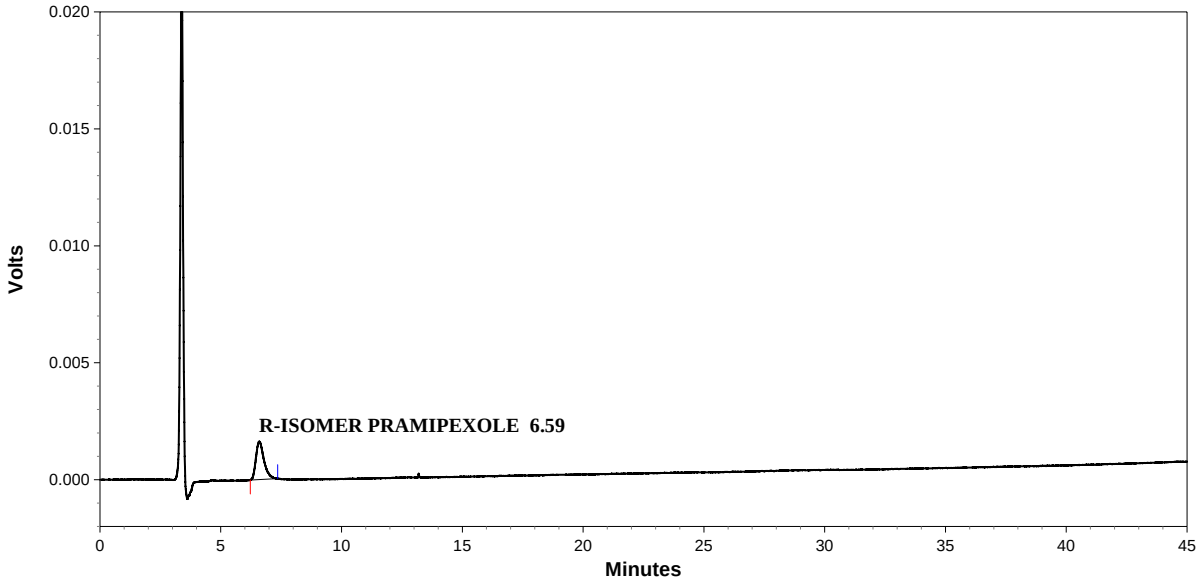


Fig.7: Linearity study chromatogram of level-4

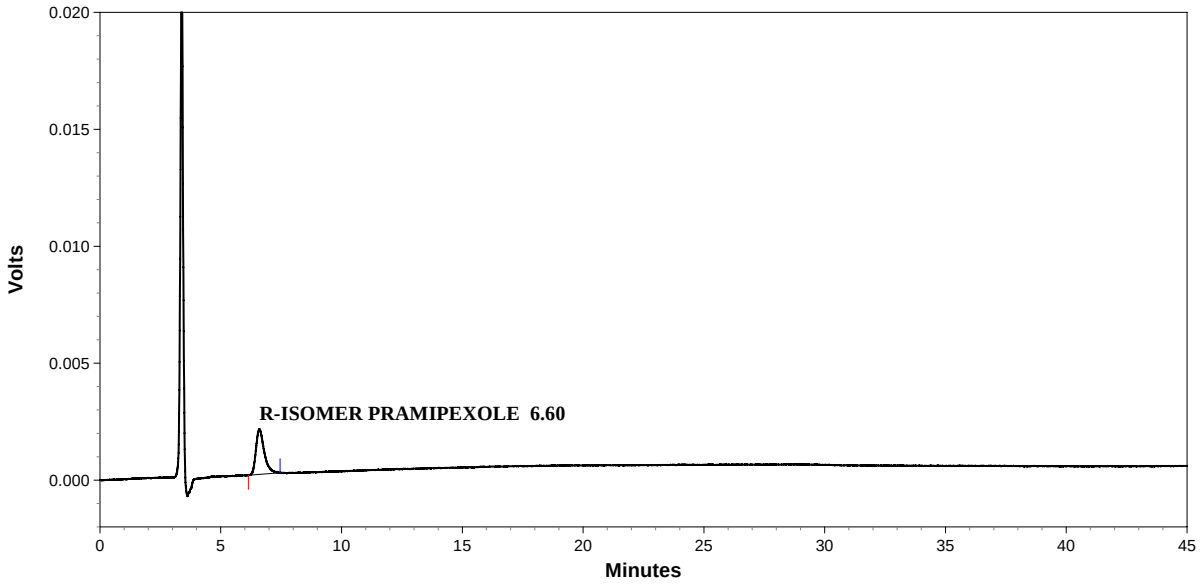
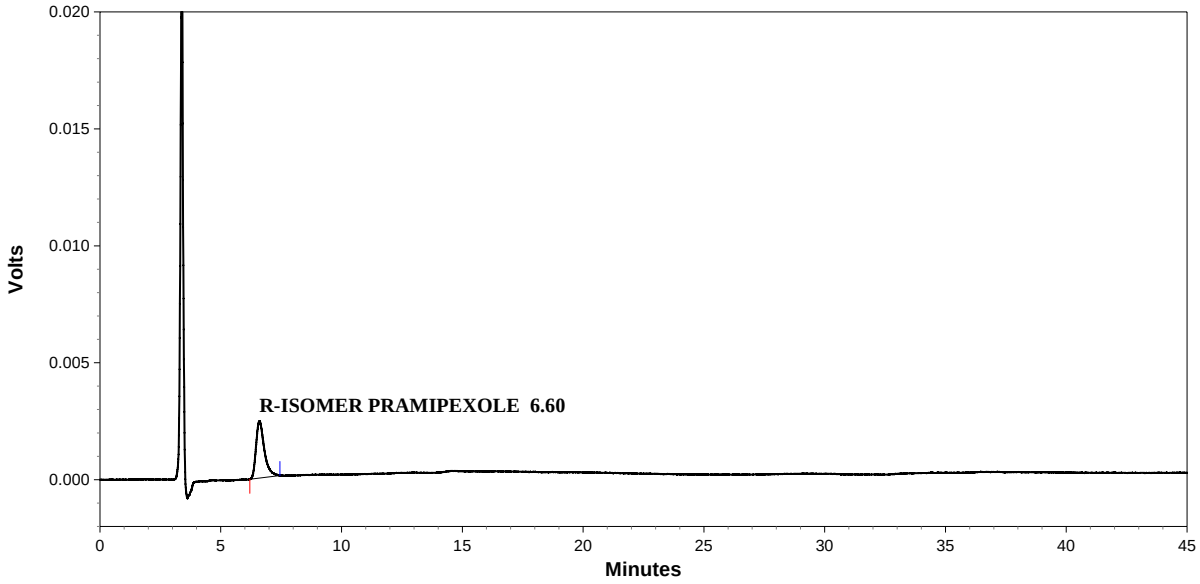


Fig.8: Linearity study chromatogram of level-5



5.3.3 Precision

Method precision was established by determining the R-isomer of six sample preparations under same conditions. Six replicate of samples were prepared by spiking the solution of R-isomer at the target concentration and injected. Mean, %RSD and 95% confidence interval for % of R-isomer result were calculated as mentioned in Table 3 and chromatogram shown in fig.9 and fig.10.

Table 3: Data of precision study

Sample preparation	**Day-1**	**Day-2**
	% R-isomer	% R-isomer
1.	0.10	0.10
2.	0.10	0.11
3.	0.10	0.10
4.	0.10	0.10
5.	0.11	0.10
6.	0.10	0.10
Mean	0.10	0.10
SD	0.0041	0.0041
% RSD (n= 6)	4.02	4.02
95% Confidence interval (CI)	0.003	0.003
Overall % RSD (n =12) = 3.83		

Fig.9: Chromatogram for method precision sample (spiked)

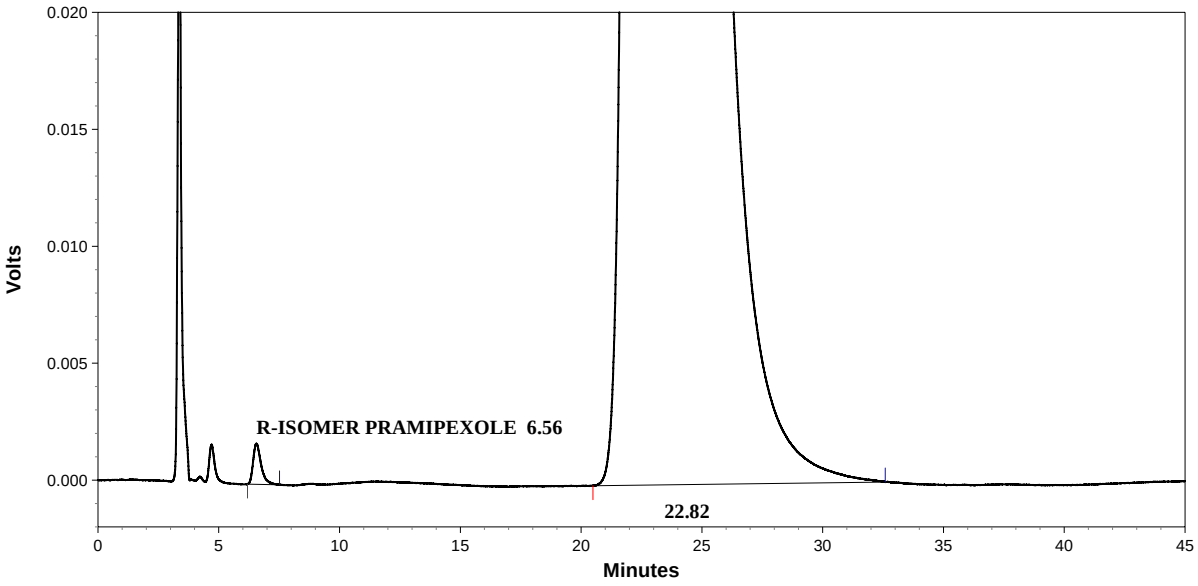
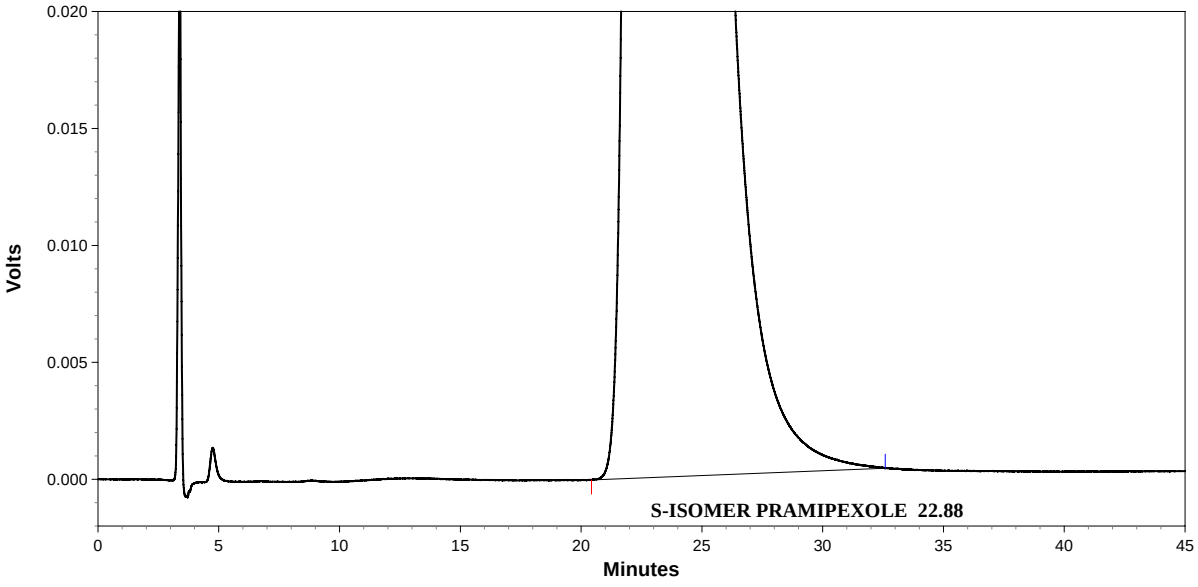


Fig.10: Chromatogram for method precision sample (as such)



5.3.4 Accuracy:

The accuracy of the method was established at three levels in the range of LOQ to 150% of specification limit. Above levels were prepared in triplicate. Each solution was injected in singlet. % Recovery was calculated at each level and recorded in table 4 and supporting chromatograms shown in fig.11 to fig.13.

Table 4: Recovery study

Level	Amount found (mg/ml)	Amount added (mg/ml)	Recovery (%)	Mean (%)	% RSD
Level-1 (LOQ)	0.2519	0.2500	100.8	101.7	0.82
	0.2560	0.2500	102.4		
	0.2549	0.2500	102.0		
Level-2 (100%)	0.5053	0.5001	101.0	102.6	1.87
	0.5103	0.5001	102.0		
	0.5237	0.5001	104.7		
Level-3 (150%)	0.7724	0.7501	103.0	105.6	2.17
	0.8004	0.7501	106.7		
	0.8038	0.7501	107.2		

Fig.11: Chromatogram of accuracy study level-1 (LOQ)

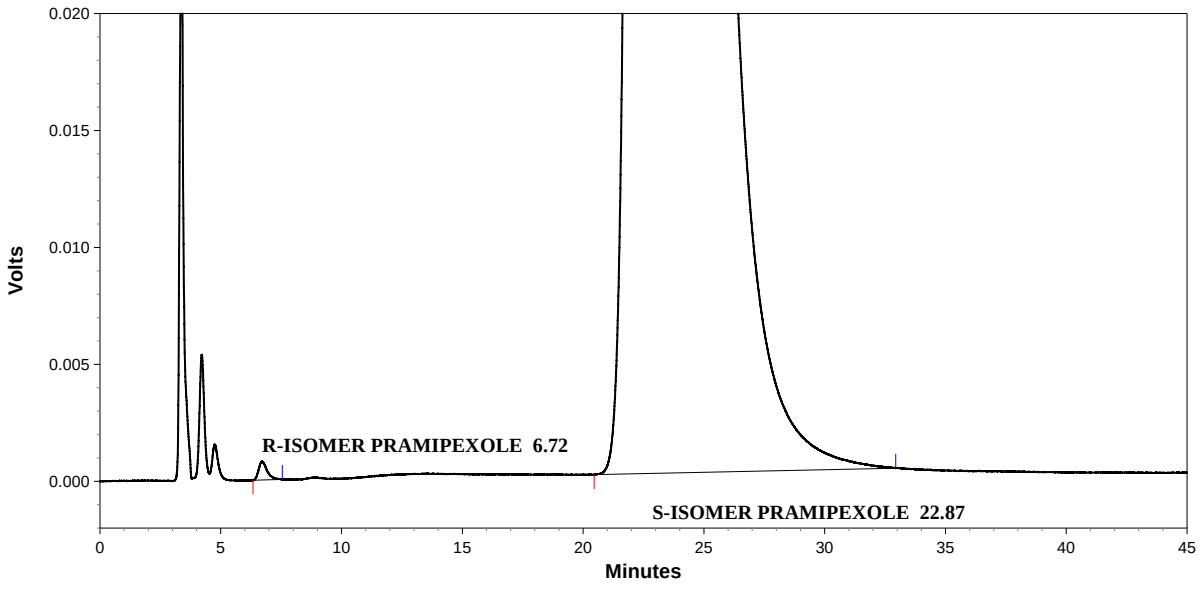


Fig.12: Chromatogram of accuracy study level-2 (100%)

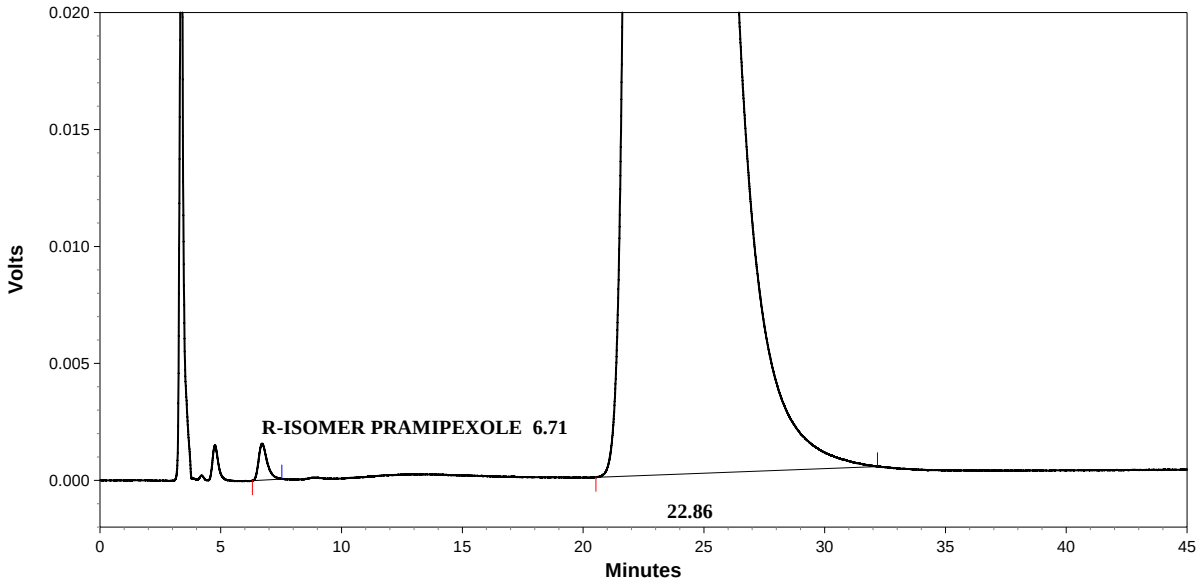
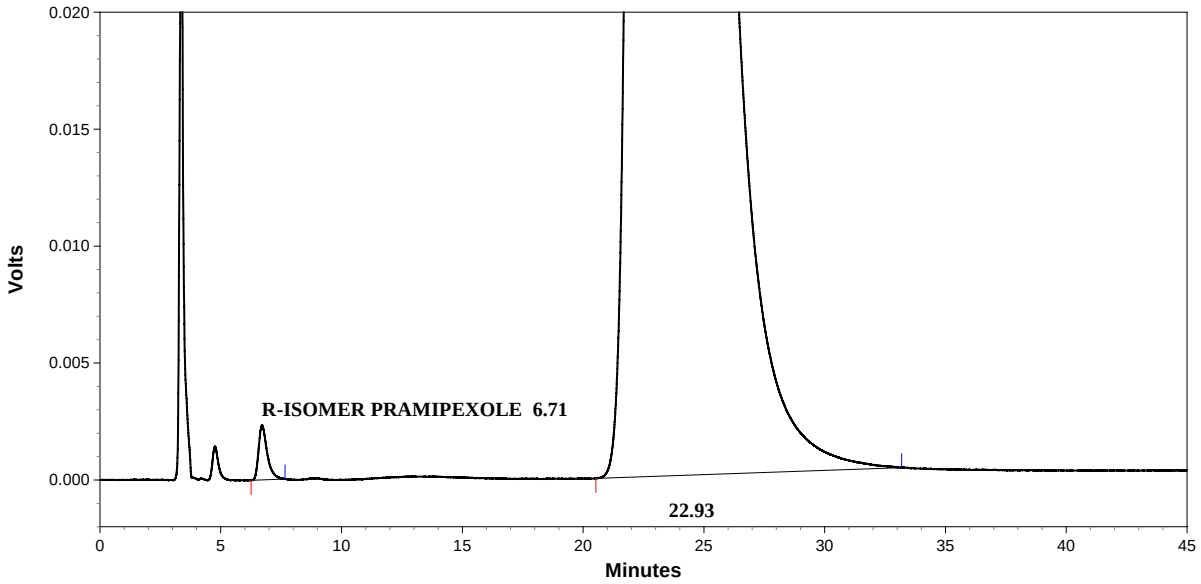


Fig.13: Chromatogram of accuracy study level-3 (150%)



5.3.5 Solution stability study:

The standard and sample preparations were prepared at test concentration and initial % R-isomer content was determined. Standard and sample preparations were stored up to 74 hours at room temperature and at 2 -8°C. The % R-isomer content obtained at different time interval were compared with the initial % R-isomer content value and recorded. The results are recorded in table 5 and table 6.

Table 5: Solution stability for standard

Standard Preparation			
Time	**Condition**	**% R-isomer content**	**Absolute difference (%)**
Initial	Room temperature	100.0	Not applicable
After 30 hours	Room temperature	100.4	0.40
After 74 hours	Room temperature	100.9	0.90

Table 6: Solution stability for sample

Sample Preparation			
Time	**Condition**	**% R-isomer content**	**Absolute difference (%)**
Initial	Room temperature	0.10	Not applicable
After 27 hours	Room temperature	0.10	0.00
After 71 hours	Room temperature	0.10	0.00

5.3.6 Robustness:

The robustness of the method was established by making deliberate minor variations in the following method parameters.

(A) Change in flow rate by ± 10% (0.54 ml/min and 0.66 ml/min)

(B) Change in the minor components in the mobile phase by ± 2% absolute or 30% relative whichever is lower (Buffer: Acetonitrile, 68:32 and Buffer: Acetonitrile, 72:28)

(C) Change in column oven temperature + 5°C (Column temperature 30°C)

(D) Change in buffer pH by ± 0.2 (pH 8.3 and 8.7)

(E) Change in column lot.

Sample was prepared by spiking the R-isomer at specification level

The effect of changes was observed on system suitability and recorded in table 7. The effect of changes was observed on % R-isomer content and recorded in table 8.

Table 7: Robustness and system suitability

Condition	System suitability parameters	
	%RSD	Resolution
Limits:	NMT 5.0	NLT 2.0
A) Change in flow rate		
Normal condition (0.6 ml per minute)	1.54	7.72
Change in flow rate by 10% (0.54 ml per minute)	2.28	7.75
Change in flow rate by 10% (0.66 ml per minute)	2.29	7.00
B) Change in minor component in the mobile phase		
Normal condition (Buffer:Acetonitrile (70:30))	1.54	7.72
Change in Acetonitrile composition by + 2% absolute (Buffer:Acetonitrile (68:32))	0.5	7.39
Change in Acetonitril composition by - 2% absolute (Buffer:Acetonitrile (72:28))	2.02	7.52
C) Change in column oven temperature		
Normal condition (25°C)	1.54	7.72
Change in oven temperature by + 5°C (30°C)	1.3	8.65
D) Change in pH of mobile phase		
Normal condition (pH 8.5)	1.75	7.71
Change in pH of buffer by + 0.2units (pH 8.7)	2.22	8.88
Change in pH of buffer by - 0.2units (pH 8.3)	2.35	7.51
E) Change in column lot		
Normal column lot: CH/AD-RH/150-4.6/03	4.44	8.02
Changed column lot: CH/AD-RH/150-4.6/02	2.26	7.80

Table 8: Robustness and system suitability

Condition	% R-isomer
A) Change in flow rate	
Normal condition (0.6 ml per minute)	0.10
Change in flow rate by – 10% (0.54 ml per minute)	0.11
Absolute difference from normal condition	**0.01%**
Change in flow rate by + 10% (0.66 ml per minute)	0.11
Absolute difference from normal condition	**0.01%**
B) Change in minor component in the mobile phase	
Normal condition (Buffer: Acetonitrile (700:300))	0.10
Change in Acetonitrile composition by – 2% absolute (Buffer: Acetonitrile (720:280))	0.12
Absolute difference from normal condition	**0.02%**
Change in acetonitrile composition by + 2% absolute (Buffer: Acetonitrile (680:320))	0.11
Absolute difference from normal condition	**0.01%**
C) Change in buffer pH by ± 0.2 units	
Normal condition (buffer pH-8.5)	0.10
Change in buffer pH by +0.2 unit (pH-8.7)	0.11
Absolute difference from normal condition	**0.01%**
Change in buffer pH by -0.2 unit (pH-8.3)	0.11
Absolute difference from normal condition	**0.01%**
D) Change in column oven temperature	
Normal condition (25°C)	0.10
Change in oven temperature by + 5°C (30°C)	0.11
Absolute difference from normal condition	**0.01%**
E) Change in column lot	
CH/AD-RH/150-4.6/03 Lot no. ADRHCD-FE053	0.10
CH/AD-RH/150-4.6/02 Lot no. ADRHCD-KE019	0.10
Absolute difference from normal condition	**0.0%**

6. Quantitation and calculation formulae

6.1 Relative Standard Deviation (%)

$$\text{Relative Standard Deviation (\%)} = \frac{SD \times 100}{\overline{X}}$$

By Microsoft excel

Average = AVERAGE (Number 1)

Standard Deviation = STDEV (Number 1)

6.2 Calculation formulae in linearity

$$\text{Correlation coefficient (r)} = \frac{n\ (\sum xy)\ -\ (\sum x)(\sum y)}{\sqrt{\{[n\sum x^2 - (\sum x)^2]\ [n\sum y^2 - (\sum y)^2]\}}}$$

Correlation coefficient (r) = CORREL (Array 1, Array 2)

$$\text{Slope of regression line} = \frac{n\ (\sum xy)\ -\ (\sum x)(\sum y)}{n\sum x^2 - (\sum x)^2}$$

Slope of regression line = SLOPE (Array 1, Array 2)

$$\text{y- intercept} = A = \overline{y} - B\overline{x}$$

y- intercept = INTERCEPT (Array 1, Array 2)

$$\text{Residual sum of squares} = \sum (Y_{original} - Y_{calculated})^2$$

Where,

$Y_{calculated} = A + Bx$

n = Number of measurements

x = Individual concentration

y = Individual area

$\bar{x}$ = Average concentration

$\bar{y}$ = Average area

Residual sum of squares = SUMXMY2 (Array 1, TREND (Array 1, Array 2, Array2))

6.3 Accuracy (% Recovery)

$$\% \text{ Recovery} = \frac{\text{Ammount of sbustance (mg) found}}{\text{Ammount of substance (mg) added}} \text{ x } 100$$

6.4 % R-isomer in pramipexole

$$\% \text{ R-isomer} = \frac{\text{Asp}}{\text{Astd}} \times \frac{\text{Std. wt}}{\text{Dilution}} \times \frac{\text{Dilution}}{\text{Spl Wt}} \times \text{Std. assay \% (as such)}$$

$$\% \text{ R-isomer} = \frac{39940}{38682} \times \frac{2.501}{50} \times \frac{5}{50} \times \frac{5}{50} \times \frac{50}{25.02} \times 99.62$$

% R-isomer = 0.10

Asp	:	Area of R-isomer in sample
Astd	:	Mean area of diluted standard
Std. wt	:	Std weight in mg
Spl wt	:	Sample weight in mg

6.5 95% Confidence Interval (CI)

95% Confidence Interval (CI) = $\pm \frac{t\alpha \times SD}{\sqrt{n}}$

Where,

SD = Standard Deviation

$\overline{X}$ = Average concentration

n = Number of measurements

t_α = t value at n-1 from t table (two tail)

95% Confidence Interval (CI) = SD (2.57058)/SQRT (6)

References

[1] Sifrol, INN-Pramipexole; http://www.emea.europa.eu/humandocs/PDFs/EPAR/Sifrol/059197EN6.pdf

[2] Clarke's analysis of drug and poison in pharmaceuticals, body fluids and postmortem material 3 rd edition 2004 p.1468

[3] http://www.Mirapex.com

[4] A Pathare DB, Jadhav AS, Shingare MS J. Pharm Biomed Anal.41-4(2006)1152-1156

[5] ICH guideline, Q3B (R2) step 4, Impurities in new drug products (2006)

[6] US Pharmacopeia, USP 31 NF 26, 2008

[7] European Pharmacopeia, 6th edition, 2008

[8] British Pharmacopeia, 2008

[9] Indian Pharmacopeia, 2007

[10] Japanese Pharmacopeia, 15th edition (2006)

[11] Pharmacopeia of the people's republic of china (2005)

[12] ICH guideline, Q2 (R1) step 4, Validation of Analytical Procedures: Text and Methodology (2005)

[13] Column instruction manual, Daicel chemical industries limited.

INTRODUCTION TO UPLC

History of chromatography can give an idea about improvement in technology from conventional column chromatography to high performance liquid chromatography and finally at this stage an ultra performance liquid chromatography or in other way a combination of pressurized chromatographic technology and sub 2 (two) micron particle size of stationary phase technology leads to advance Ultra Performance Liquid Chromatography (UPLC) or Rapid Resolution Liquid Chromatography (RRLC) technology.

Technology of sub 2 (two) micron particle size leads many modifications in hardware part of the system like reduction of system volume, higher pump pressure capacity, injector and needle part, and cell volume of detector as well as in software area, data acquisition rate or capacity was increased for sufficient data collection.

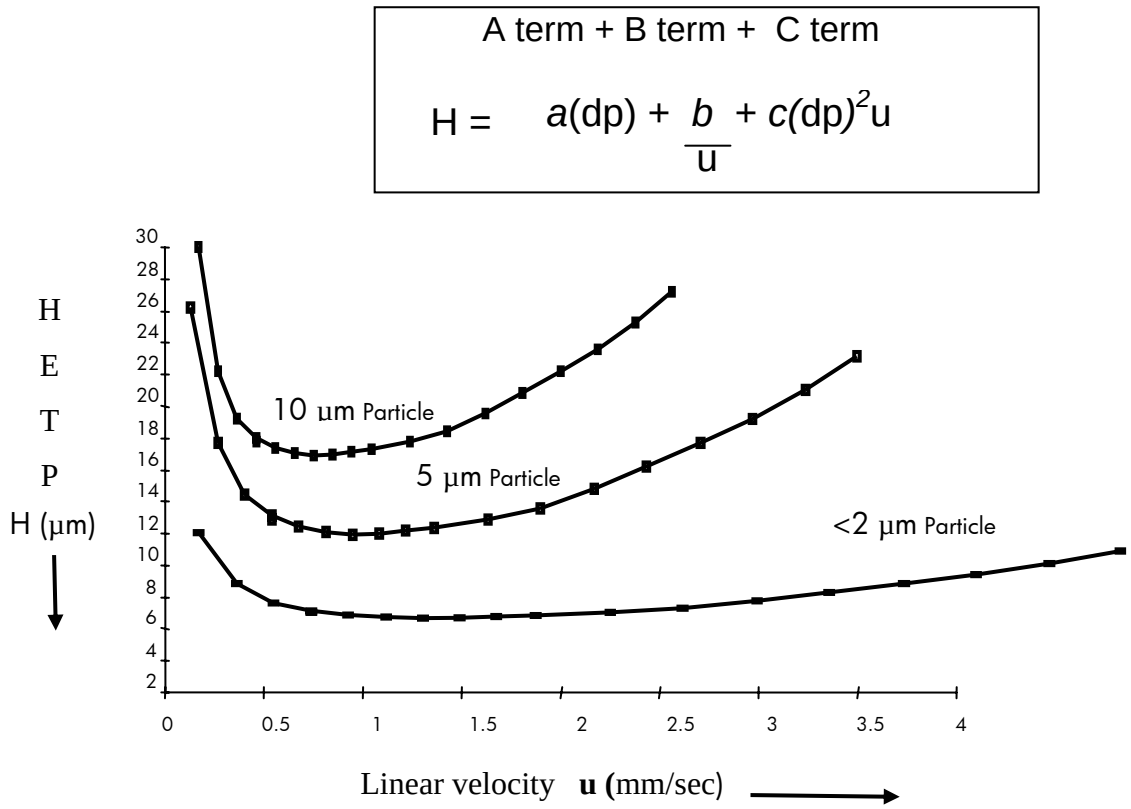
In brief detail, small particle size columns leads to increase in pump pressure so that area was improved and for accurate and precise injection volume needle in needle technology with teflon material was came into the picture. Detector cell volume was reduced for better signals and resolution.

Smaller particle size of 2 micron technology altered the machine and its application for faster way of analysis in current scenario of separation science. Requirement of this technology can be explained by van deemter equation [1,4] and plot as shown in fig 1. From this plot it reveals that there is minimum HETP against the linear velocity with the almost constant relation or maximum the theoretical plates can be achieved with particle size less than 2 micron. Finally as a known fact increasing in N leads to increase in Resolution as shown in formula;

$$Rs = \frac{\sqrt{N}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k}{k+1} \right)$$

System Efficiency **Selectivity** **Retentivity**

Fig.1: Van Deemter plots-influence of particle size



Now, for method conversion from HPLC to UPLC or for comparison of both the technology following aspects needs to take in consideration [2-3].

- Ratio of column length to particle size (L/dp) needs to keep constant.
  i.e. 150 mm/5 µm = 30,000 is closest to 50mm/1.7 µm = 29,500

- Column selection should be based on same basic column chemistry
  i.e. C_{18} column should be replaced by C_{18} column

- 5 µm to 1.7 µm particle size leads to increase in speed of 9X along with 9X pressure
- 3 µm to 1.7 µm particle size leads to increase in speed of 3X along with 3X pressure

- 5 µm to 1.7 µm particle size leads to increase in peak height of 1.7X
- 3 µm to 1.7 µm particle size leads to increase in peak height of 1.3X

- 5 µm to 1.7 µm particle size leads to decrease in peak width of 0.6X
- 3 µm to 1.7 µm particle size leads to decrease in peak width of 0.8X

- Column efficiency (N) is inversely proportional to dp

$$N \propto \frac{1}{dp}$$

i.e. 5 µm to 1.7 µm particle size leads to increase in column efficiency (N) 3X but

$$Rs \propto \sqrt{N}$$

So, resolution also increase by 1.7X

Based on above fact practically an example for chromatogram comparison against column dimension for rune time and resolution is shown in fig 2.

Remark

Here, X is used to express the mathematical relation in multi fold.

e.g. pressure increased by 3X i.e. pressure increase by three times

Method Development

Method development in UPLC remains same as of HPLC but few areas of chromatographic conditions are different e.g. for gradient elution column equilibration time is very less as compare to HPLC due to lower column volume.

Advance technology in column filled material for HPLC as well as UPLC allows higher pH and temperature stability for column for wider choice of mobile phase for different applications. e.g. pH of mobile phase or its buffer can selected based on compound chemical nature and that can be explain by fig.3 for reversed phase retention plot.

Fig.2 Chromatogram comparison against column dimension

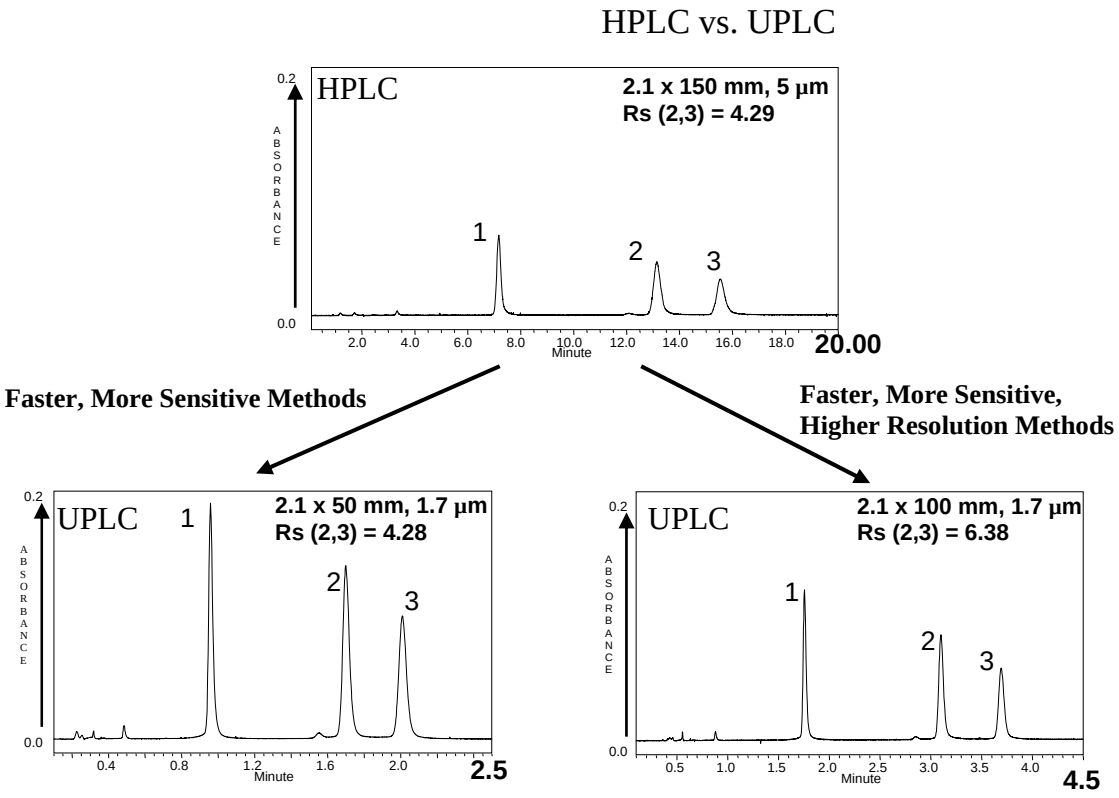
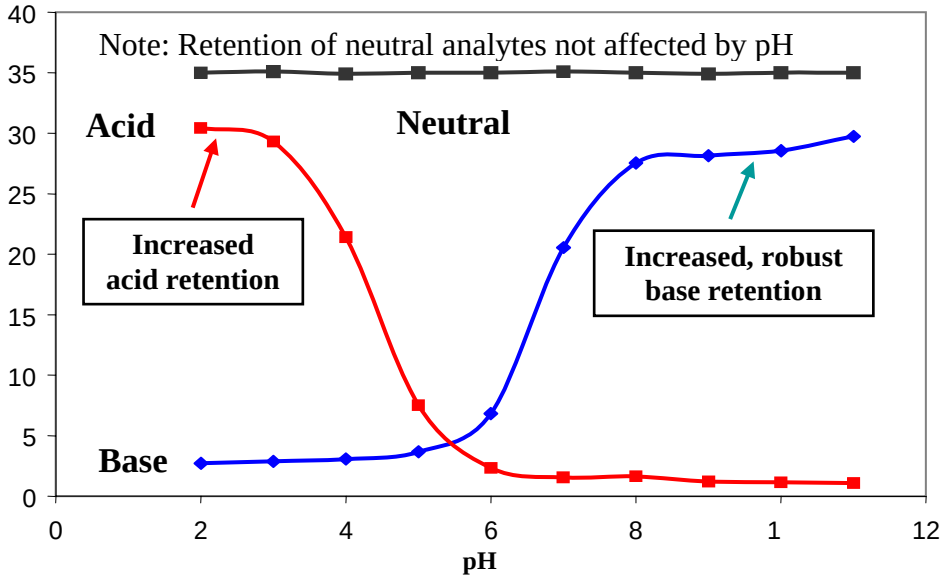


Fig.3 Reversed phase retention plot



From the above plot and fundamental theory of solvent gives following information for development consideration.

Facts for basic compound

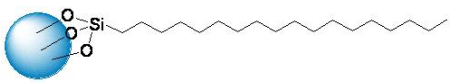
1. Alkaline pH increases retention of basic analytes
2. Methanol increases retention of all components compared to acetonitrile
3. Similar basic analytes differ little in selectivity, respective to one another, when they are either fully charged or uncharged
4. Largest selectivity differences between bonded phases occur with methanol and analytes in their unionized state

Facts for acid compound

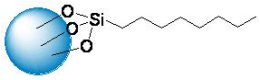
1. Acidic pH increases retention of acidic analytes
2. Methanol increases retention of all components compared to acetonitrile
3. Large differences in selectivity are observed when change in pH alters charge state
4. Largest selectivity differences between bonded phases occur with methanol and analytes in their unionized state

Column chemistry for known columns UPLC are shown in fig 4.

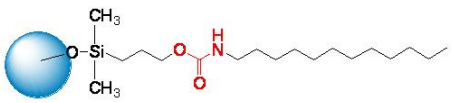
Fig.4: Column chemistry of UPLC column



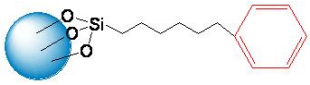
ACQUITY UPLC™ BEH C18



ACQUITY UPLC™ BEH C8



ACQUITY UPLC™ BEH Shield RP18



ACQUITY UPLC™ BEH Phenyl

By many recent research and development, UPLC presents the ability to extend and expand the utility of separation science at a time when many scientists have reached separation barriers, pushing the limits of conventional HPLC. New chemistry and instrumentation technology can provide more information per unit of work as UPLC begins to fulfil the promise of increased speed, resolution, and sensitivity predicted for liquid chromatography. As this is the concept for the scientist many of industries may take time to use in routine but it can be the future of the liquid chromatography. Hence present research work includes the extended area of HPLC to UPLC as a part of technology updating or a balance form of present HPLC and improved LC or UPLC for future scope for separation science.

References

[1] Dao T.-T. Nguyen, Davy Guillarme, Serge Rudaz, Jean-Luc Veuthey, Journal of Chromatography A, 1128 (2006) 105–113

[2] UPLC waters seminar presentation at Singapore (2006)

[3] http://www.waters.com

[4] Michael E. Swartz, Journal of Liquid Chromatography and Related Technologies 28(2005)1253-1263

Section-I: UPLC Method development and validation for determination of related impurities in Torsemide tablets

1. Introduction to torsemide tablets

Torsemide is a diuretic of the pyridine-sulfonylurea class. Its chemical name is 1-isopropyl-3-[(4-m-toludino-3-pyridyl)sulfonyl]urea, and N-[[(1-methylethyl)amino] coarbonyl]-4-[(3-methylphenyl)amino]-3-pyridinesulfonamide. Torsemide is a loop diuretic used for oedema associated with heart failure, including pulmonary oedema and with renal and hepatic disorders. It is also used in the treatment of hypertention, either alone or with other antihypertensive.Its molecular weight is 348.43 g/mole with molecular formula of $C_{16}H_{20}N_4O_3S$. Its dissociation constant (pKa) reported is 7.1 in Demadex® complete product information while in Merck index pKa = 6.44 is reported. Its melting point is 163°C to 164°C with decomposition [1-3]

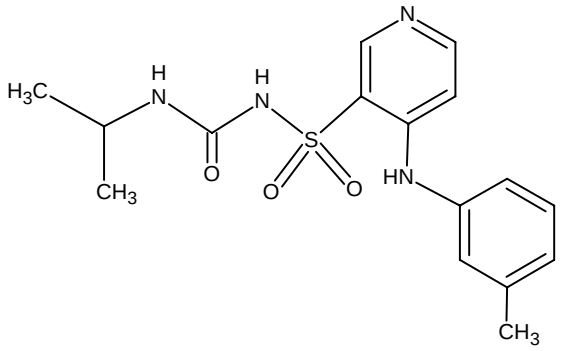


Fig.1: Torsemide

Innovator: Roche pharmaceuticals, USA

Strengths: Torsemide tablets are available in four strengths as 5 mg, 10 mg, 20 mg, and 100 mg of Torsemide as an active ingredient for oral administration.

1.1 Clinical pharmacology

Mechanism of Action: Micro puncture studies in animals have shown that Torsemide from within the lumen of the thick ascending portion of loop of Henle, where it inhibits the $Na^+/K^+/2Cl^-$ carrier system. Clinical pharmacology studies have confirmed this site of action in humans, and effects in other segments of the nephron have not been demonstrated. Diuretic activity thus correlates better with the rate of drug excretion in the urine than with the concentration in the blood.

Torsemide increases the urinary excretion of sodium, chloride and water but it does not significantly alter the glomerular filtration rate, renal plasma flow or acid base balance.

1.2 Pharmacokinetic and metabolism

The bioavailability of Demadex® tablets is approximately 80% with little intersubject variation: the 90% confidence interval is 75% to89%. The drug is absorbed with little first pass metabolism and the serum concentration reaches its peak (C_{max}) within 1 hour after oral administration. C_{max} and area under the serum concentration-time curve(AUC) after oral administration are proportional to dose over the range of 2.5 mg to 200 mg. Simultaneous food intake delays the time to C_{max} by about 30 minutes but overall bioavailability (AUC) and diuretic activity are unchanged. Absorption is essentially unaffected by renal or hepatic dysfunction.

The volume of distribution in adult is 12 liters to 15 liters in adults or in patients with mild to moderate renal failure or congestive heart failure. In patient with hepatic cirrhosis, the volume of distribution is approximately doubled.

1.3 Contraindications

Demadex® is contraindicated in patients with known hypersensitivity to Demadex® or sulfonylureas.

2. Literature review

The literature reviews regarding Torsemide suggest that various analytical methods were reported for drug substance as well as in pharmaceutical formulation and in various biological fluids. The literature reviews for analysis of torsemide are as under.

2.1 Begona Barroso M., Alonso R.M., Jimenez R.M have developed a high performance liquid chromatographic method with amperometric detection for the determination of the loop diuretic 1-Isopropyl-3-[4-(3-methylphenylamino)-3-pyridinesulphonyl] urea, torsemide, using a μ-Bondapak C_{18} column and a mobile

phase water :acetonitrile (65 :35), 5 mM in potassium dihydrogen phosphate/ dipotassium hydrogen phosphate at pH 5.3. Eluant is monitored at +1300 mV with an amperometric detector equipped with a glassy carbon working electrode. The method showed a determination limit of 8 ppb and reproducibility in terms of relative standard deviation lower than 2% in intra-day assays and 5 % in inter-day assays. The HPLC-EC method was applied to urine samples obtained from a healthy volunteer. Concentration levels of torsemide at different time intervals were monitorized and results were in agreement with the pharmacokinetic parameters of this diuretic. The determination of torsemide in urine required a liquid-liquid extraction prior to chromatographic analysis due to the interferences found in urine matrix. With this simple clean-up procedure, recoveries around 70% were achieved. Torsemide was directly determined in tablets after dissolution of the powder in a methanolic solution.

2.2 Marothu Vamsi Krishna and Dannana Gowri Shankar have developed a simple and cost effective spectrophotometric method for the determination of torsemide in pure form and in pharmaceutical formulations. The method is based on the formation of blue colored chromogen when the drug reacts with Folin-Ciocalteu (F-C) reagent in alkaline medium. The colored species has an absorption maximum at 760 nm and obeys beer?s law in the concentration range 30-150 µg mL^{-1}. The absorbance was found to increase linearly with increasing concentration of TSM, which is corroborated by the calculated correlation coefficient value of 0.9999(n=8). The apparent molar absorptivity and sandell sensitivity were $1.896x10^{3}$ L mol^{-1} cm^{-1} and 0.183 µg cm^{-2}, respectively. The slope and intercept of the equation of the regression line are $5.4x10^{-3}$ and $1.00x10^{-4}$ respectively. The limit of detection was 0.94. The optimum experimental parameters for the reaction have been studied. The validity of the described procedure was assessed. Statistical analysis of the results has been carried out revealing high accuracy and good precision. The proposed method was successfully applied to the determination of TSM in pharmaceutical formulations.

2.3 Kwang-Hyeon Liu1 and et al have established a rapid and sensitive method to assay torsemide in plasma was developed using a simple liquid-liquid extraction technique followed by HPLC. Torsemide and the internal standard furosemide were extracted

from 0.5 mL of plasma using ethyl acetate in the presence of 0.1M HCl. The analysis of the extracts was performed on a monolithic silica column with ultraviolet spectrophotometric detection. The calibration curve was linear over the concentration range of 0.05-5 μg mL^{-1} in plasma. Recoveries were reasonable for routine analyses (>80%); the limit of quantification was 0.05 μg mL^{-1} with a signal-to-noise ratio of 5. The coefficient of variation of the assay precision was less than 6.1%, and the accuracy exceeded 98%. This method was used to measure the torsemide concentration in plasma from healthy subjects after a single 20-mg oral dose of torsemide. This method provides a very simple, sensitive, and accurate way to determine torsemide concentrations in plasma.

2.4 Dragica Zendelovska, Trajce Stafilov have developed a reverse phase high-performance liquid chromatography (RP-HPLC) methods for determination of diuretics in different human body fluids (whole blood, plasma, serum or urine). Sample preparation procedures, including solid-phase extraction, liquid-liquid extraction, dilution, precipitation as well as automated RP-HPLC procedures, are discussed in order to present the advantages and disadvantages of each type of sample preparation. Also, values of analytical recovery of each procedure used for sample preparation are summarized. The most important RP-HPLC parameters detection mode, stationary phase, mobile phase, sensitivity, *etc*.) are also summarized and discussed.

2.5 H-J Guchelaarl, L. chandil, O. Schoutenl, W.A. van den Brandl have developed a simple and adequate HPLC method for screening of human urine for the following 17 diuretic drugs: acetazolamide, bendrofluazide, bumetanide, canrenoic acid, chlorothiazide, chlorthalidone, clopamide, epitizide, etacrynic acid, furosemide, hydrochlorothiazide, indapamide, mefruside, piretanide, spironolactone, torasemide, and triamterene. The assay involves extraction from two 2 mL urine samples with ethyl acetate at pH = 5, washing with a phosphate buffer at pH = 6 and analysis by HPLC using a reversed phase C18 column and ultraviolet detection with a diode array detector for all drugs (except triamterene) using two eluents consisting of water,

triethylamine, phosphoric acid and acetonitrile at different ratios and different pH values. Triamterene is determined by direct injection of diluted urine onto the column and is measured by fluorescence detection. The recoveries of the diuretic drugs were determined at two different concentrations and ranged from 43–110% (median: 87%) which is sufficient to detect abuse of these drugs. The repeatability of the assay ranged from 1–12% (median: 5.5%).

3. Aim of present work

Literature reveals that the torsemide drug substance is official in US pharmacopeia as well as in British pharmacopeia but its tablet formulation is not official [4, 5]. An assay method has been described for determination of torsemide in tablet formulation by HPLC EC method [6]. A spectrophotometric method reported for determination of torsemide in bulk drug and formulation using Folin-Ciocalteu reagent as color forming reagent [7]. Some methods are also available for determination of torsemide in urine and biological fluids [8-10]. But none of method available for determination of related impurities for torsemide tablets. The present communication describes method for determination of related impurities for Torsemide tablets with established relative response factor (RRF) and relative retention time (RRT) for known impurities. Moreover, analytical column with sub 2-micron particle size technology was explored for better chromatography.

Simple and precise analytical method for determination of related impurities for torsemide tablets was developed and validated as per ICH guideline. Hence, this faster LC method could be of use for formulation development and stability testing as well as at quality control laboratory for routine use.

The aim and scope of the proposed work are as under

- LC method development for determination of related impurities in torsemide tablets formulation
- Forced degradation study under stress condition
- To resolve all known impurities and generated during the force degradation studies

- Perform analytical method validation for the proposed method as per ICH guideline and method applicability to market samples including IFF.

4. Experimental

4.1 Materials, chemicals and reagents

Materials, chemicals and reagents were used as mentioned in Table 1 and Table 2.

Table 1: Materials

Name	B.No./Lot No	Potency / Strength
Torsemide related compound A, USP RS (imp A) [4-[(3-methylphenyl)amino]-3-pyridinesulfonamide]	F0B071	100.0 %
Torsemide related compound B, USP RS (imp B) [N-[(n-butylamino)-carbonyl]-4-[(3-methylphenyl)amino]-3-pyridinesulfonamide]	F0B083	100.0 %
Torsemide related compound C, USP RS (imp C) [N-[(ethylamino)carbonyl]-4-[(3-methylphenyl)amino]-3-pyridinesulfonamide]	F0B078	100.0 %
Torsemide , USP RS	G0E293	99.6 %
Torsemide working standard, TRC, India	TO 0011005	99.5 %
Placebo, TRC, India	001	--
Torsemide tablets, TRC, India	003	5 mg
Torsemide tablets, Tide-10, TPL India	C8938003	10 mg
Torsemide tablets, Demadex, Roche Lab, USA	E6772	10 mg
Torsemide tablets, Torsinex-10, Micro lab, India	TNXH0020	10 mg
Torsemide tablets, Dytor-10, Cipla, India	D82152	10 mg

Table 2: Chemicals and reagents

Sr. No.	Chemical/Reagent	Grade
1	Potassium dihydrogen phosphate	AR grade (Rankem, India)
2	Ortho-phosphoric acid	GR grade (Merck, India)
3	Methanol	HPLC grade (Merck, India)
4	Acetonitrile	HPLC grade (Merck, India)
5	Mill-Q-Water	By Milli-Q system (Millipore, USA)

4.2 Instrumentation

The LC system of Waters acquity UPLC with PDA was used for this entire study and chromatographic separation was achieved on Zorbax SB-C18 (50 mm X 4.6mm 1.8µm) column as stationary phase with binary gradient mode.

4.3 Standard stock solution

Transferred about 1.5 mg of each of Torsemide USP reference standard and USP Torsemide related compound A, Torsemide related compound B and Torsemide related compound C reference standard into 50ml volumetric flask and 25ml methanol was added to dissolve with aid of sonication and diluted to volume with diluent (30µg/ml of Torsemide and its related compound A, B and C) Fig.2.

4.4 Standard solution

In a 25ml volumetric flask, 1 ml of standard stock solution was diluted to volume with diluent (1.2 µg/ml of torsemide and its related compound A, B and C) Fig.3.

4.5 Diluted standard solution

Transferred about 2.0 mg of torsemide USP working standard into 100ml volumetric flask and 50ml methanol was added to dissolve with aid of sonication and diluted to volume with diluent. In a 50ml volumetric flask, 1 ml of standard stock solution was diluted to volume with diluent Fig.4.

4.6 Resolution solution

Transferred about 20 mg of torsemide working standard into 50ml volumetric flask, 25ml of diluent was added and sonicated to dissolve, 2 ml of standard stock solution was added and diluted to volume with diluent (400µg/ml of Torsemide and 1.2 µg/ml of Torsemide related compound A, B and C] Fig.5.

4.7 Sample solution

Twenty tablets were crushed to fine powder by mortar and pestle. Sample powder equivalent to about 40mg of torsemide was weighed and transferred to 100 ml volumetric flask, 50ml of diluent was added and sonicated for 25 minute with intermittent swirling, diluted to volume with diluent (400µg/ml of torsemide) and filtered with 0.22 µm PVDF membrane filter. As such sample preparation as per fig.6.

Fig.2: Chromatogram of standard stock preparation

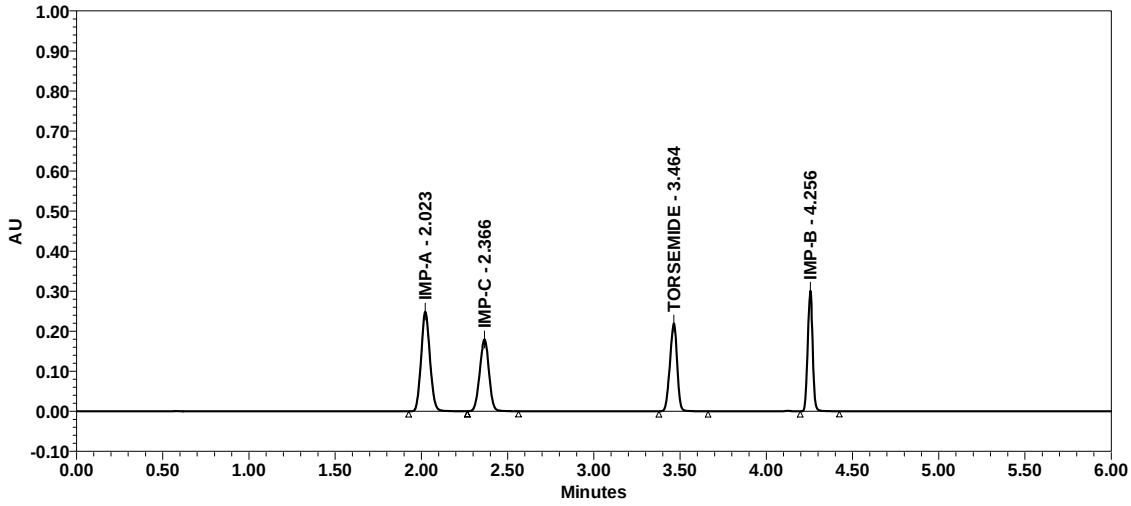


Fig.3: Chromatogram of standard preparation

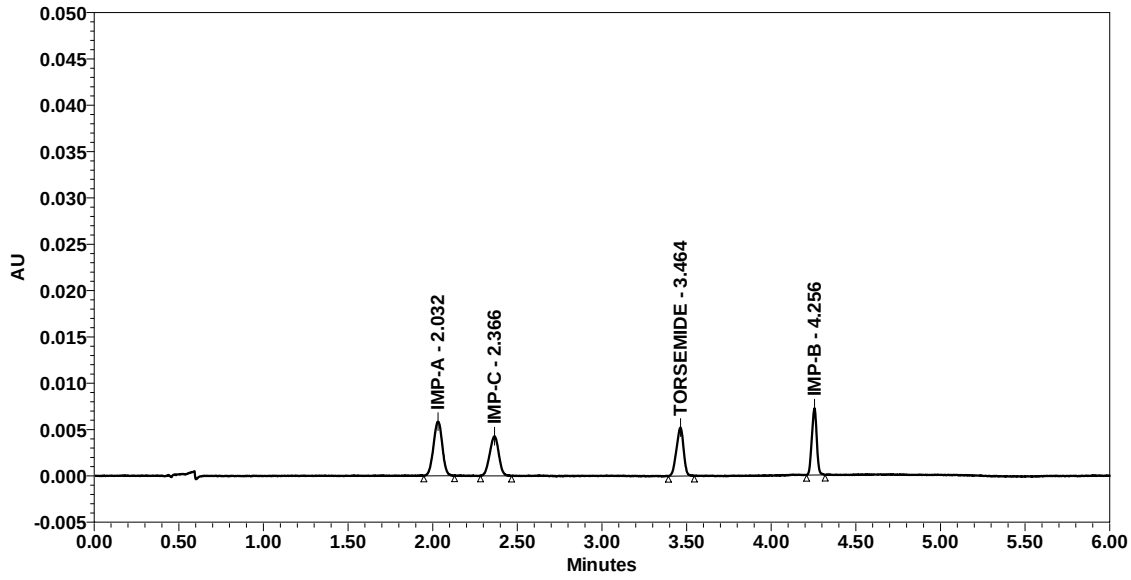


Fig.4: Chromatogram of diluted standard preparation

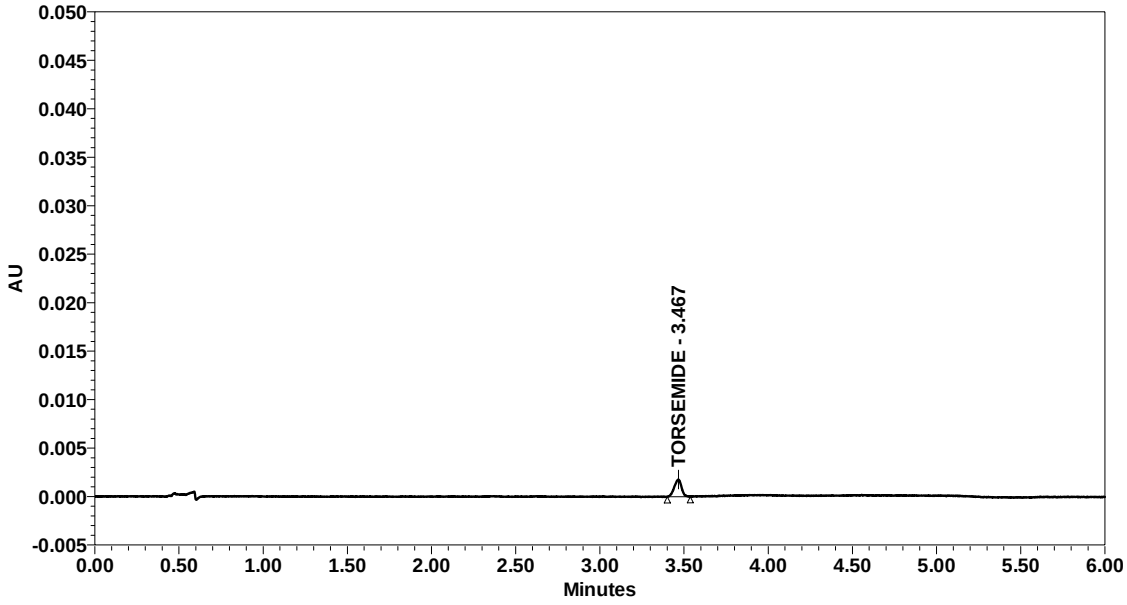


Fig.5: Chromatogram of resolution solution

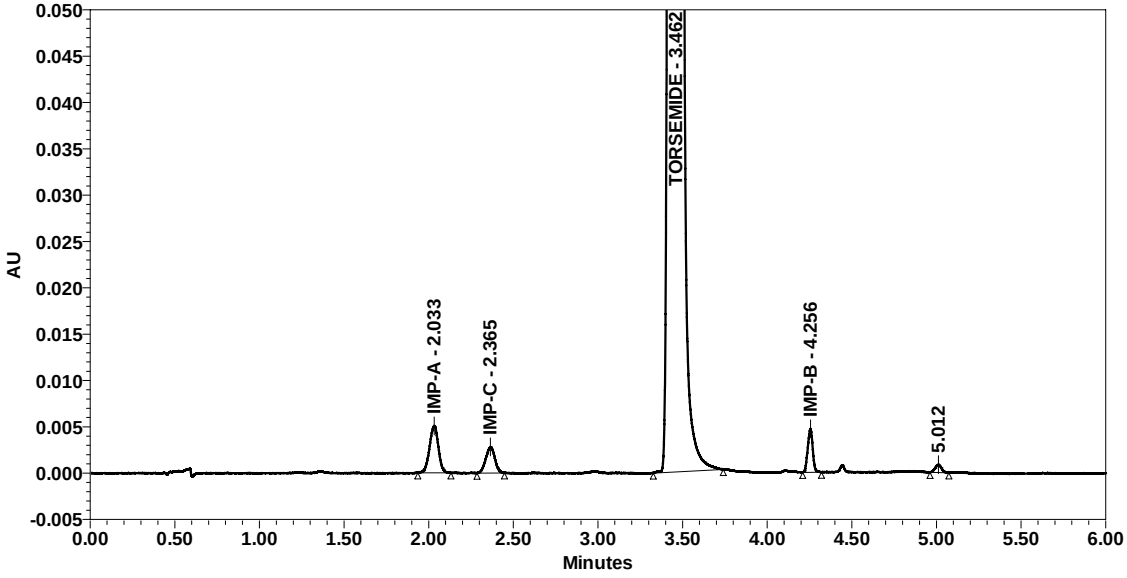
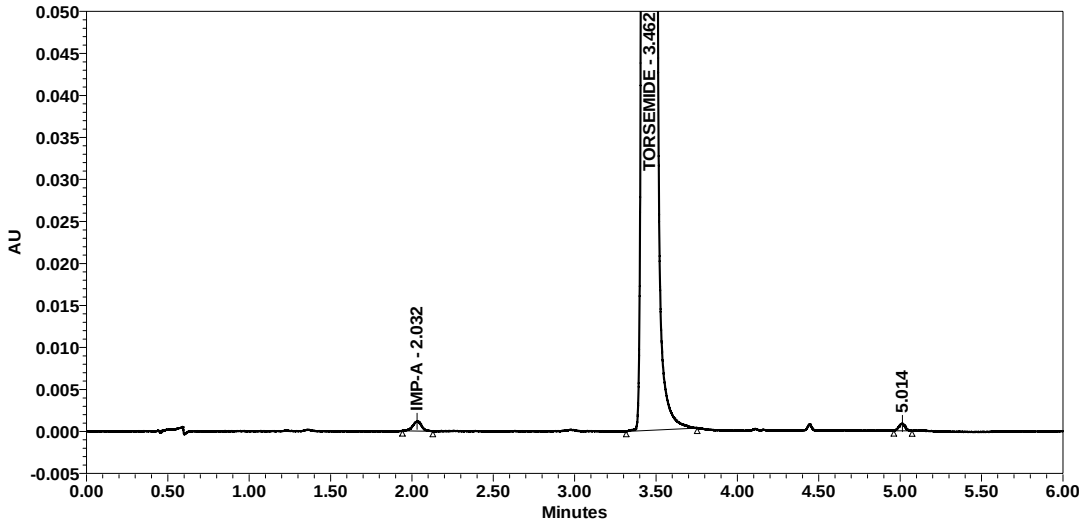


Fig.6: Chromatogram of sample solution (as such preparation)



5. Result and discussion

5.1 Development and optimization of the LC method

Method development was started with same mobile phase as mentioned in the monograph of torsemide USP as a part of chromatographic conditions but peak of impurity B was eluted in very late eluting region so, run time is little high for analysis and flow rate can not be increased further more as due to buffer and methanol gave high pump pressure. So, on this fact methanol was replaced by acetonitrile with composition optimization or in other words, torsemide USP related substances method for its chromatographic condition can of use but it is of very long run time of about 40 minutes [Fig.7A]. While instead of methanol in mobile phase acetonitrile gives better peak shape with its sharpness and run time also reduced and chromatographic separation can achieved in 25 minutes [Fig.7B].

But scientist is always looking for better technology for betterment or for improved chromatography. As a part of that UPLC method with Zorbax column of sub 2 micron particle size technology was explored to reduce the run time to 6 minutes [Fig.7C].

Fig.7A: HPLC Chromatogram with Torsemide USP HPLC method

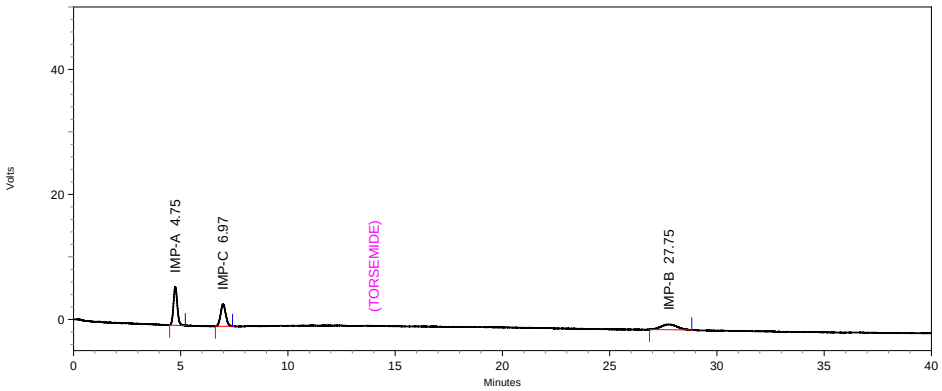


Fig.7B: HPLC Chromatogram with modified HPLC method

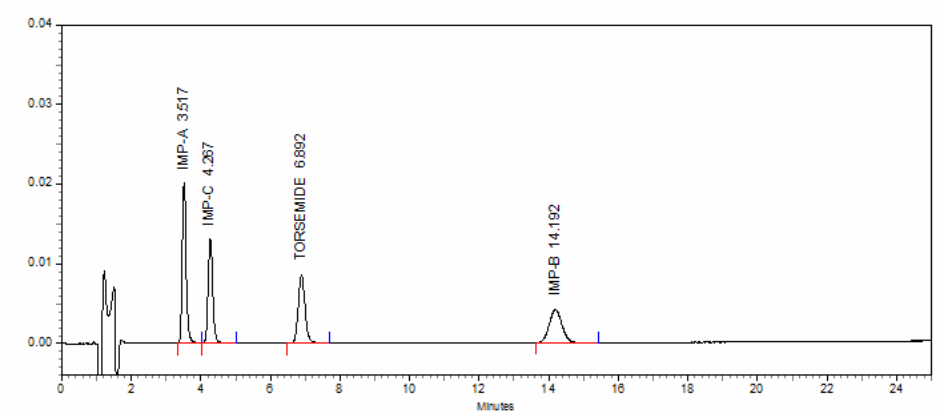
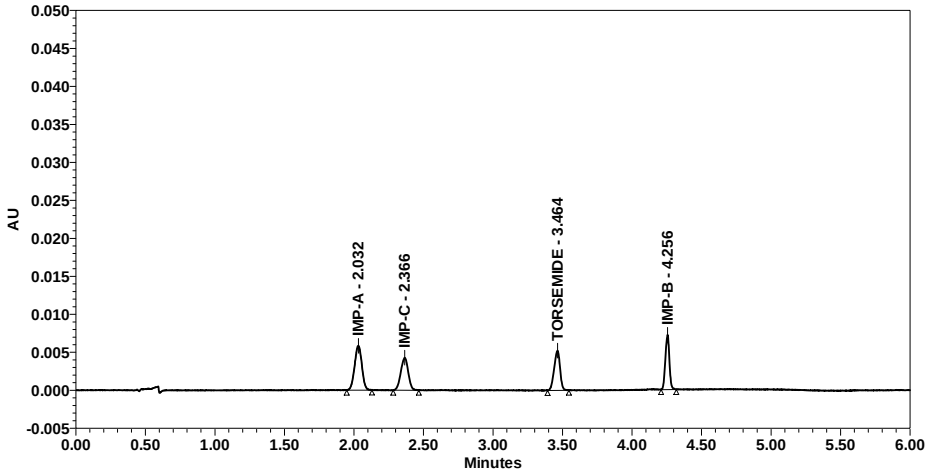


Fig.7C: UPLC Chromatogram



5.2 Separation for known impurities

Impurity stock solution of torsemide and its USP related compound A, B and C was used for method development parameters like column selection and gradient optimization (30µg/ml of torsemide and its related compound A, B and C). Once desired separation for known impurities and main component was achieved forced degradation was performed. Finally proposed method with chromatographic condition as per table no. 3 was subjected to method validation as per ICH guideline with consideration of sample concentration to achieve LOQ below the reporting threshold of impurities [11].

Table 3: Chromatographic condition

Buffer	Buffer, 20 mM potassium dihydrogen phosphate, pH was adjusted to 3.5 with orthophosphoric acid, filtered through 0.22µm PVDF membrane		
Mobile phase-A	Buffer : Acetonitrile, 90:10 (v/v)		
Mobile phase-B	Buffer : Acetonitrile, 50:50 (v/v)		
Diluent	Buffer : Acetonitrile : Methanol, 25:25:50 (v/v/v)		
Column oven temp.	30°C		
Detection	UV at 288 nm		
Injection volume	6 µl		
Gradient programme	**Time (min)**	**Mobile phase-A (%)**	**Mobile phase-B (%)**
	0.0	75	25
	2.5	65	35
	4.0	25	75
	4.6	25	75
	5.0	75	25
	6.0	75	25

5.3 Forced degradation Study

Forced degradation was performed on torsemide tablets to achieve desired degradation and placebo as well as Torsemide drug substance were treated with similar conditions as mentioned below, based on development trials optimized forced

degradation conditions were established. Final sample concentration was achieved 400µg/ml of torsemide with diluent as proposed sample concentration.

5.3.1 Oxidative degradation

Torsemide sample powder equivalent to 40mg of torsemide was subjected to oxidative stress condition by 5ml of 3% v/v H_2O_2 solution in 100ml volumetric flask and heated on water bath at 100°C for 30 min and sample solution was prepared as per procedure.

5.3.2 Acid degradation

Torsemide sample powder equivalent to 40mg of torsemide was subjected to acidic stress condition by 5ml of 1N HCl solution in 100ml volumetric flask and heated on water bath at 100°C for 30 min and sample was neutralised with 1N NaOH and sample solution was prepared as per procedure.

5.3.3 Base degradation

Torsemide sample powder equivalent to 40mg of torsemide was subjected to alkaline stress condition by 5ml of 1N NaOH solution in 100ml volumetric flask and heated on water bath at 100°C for 30 min and sample was neutralized with 1N HCl and sample solution was prepared as per procedure.

5.3.4 Thermal degradation

Torsemide tablets were kept in temperature controlled oven at 100 °C for 30 hours and sample solution was prepared as per procedure.

5.3.5 Photolytic degradation

Torsemide tablets were kept in photo stability chamber to expose it for 1.2 million lux hours and sample solution was prepared as per procedure

5.4 Method applicability to market samples

Method was success fully applicable to market samples for evaluation of known impurities with their UV spectral confirmation as mentioned in table 4.

Table 4: Impurity profile for different make samples

Sample Name	B.No./Lot No	Expiry Date	Imp A (%)	Imp B (%)	Imp C (%)
Torsemide tablets, Tide-10, TPL India	C8938003	Feb 2010	0.09	BQL	BQL
Torsemide tablets, Demadex, Roche Lab, USA	E6772	Apr 2009	0.37	BQL	BQL
Torsemide tablets, Torsinex-10, Micro lab, India	TNXH0020	Apr 2009	0.09	BQL	BQL
Torsemide tablets, Dytor-10, Cipla, India	D82152	Mar 2011	0.07	BQL	BQL

BQL = Below Quantitation Limit

(Limit of quantitation = 0.025%)

5.5 Method Validation

Method validation approach:

The method for the determination of related impurities for torsemide tablets of 5 mg, 10 mg, 20 mg, and 100 mg is same. Sample concentration for all strength is 400 µg/ml of torsemide and complete validation was performed on lower strength (5 mg) and linearity was performed by 5 levels of concentration. Recovery was performed by 3 replicates x 3 concentration. Filter media interference study was covered in accuracy study.

5.5.1 Specificity

Interference from blank was not observed at any peak of interest and peak purity of all known impurities in spiked sample (as per method precision) observed within the acceptance criteria and forced degradation data as discussed in section 5.3 summarized in table 5 and chromatograms are as shown in fig.8(A) to 8(J).

Table 5: Forced degradation data

Stress condition	% of degradation
Oxidative degradation	15.40
Acid degradation	10.81
Alkali degradation	0.42
Thermal degradation	6.35
Photolytic degradation	0.45

Fig.8 (A): Peroxide degradation

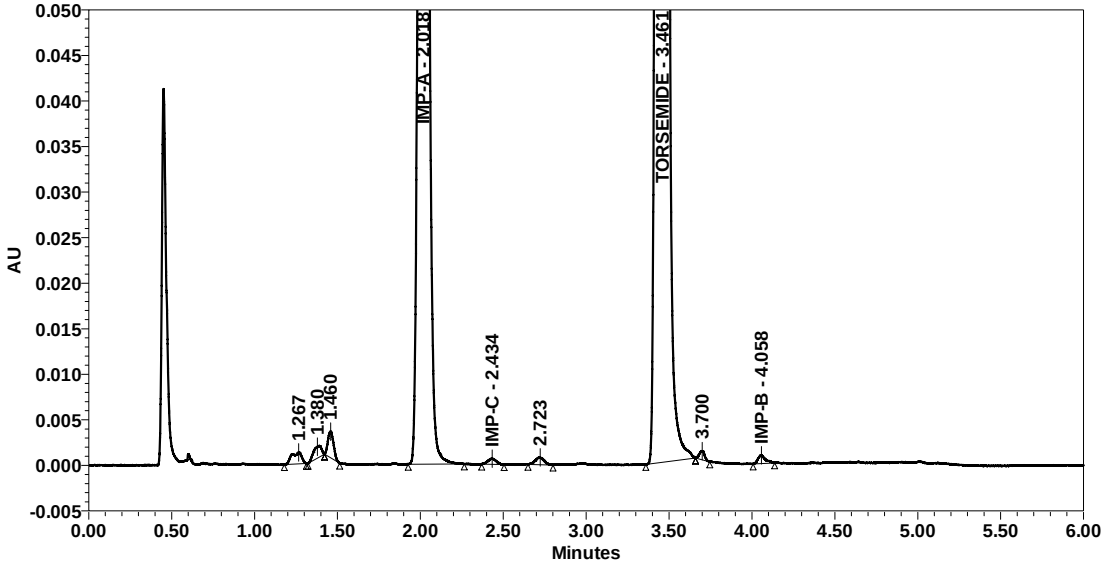


Fig.8 (B): Acid degradation

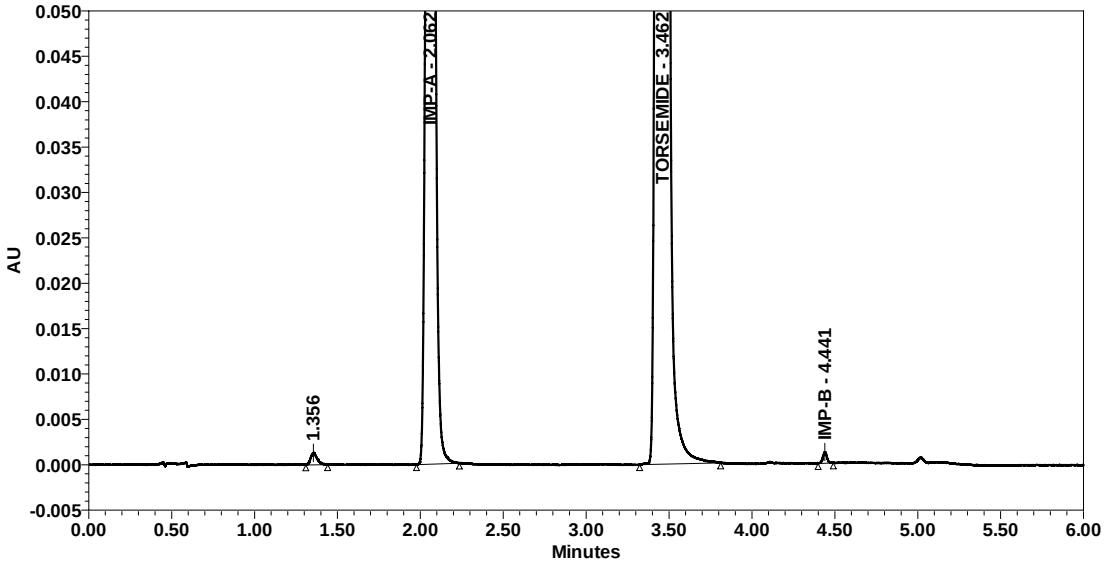


Fig.8 (C): Base degradation

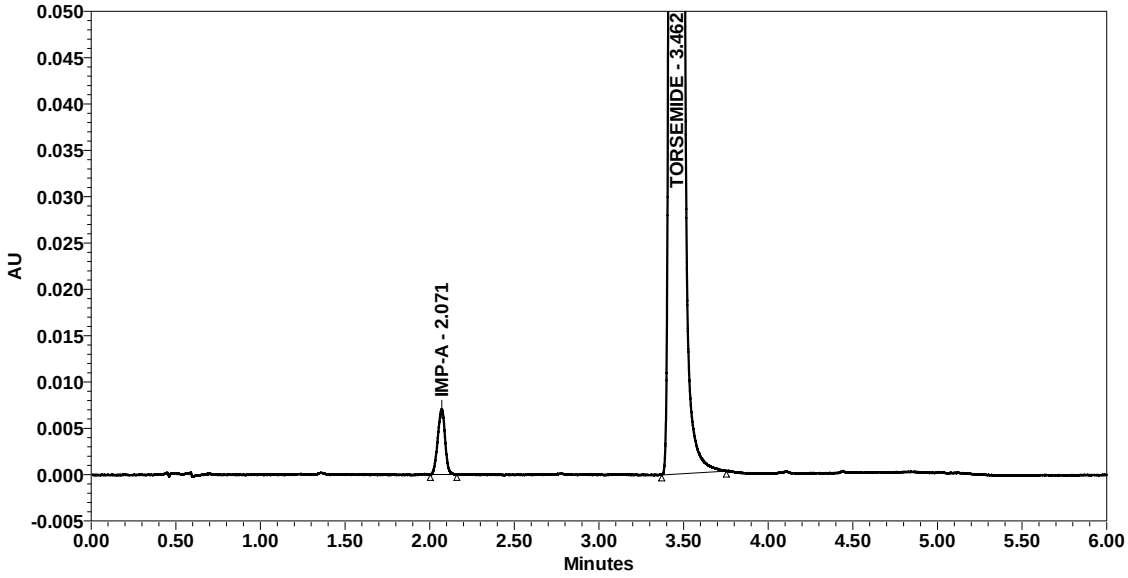


Fig.8 (D): Thermal degradation

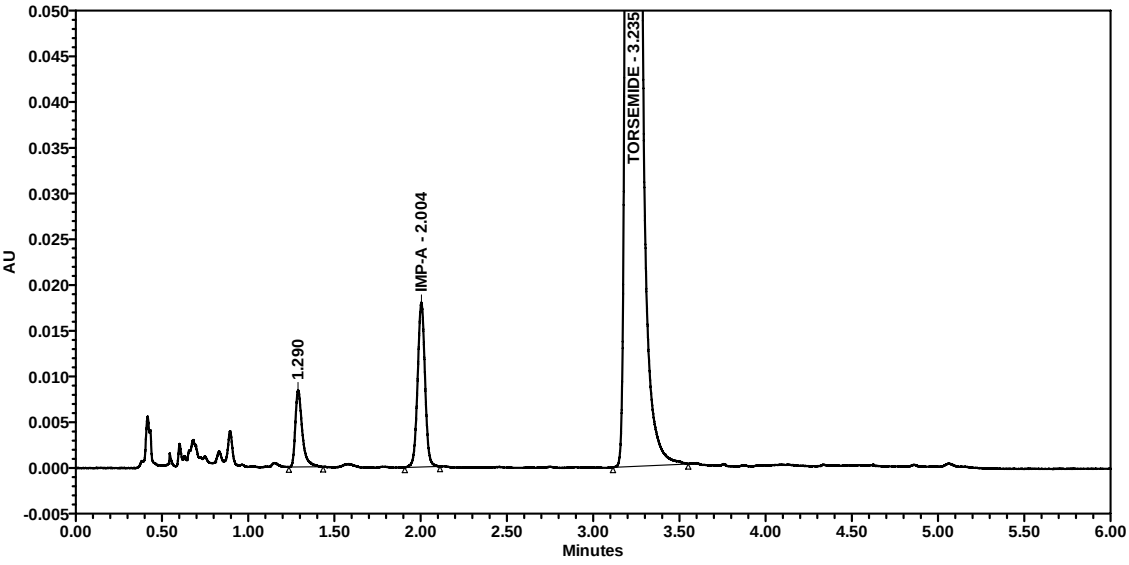


Fig.8 (E): Photolytic degradation

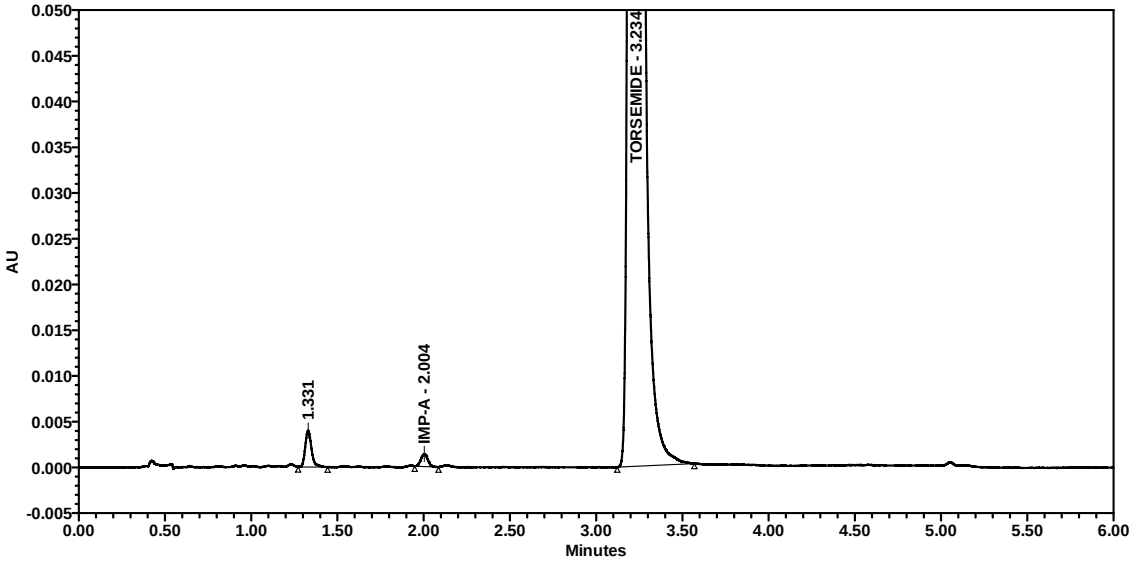


Fig.8 (F): Chromatogram of impurity-A

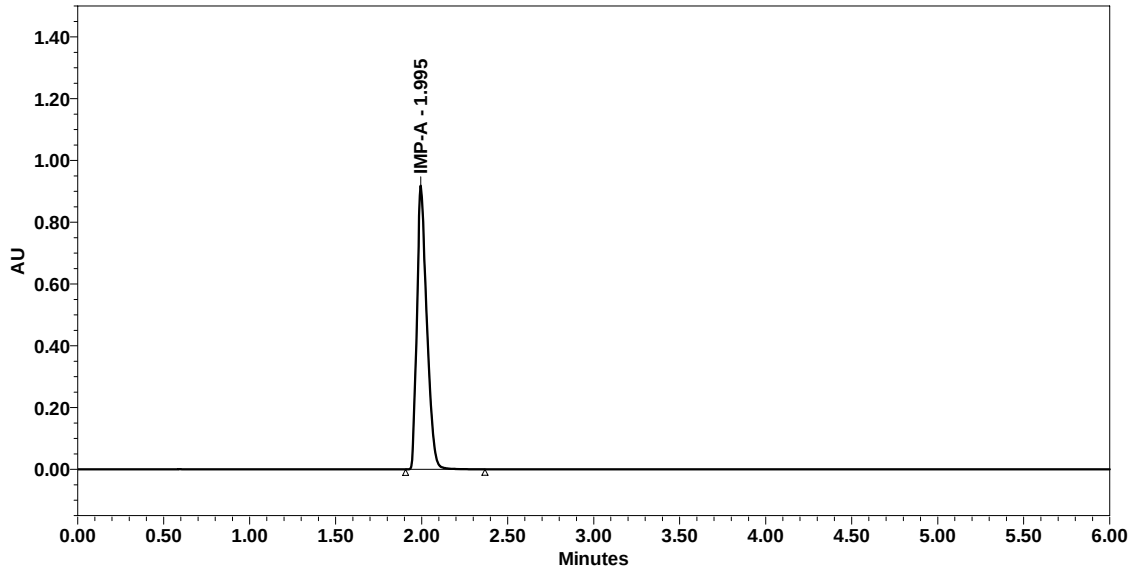


Fig.8 (G): Chromatogram of impurity-B

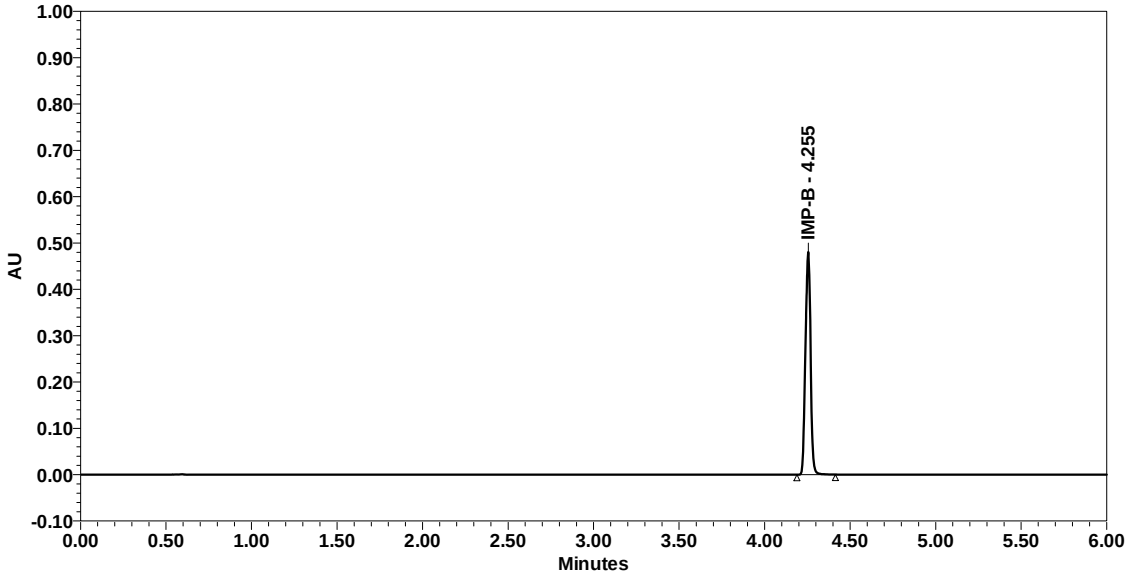


Fig.8 (H): Chromatogram of impurity-C

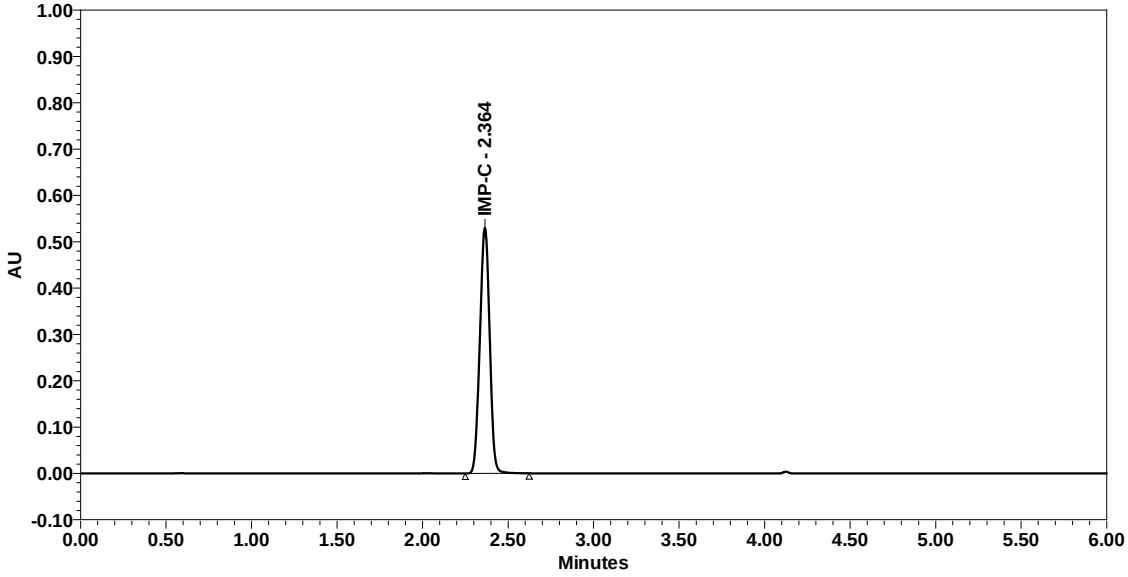


Fig.8 (I): Chromatogram of blank/diluent

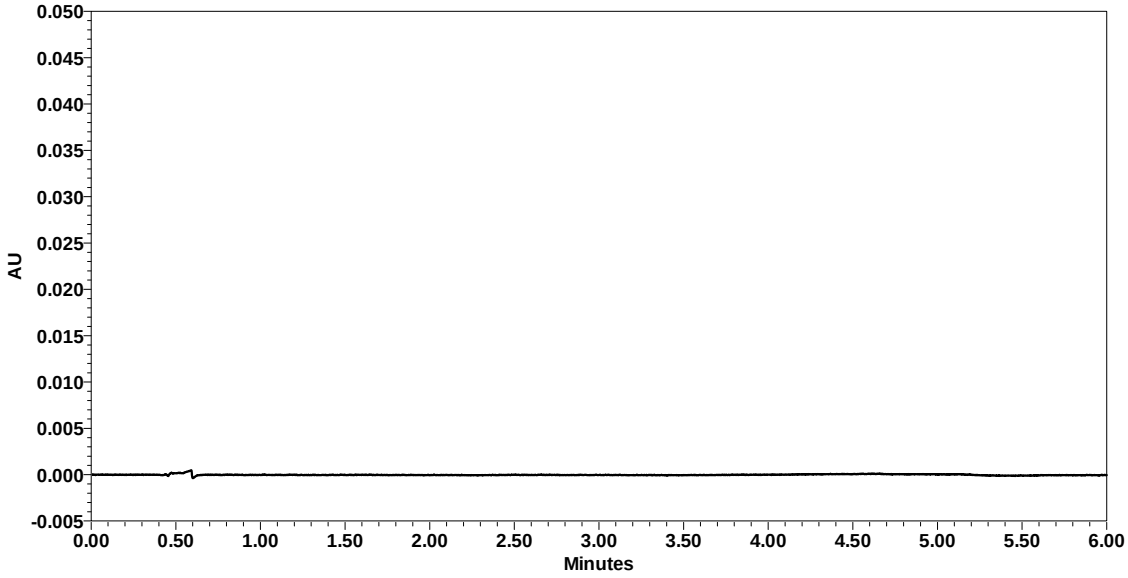
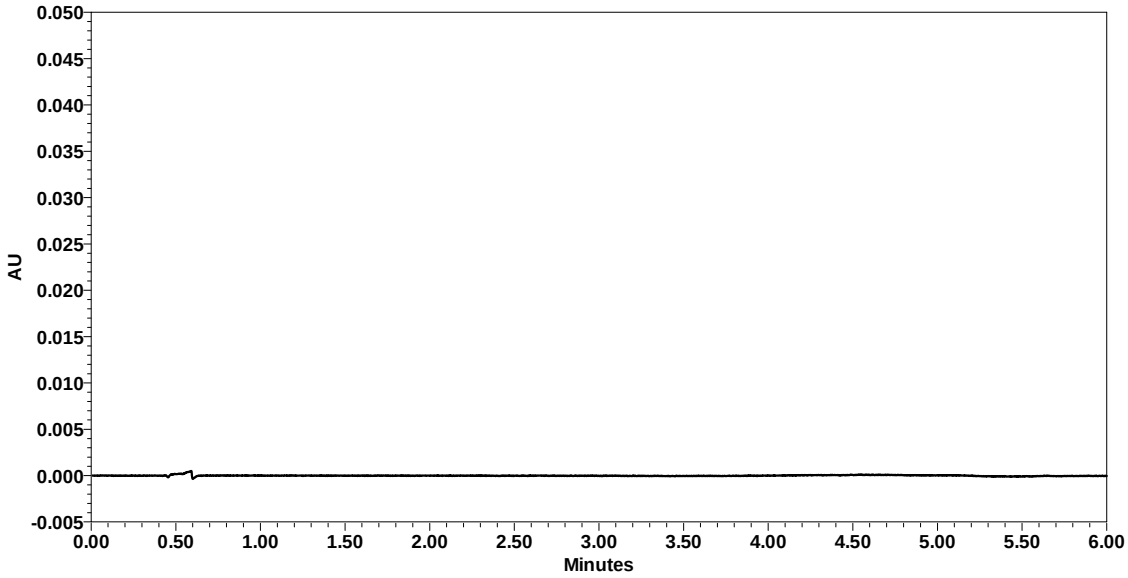


Fig.8 (J): Chromatogram of placebo



5.5.2 Linearity

Linearity and relative response factor determination

The calibration curve for Torsemide and its impurities were constructed over the absolute concentration range of 0.1-4.0 µg/ml. So, 0.025% to 1% concentration with

respect to sample solution concentration in five levels. Peak area of each impurities and Torsemide versus respective concentration was plotted and linear regression analysis performed on resultant curves. Relative response factor (RRF) was calculated for each known impurities by taking the ratio of impurity slope to Torsemide slope [12]. Statistical data summarized in table 6 and chromatograms mentioned in Fig.9 (A) to 9 (E).

Table 6: Linearity and RRT and RRF

Name	Slope	Intercept	r^2	RRT	RRF
Imp-A	17365	71	0.9996	0.587	1.357
Imp-B	12107	-19	0.9999	1.229	0.946
Imp-C	13316	8	0.9996	0.683	1.041
Torsemide	12793	-29	0.9997	1.000	1.000

RRT = Relative retention time

RRF = Relative response factor

Fig.9: Linearity chromatograms

Fig.9 (A): Linearity level-1

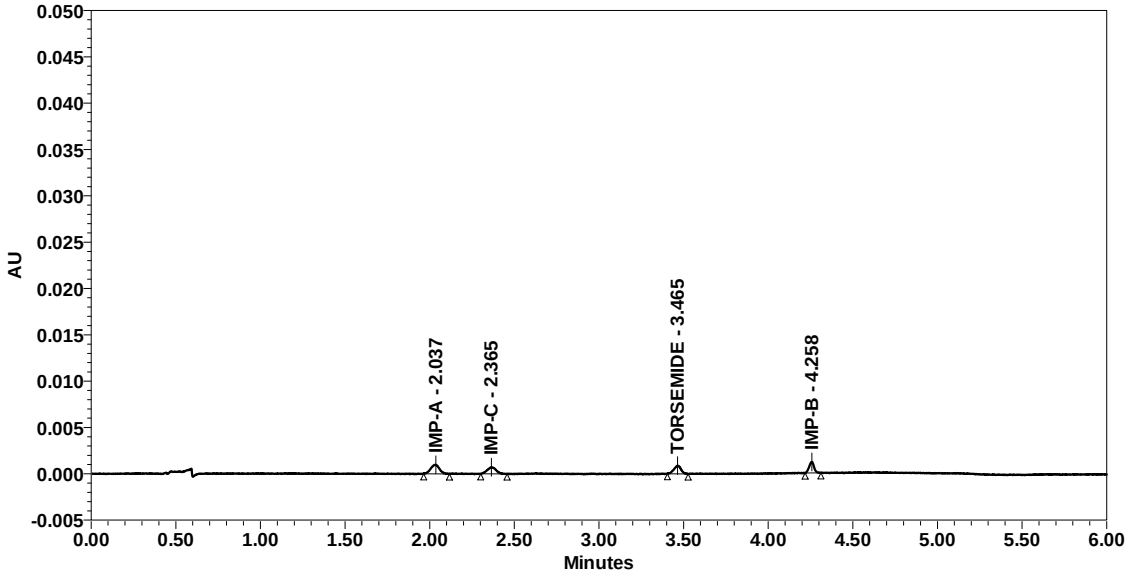


Fig.9 (B): Linearity level-2

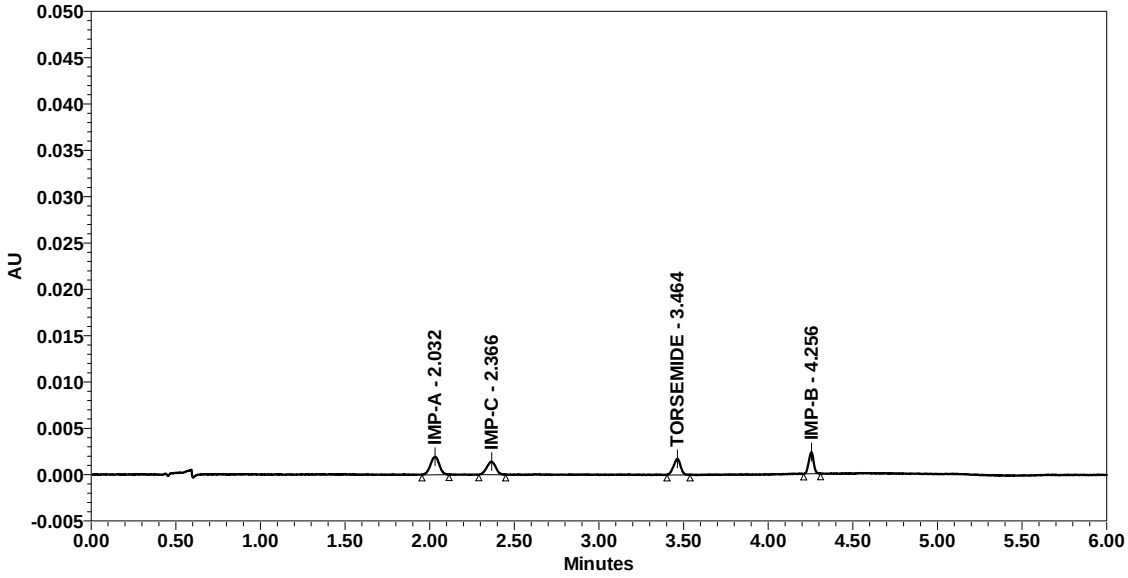


Fig.9 (C): Linearity level-3

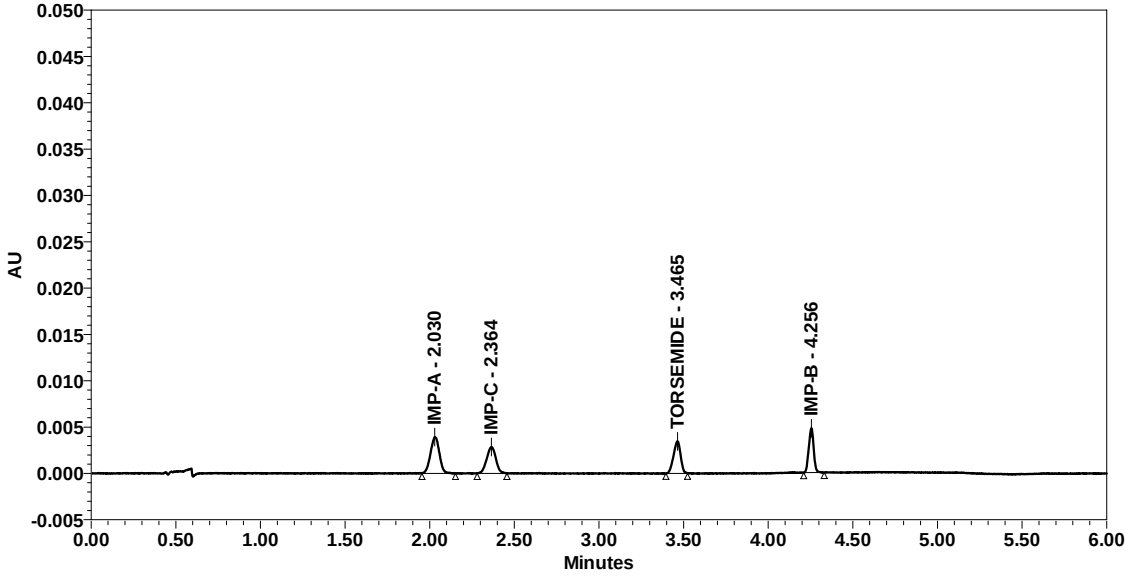


Fig.9 (D): Linearity level-4

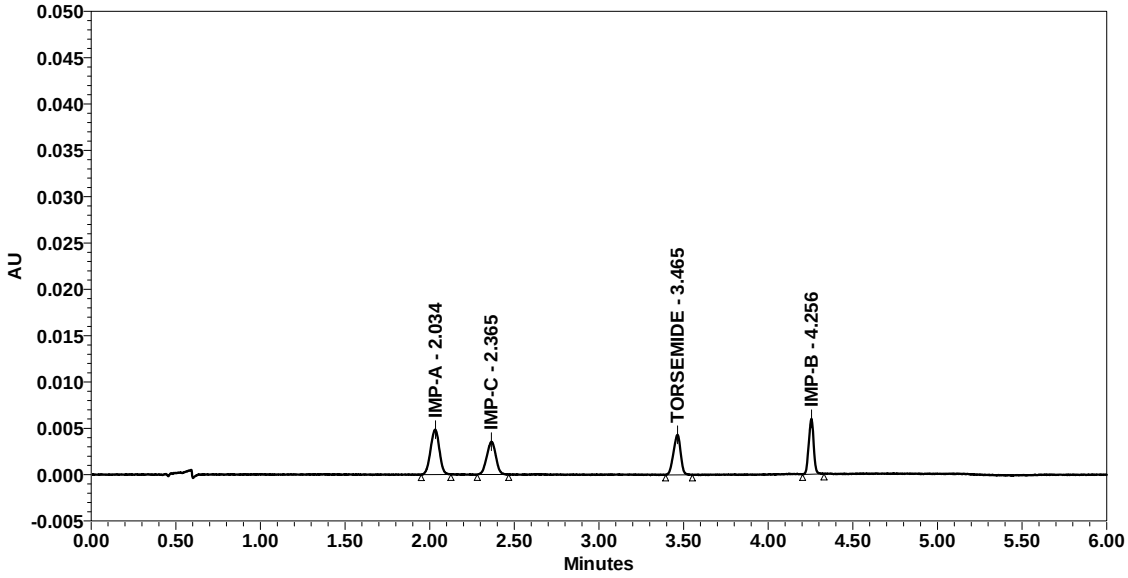
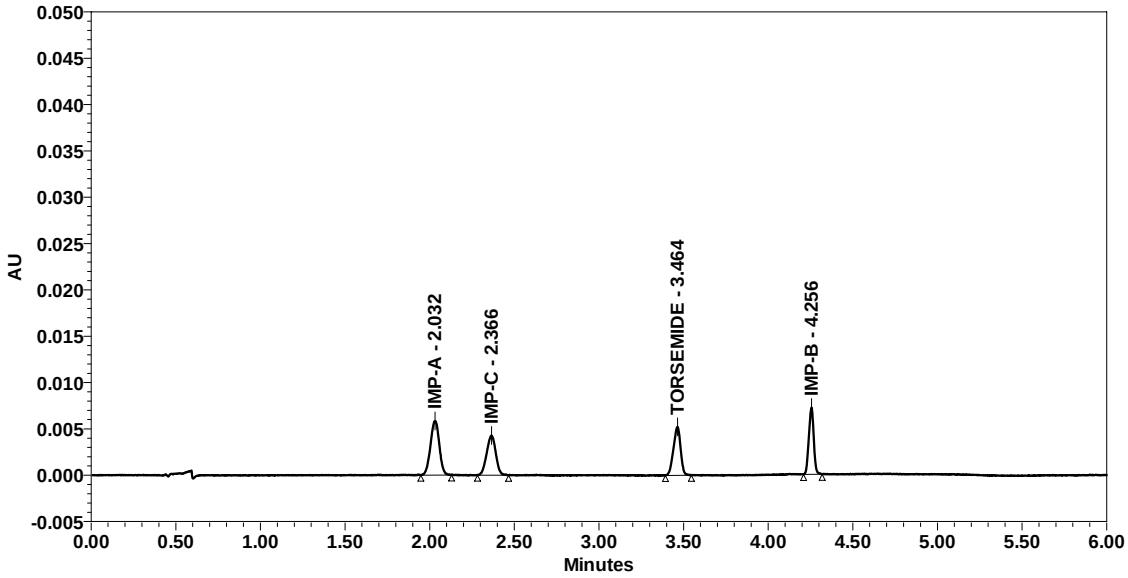


Fig.9 (E): Linearity level-5



5.5.3 Precision at LOQ

The LOQ concentrations for all known impurities were determined 0.1 µg/ml as 0.025 % with respect to sample solution concentration of Torsemide by signal to noise ratio methodology [13]. The reproducibility at LOQ was determined by six replicate injections of composite mixture of impurities and Torsemide solution as per table 7 and Fig.10.

Fig.10: Chromatogram of Precision at LOQ

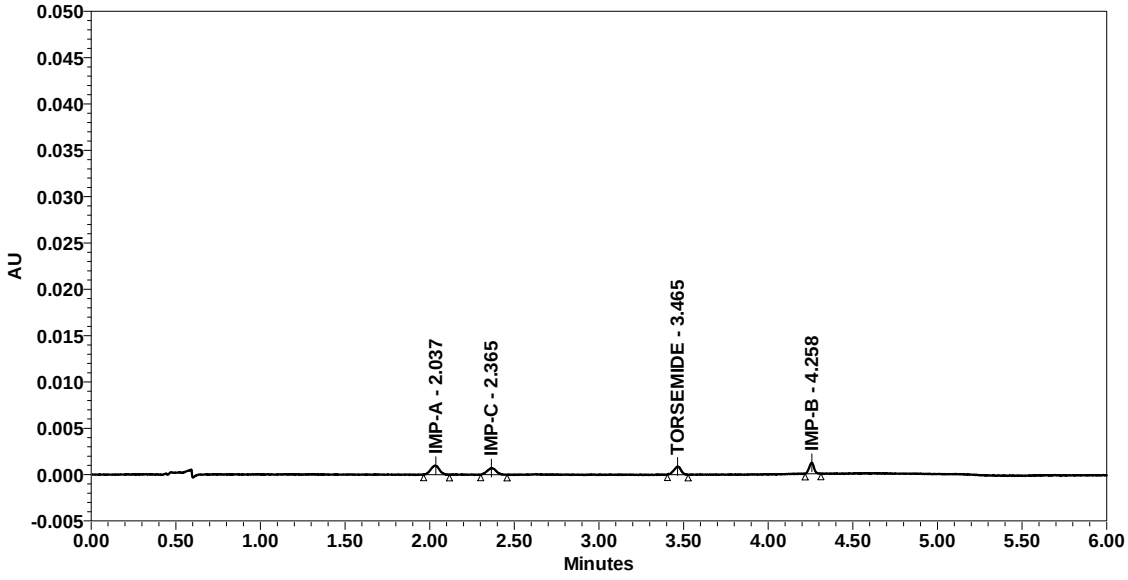


Table 7: Precision at LOQ

Name	RSD (%) Day-1	RSD (%) Day-2
Imp-A	1.10	0.73
Imp-B	1.02	0.84
Imp-C	0.87	0.89
Torsemide	0.92	0.76

5.5.4 Precision

Method precision was established by six sets of sample preparation. To demonstrate the method precision all known impurities were spiked at 0.3 % level to all six sample preparation fig.11. Data are summarized in table 8.

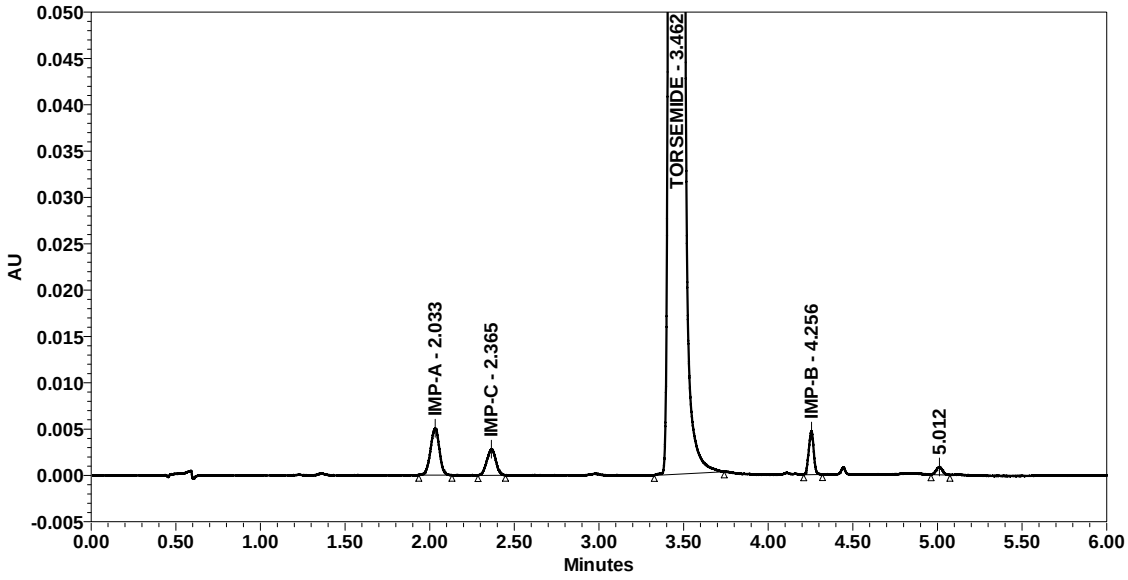
Table 8: Summary of precision day-1 and day-2

Day-1					
Set No.	Imp-A (%)	Imp-B (%)	Imp-C (%)	Unk imp (%)	Total Imp (%)
1	0.32	0.30	0.30	0.05	0.97
2	0.32	0.31	0.31	0.05	0.99
3	0.31	0.31	0.31	0.05	0.98
4	0.32	0.30	0.31	0.05	0.98
5	0.31	0.30	0.30	0.05	0.96
6	0.31	0.30	0.30	0.05	0.96
Mean	0.32	0.30	0.31	0.05	0.97
SD	0.005	0.005	0.005	0.000	0.012
%RSD	1.739	1.702	1.796	0.000	1.244
95% CI	0.0057	0.0054	0.0057	0.0000	0.0127
Day-2					
Set No.	Imp-A (%)	Imp-B (%)	Imp-C (%)	Unk imp (%)	Total Imp (%)
1	0.30	0.31	0.31	0.05	0.97
2	0.31	0.30	0.31	0.05	0.97
3	0.30	0.31	0.30	0.05	0.96
4	0.31	0.30	0.31	0.05	0.97
5	0.30	0.30	0.30	0.05	0.95
6	0.31	0.30	0.31	0.05	0.97
Mean	0.31	0.30	0.31	0.05	0.97
SD	0.005	0.005	0.005	0.000	0.008
%RSD	1.796	1.702	1.684	0.000	0.867
95% CI	0.0057	0.0054	0.0054	0.0000	0.0088

SD = Standard Deviation, %RSD = Relative Standard Deviation

Unk imp = Unknown impurity, CI = confidence interval

Fig.11: Chromatogram of method precision



5.5.5 Accuracy (Recovery)

The accuracy was evaluated by the recovery study for method which was established at three levels in the range of 0.1-4.0 µg/ml (0.025% to 1% relative to sample concentration, 3 levels x 3 preparations). Calculated amount of known impurities were added in form of solution to the sample preparation to attain lowest to highest level. Data summarized in table 9, Chromatograms shown in Fig.12 (A) to (C).

Fig.12 (A): Chromatogram of accuracy level-1

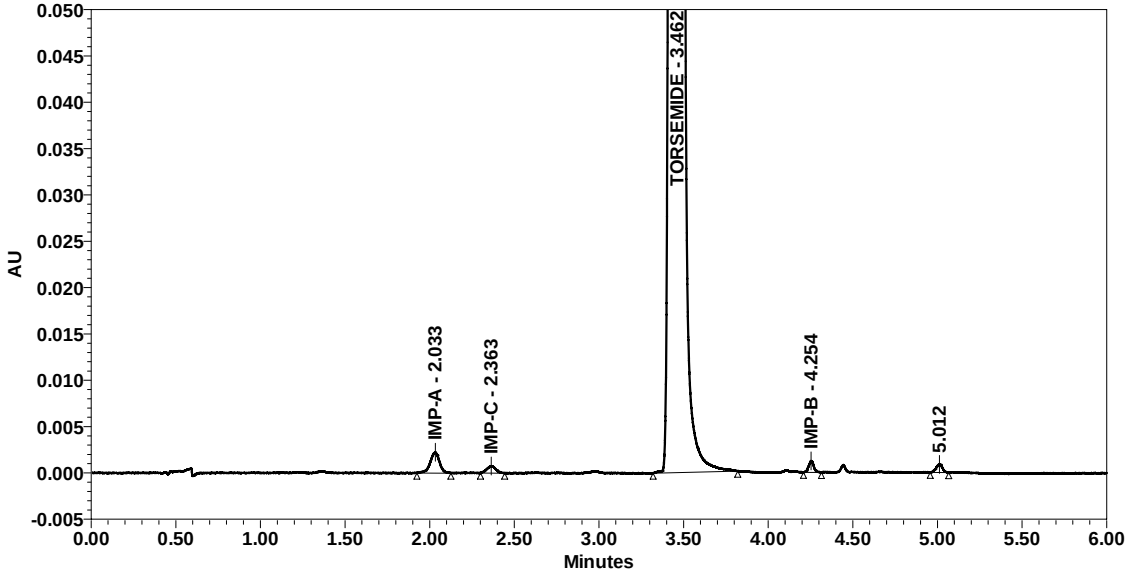


Fig.12 (B): Chromatogram of accuracy level-2

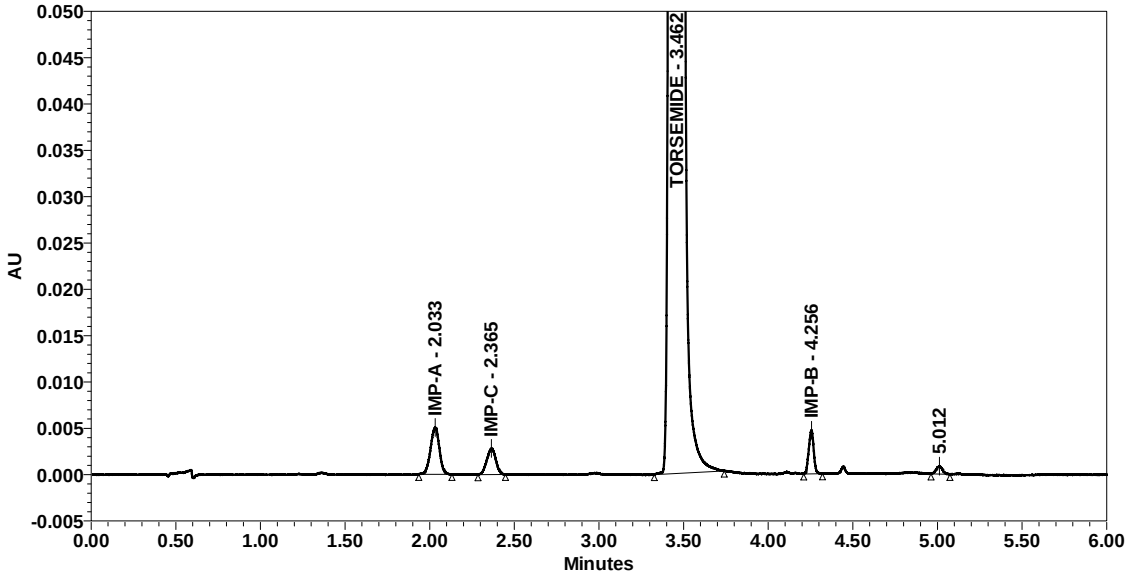


Fig.12 (C): Chromatogram of accuracy level-3

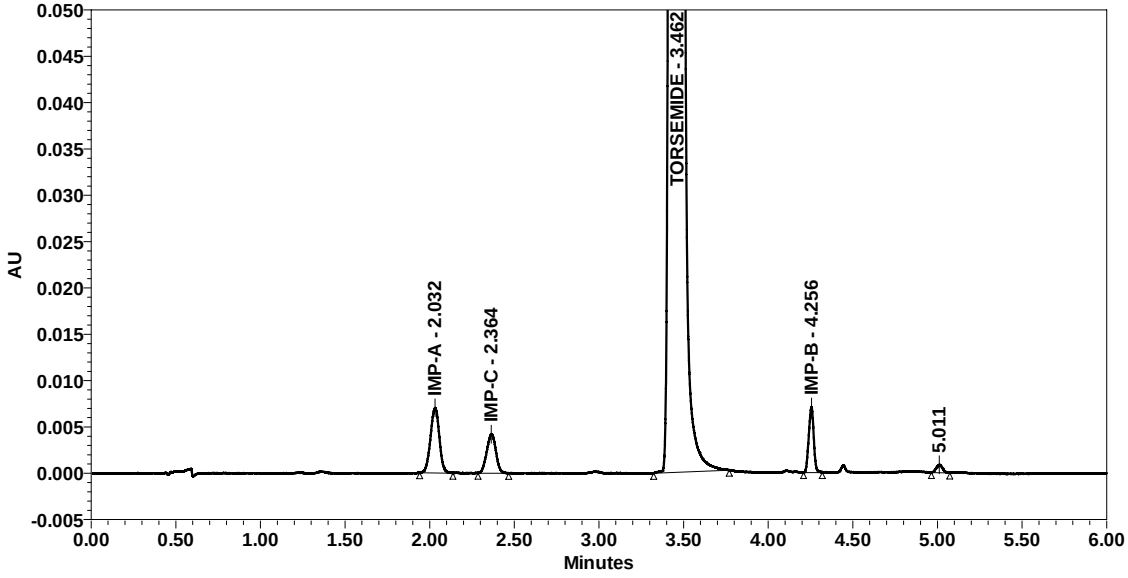


Table 9: Summary of recovery data

Name	Amount added [a] (mcg/ml)	Recovery [a] (%)	% RSD
Imp A	0.1	98.6	1.37
	1.2	100.7	1.04
	4.0	99.8	1.26
Imp B	0.1	99.7	0.79
	1.2	100.6	1.24
	4.0	99.8	0.80
Imp C	0.1	98.6	0.83
	1.2	101.3	1.67
	4.0	99.6	1.15

a = Average of three determinations

5.5.6 Solution Stability

The solution stability at 25°C was studied at different time interval against fresh standard preparation at specified time interval. The results obtain for the solution stability study at different time intervals for standard and sample preparation shown in table 10(A) and 10(B). It was concluded that the standard and sample preparation solution were found stable up to 48 hours at 25 ˚C.

Table 10(A): Sample solution stability

Sample solution stability					
Time (Hours)	Imp-A (%)	Imp-B (%)	Imp-C (%)	Unk imp (%)	Total Imp (%)
Initial*	0.32	0.30	0.30	0.05	0.97
24	0.30	0.31	0.31	0.05	0.97
48	0.31	0.30	0.31	0.05	0.97

Table 10(B): Standard solution stability

Standard solution stability				
Time (Hours)	Imp-A (µg/ml)	Imp-B (µg/ml)	Imp-C (µg/ml)	Torsemide
Initial*	1.2024	1.2036	1.2012	1.2010
24 #	1.2025	1.2030	1.2008	1.2015
48 #	1.2028	1.2034	1.2011	1.2007

***** Data taken from test preparation-1 and system suitability from method precision.

Concentration of component obtained against fresh standard preparation.

5.3.6 Robustness:

The result of robustness study of the developed method was shown in Table 11(A) and 11(B). The results have shown that during all variance conditions value of related impurities for the test preparation was within the acceptance criteria. It was in accordance with that of accepted true value from method precision study. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

Table 11(A): System suitability summary of robustness study

Condition	**System suitability parameters**	
	Asymmetry	**% RSD**
A) Change in flow rate		
Flow rate 1.0 ml/minute	1.02	0.62
Flow rate 0.9 ml/minute	1.00	0.28
Flow rate 1.1 ml/minute	1.01	0.45
B) Change in minor component in the mobile phase		
MP-A, Buffer : Acetonitrile (90:10)	1.02	0.62
MP-A, Buffer : Acetonitrile (88:12)	1.09	0.35

MP-A, Buffer : Acetonitrile (92:8)	1.13	0.21
C) Change in column temperature		
Column temperature 30°C	1.02	0.46
Column temperature 25°C	1.14	0.80
Column temperature 35°C	1.08	0.71
D) Change in column Lot No #		
Method precision	1.02	0.46
Intermediate precision	1.05	0.36

= this parameter was studied as part of precision (method precision & intermediate precision).

Table 11(B): System suitability summary of robustness study

Condition	% of Impurities				
	A	**B**	**C**	**Unk**	**Total**
A) Change in flow rate					
Flow rate 1.0 ml/minute	0.32	0.30	0.30	0.05	0.97
Flow rate 0.9 ml/minute	0.31	0.30	0.31	0.05	0.97
Flow rate 1.1 ml/minute	0.30	0.31	0.32	0.05	0.98
B) Change in minor component in the mobile phase					
MP-A, Buffer : Acetonitrile (90:10)	0.32	0.30	0.30	0.05	0.97
MP-A, Buffer : Acetonitrile (88:12)	0.32	0.30	0.30	0.05	0.97
MP-A, Buffer : Acetonitrile (92:8)	0.31	0.30	0.30	0.05	0.96
C) Change in column temperature					
Column temperature 30°C	0.32	0.30	0.30	0.05	0.97
Column temperature 25°C	0.31	0.32	0.33	0.05	1.01
Column temperature 35°C	0.31	0.31	0.31	0.05	0.98
D) Change in column Lot No*					
Method precision	0.32	0.30	0.31	0.05	0.97

Intermediate precision	0.31	0.30	0.31	0.05	0.97

6. Quantitation and calculation formulae

6.1 Relative Standard Deviation (%)

$$\text{Relative Standard Deviation (\%)} = \frac{SD \times 100}{\overline{X}}$$

By Microsoft excel

Average = AVERAGE (Number 1)

Standard Deviation = STDEV (Number 1)

6.2 Calculation formulae in Linearity and RRF

$$\text{Correlation coefficient (r)} = \frac{n\ (\sum xy)\ -\ (\sum x)(\sum y)}{\sqrt{\{[n\sum x^2 - (\sum x)^2]\ [n\sum y^2 - (\sum y)^2]\}}}$$

Correlation coefficient (r) = CORREL (Array 1, Array 2)

$$\text{Slope of regression line} = \frac{n\ (\sum xy)\ -\ (\sum x)(\sum y)}{n\sum x^2 - (\sum x)^2}$$

Slope of regression line = SLOPE (Array 1, Array 2)

$$\text{y- intercept} = A = \overline{y} - B\overline{x}$$

y- intercept = INTERCEPT (Array 1, Array 2)

$$\text{Residual sum of squares} = \sum (Y_{original} - Y_{calculated})^2$$

Where,

$Y_{calculated} = A + Bx$

n = Number of measurements

x = Individual concentration

y = Individual area

$\bar{x}$ = Average concentration

$\bar{y}$ = Average area

Residual sum of squares = SUMXMY2 (Array 1, TREND (Array 1, Array 2, Array2))

$$\text{Relative Response Factor} = \frac{\text{Slope of regression line for impurity}}{\text{Slope of regression line for standard}}$$

6.3 Accuracy (% Recovery)

$$\% \text{ Recovery} = \frac{\text{Ammount of sbustance (mg) found}}{\text{Ammount of substance (mg) added}} \text{ x } 100$$

6.4 % impurity in Torsemide Tablets

$$\% \text{ Impurity} = \frac{\text{Asp}}{\text{Astd}} \times \frac{\text{Std. wt}}{\text{Dilution}} \times \frac{\text{Dilution}}{\text{Spl Wt}} \times \frac{\text{Avg Wt.}}{\text{LC}} \times \frac{1}{\text{RRF}} \times \text{Std. assay \% (as such)}$$

e.g.

$$\% \text{ Impurity-A} = \frac{22115}{5048} \times \frac{2.001}{100} \times \frac{1}{50} \times \frac{100}{605.6} \times \frac{75.7}{5} \times \frac{1}{1.357} \times 99.5$$

$\% \text{ Impurity-A} = 0.32$

$$\% \text{ Impurity-B} = \frac{14403}{5048} \times \frac{2.001}{100} \times \frac{1}{50} \times \frac{100}{605.6} \times \frac{75.7}{5} \times \frac{1}{0.946} \times 99.5$$

$\% \text{ Impurity-B} = 0.30$

$$\% \text{ Impurity-C} = \frac{15927}{5048} \times \frac{2.001}{100} \times \frac{1}{50} \times \frac{100}{605.6} \times \frac{75.7}{5} \times \frac{1}{1.041} \times 99.5$$

$\% \text{ Impurity-C} = 0.30$

$$\% \text{ unknown} = \frac{2579}{5048} \times \frac{2.001}{100} \times \frac{1}{50} \times \frac{100}{605.6} \times \frac{75.7}{5} \times \frac{1}{1} \times 99.5$$

% unknown = 0.05

% Total impurities = Total known impurities + Total unknown impurities

% Total impurities = (0.32 + 0.30 + 0.30) + (0.05)

% Total impurities = 0.97

Asp	:	Area of impurity in sample
Astd	:	Mean area of diluted standard
Std. wt	:	Std weight in mg
Spl wt	:	Sample weight in mg
Avg wt	:	Average weight in mg
LC	:	Label claim per tablet (mg)

6.5 95% Confidence Interval (CI)

$$95\% \text{ Confidence Interval (CI)} = \pm \frac{t\alpha \times SD}{\sqrt{n}}$$

Where,

SD = Standard Deviation

$\overline{X}$ = Average concentration

n = Number of measurements

t_α = t value at n-1 from t table (two tail)

95% Confidence Interval (CI) = SD (2.57058)/SQRT (6)

References

[1] Merck Index 14th edition, Merck & Co.INC. 2006 p.9551

[2] Martindale The Complete Drug Reference, Pharmaceutical press 35th ed 2007 p. 1273-1274

[3] Physicians' Desk Reference, Thomson Healthcare INC., PDR 62 ed 2008 p.2723-2724

[4] US pharmacopeia 31 NF 26 2008, p.3436

[5] British pharmacopeia 2008, p.2170-2171

[6] Begona Barroso M., Alonso R.M., Jimenez R.M, Journal of liquid chromatography & related technologies, 19 (1996) 179-186

[7] Marothu Vamsi Krishna, Dannana Gowri Shankar E-J.Chem, 5 (2007) 473-478

[8] Kwang-Hyeon Liu1 and et al, Chromatographia, 60 (2004) 639-643

[9] Dragica Zendelovska, Trajce Stafilov, Acta Pharm. 56 (2006) 115-142

[10] H-J Guchelaarl, L. chandil, O. Schoutenl, W.A. van den Brandl, Fresenius journal of Analytical Chemistry, 363 (1999) 700-705

[11] ICH guideline, Q3B (R2) step 4, Impurities in new drug products (2006)

[12] Behnam Davani, Karen A. Russo, Andrzej Wilk, Lokesh Bhattacharya
USP PF 31(2) (2005) 626-636

[13] ICH guideline, Q2 (R1) step 4, Validation of Analytical Procedures: Text and Methodology (2005)

GLOSSARY

SPECIFICITY

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).
This definition has the following implications:

Identification: To ensure the identity of an analyte.

Purity Tests:

To ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency):

To provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.
This is sometimes termed trueness.

PRECISION

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision .

Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

QUANTITATION LIMIT

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

RANGE

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

ROBUSTNESS

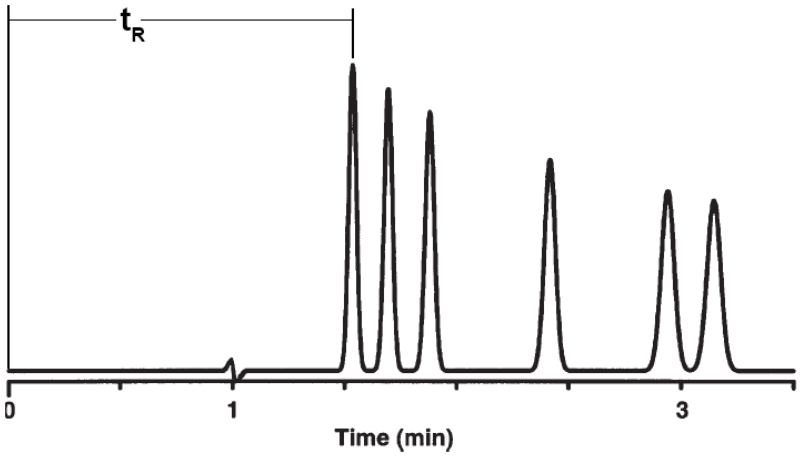
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Retention time	$\mathbf{t_R}$	Time from injection to appearance of peak maximum $\mathbf{t_R = t_m\,(1+k)}$
Column dead time	$\mathbf{t_m}$	Time for elution of earliest possible band (or mobile phase molecules); also symbolized as t_0. $\mathbf{t_m = V_m/F}$
Retention factor	**k**	Fraction of a sample compound distributed into stationary phase divided by fraction in mobile phase; also symbolized as k $\mathbf{k = (t_R - t_m) / t_m}$

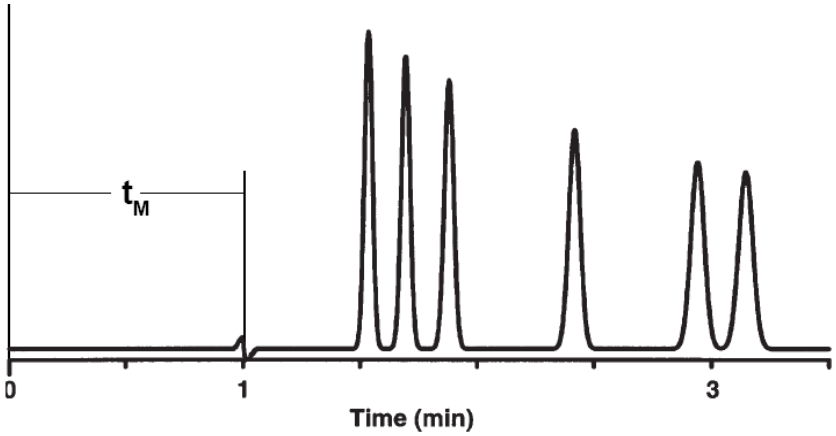
Column dead volume	**V_m**	Volume of mobile phase inside column **$V_m = t_m F$** **$V_m \approx 0.7 (p/4) L d_c^2$** **$\approx 0.5 L d_c^2$** (F is the mobile phase flow rate in mL/min; t_0 is in minutes; d_c is the column i.d. in cm; and V_m is in mL)
Reversed-phase separation		Separation that uses an aqueous mobile phase (with added organic) and a column with alkyl-bonded-phase packing such as C8 or C18.
Normal-phase separation		Separation with silica or polar-bonded phase Column
Column plate number	**N**	A column parameter that measures column efficiency or the narrowness of sample bands **$N = 16 (t_R / W)2$** (W is baseline bandwidth)
Column plate height (HETP value)	**H**	A column parameter that measures column efficiency per unit length of column $H = L / N$
Resolution	**Rs**	A separation parameter that measures how well two adjacent bands are separated **$R_s = (t_2 - t_1) / (1/2)(W_1 + W_2)$**
Separation factor	α	Ratio of k values for two adjacent bands **$\alpha = k_2 / k_1$**

Separation selectivity		Relative band separation (value of α) provided by some combination of mobile phase and column packing.
Separation efficiency		Relative bandwidths (value of N) provided by some column and separation conditions (flow rate, column length, and temperature)
Column pressure	**P**	Pressure drop across column during separation (psi)

Retention Time t_R

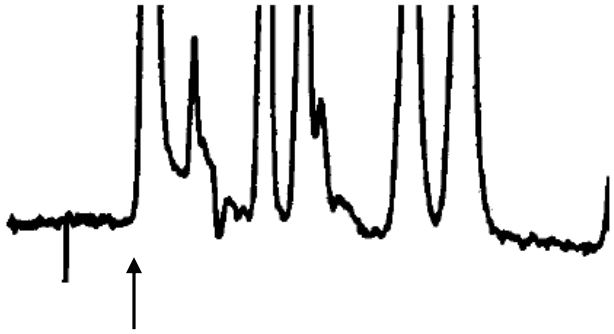


Column dead-time t_M



Estimating t_M

1 by the chromatogram:



2. By injecting an non-retained solute (uracil, NaNO3, etc.)

3. By calculation:

internal volume, V_M » 0.5 L d_C

2 (» 0.1 x L for 4.6-mm i.d.)

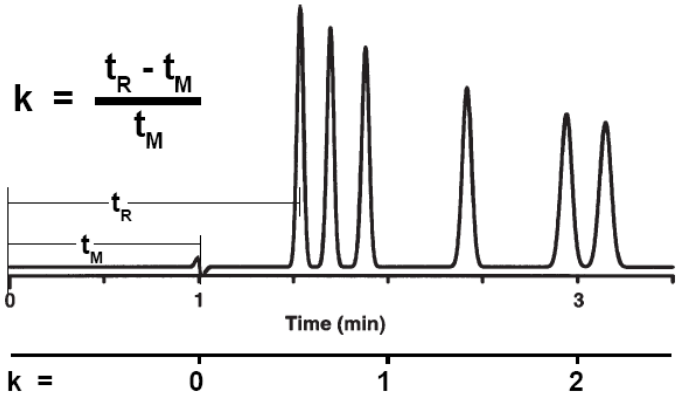
L is column length, d_C is column i.d. (both in cm)

V_M is column volume in mL, F is flow in mL/min

$t_M = V_M / F$

Retention factor k

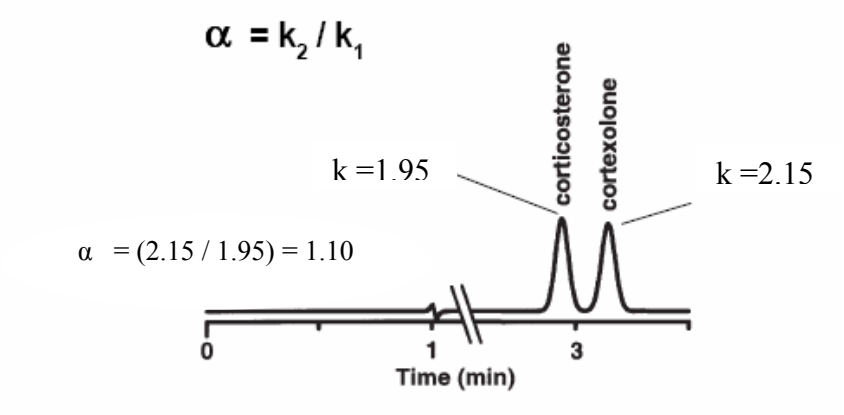
Retention factor, defined as the ratio of amount of X in the stationary phase to amount of X in the mobile phase, can be computed by dividing the corrected retention time ($t_R - t_m$) by t_m.



Retention factor can be estimated quickly by using tm as a ruler and measuring retention time from tm in units of tm. k does not change with flow rate or column dimensions, but is influenced by column chemistry and mobile phase composition.

Selectivity α

Selectivity, α is defined as the ratio of k-values for adjacent peaks. By convention α ≥1.0.Thes shortcoming of using α to measure separation is that it does not take the band width into account.



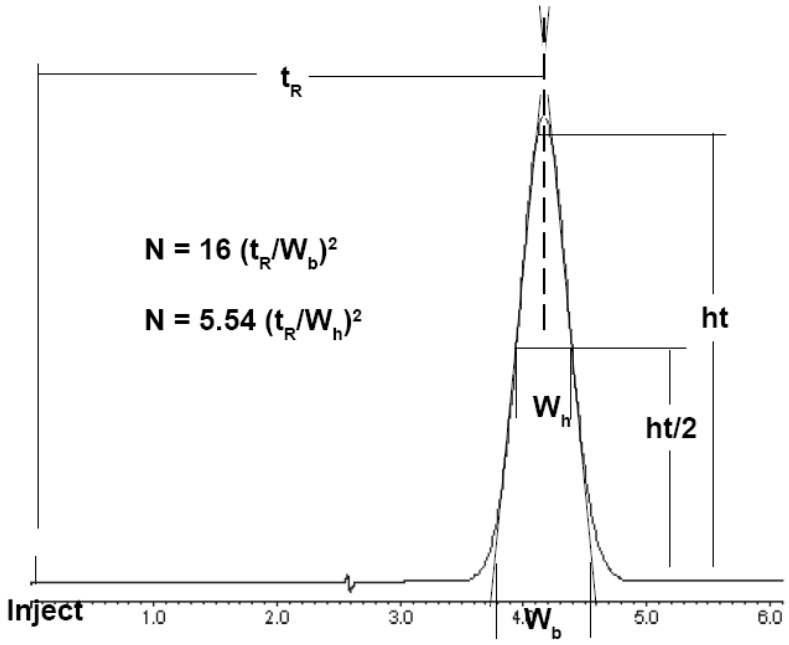
Column plate number N

Band width is measured as the column plate number. The easiest way to measure N is using the half-height method. Alternatively, the baseline technique can be used to determine N. N is an expression of the standard deviation of a peak.

N for commercial columns tested under ideal conditions (e.g., using toluene) will be in the range of 80,000 plates/meter for 5 µm columns and 100,000 plates/meter for 3 µm columns.In practical use, however, the plate number often will be much lower. This equation allows us to estimate practical expectations for N in real applications. For example, a 25 cm, 5 µm column would be expected to generate about 15,000 plates (vs a test value of perhaps N =20,000).

Estimating N

N » 3000 L / dp
where:
L = column length (cm)
dp = particle diameter (mm)

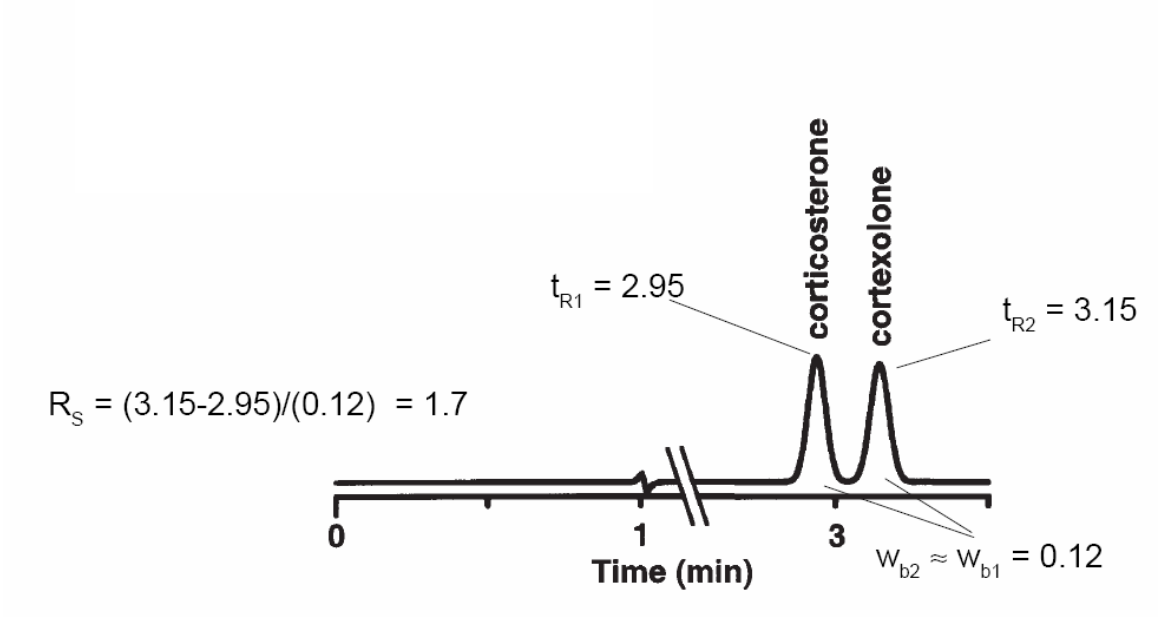


Whereas k, N, and α give us measurements of peak characteristics, we're most interested in whether our peaks are separated. The upper left chromatogram is too-well separated; time is wasted. The upper right one looks good. The lower left separation is insufficient for many purposes, but may be adequate for qualitative analysis or chiral assays. The lower right separation is too poor for any use except determining that two compounds are present. The requirements of each method dictate the resolution required for satisfactory results.

Resolution Rs

Resolution can be calculated by;

$Rs = (t_{r2} - t_{r1})/ 0.5(W_{b1} + W_{b2})$



Another way to look at resolution is to express Rs in terms of N, α, and k. k measures retention, and is influenced mainly by solvent strength. An α is most strongly affected by chemical changes in the column or mobile phase. N is a "physical" (kinetic) rather than "chemical" (thermodynamic) parameter and changes resolution only in relation to its square root.

Here we see the influence of each factor independently expressed.

$$R_s = \frac{t_{r2} - t_{r1}}{0.5(w_{b1} + w_{b2})}$$

$$R_s = (1/4)\, N^{1/2}\, (\alpha - 1)\, [k / (1 + k)]$$

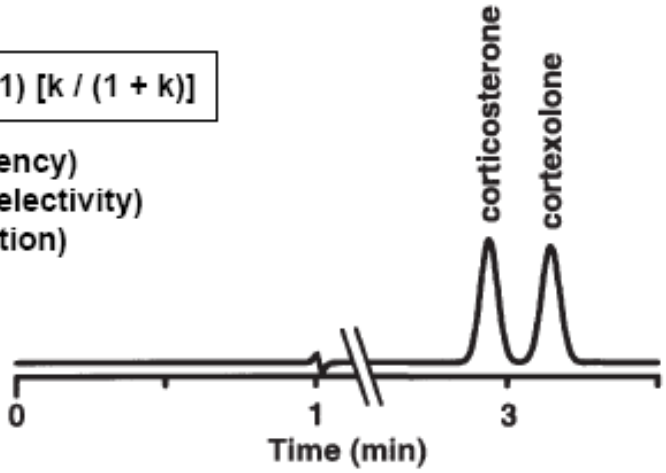
N = plate number (efficiency)
α = separation factor (selectivity)
k = capacity ratio (retention)

Where:

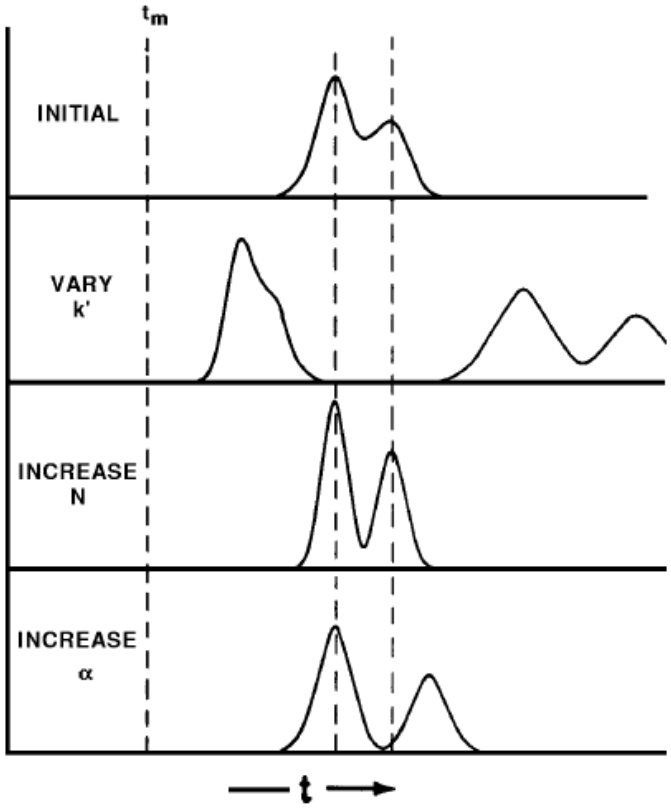
$N = 16\,(t_R/w_b)^2$

$\alpha = k_2/k_1$

$k = (t_R - t_M)/t_M$



How resolution varies with N, α, k



Resolution depends on the efficiency of the chromatographic system (as measured by the plate number, N), on the selectivity of the chromatographic system (as measured by the separation factor, a) and on the retention of the chromatographic system (as measured by the retention factor, k) for the analytes of interest. Overall, efficiency and retention (or range of retentions for various analytes) determine the number of peaks which can be separated. The selectivity determines whether a specific pair of peaks can be separated.

If α and N are held constant, then the relationship between Resolution and retention becomes Rs α k/(1 + k). At low k-values, resolution is poor. As k is increased, a point is reached at which resolution is only marginally increased for further increases in k. Sensitivity (peak height) becomes poorer as peaks get broader and are more strongly

retained. The factor k/(1+k) can be thought of as the fraction of analyte molecules which interact with the column packing at any given time.

The best place to work to obtain significant changes in resolution with reasonable changes in retention (or solvent strength) is in the $1 < k < 20$ or better yet, $2 < k < 5$ region.

Resolution depends on efficiency, selectivity and retention

$$RS = (1/4)\, N^{1/2}\, (\alpha - 1)\quad [k / (1 + k)]$$

Efficiency selectivity retention

- The number of peaks that can be resolved depends on efficiency and retention.
- Whether specific compounds can be resolved depends on selectivity.