DEVELOPMENT AND VALIDATION OF ASENAPINE AND ITS METABOLITE BY BIOANALYTICAL METHODS USING LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROSCOPY(LC-MS/MS)

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032.

In partial fulfillment of the requirements for the award of Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted
By

Reg No: 261230955

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

APRIL 2014

DEVELOPMENT AND VALIDATION OF ASENAPINE AND ITS METABOLITE BY BIOANALYTICAL METHODS USING LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROSCOPY(LC-MS/MS)

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032.

In partial fulfillment of the requirements for the award of Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted
By

V.Mohanam

(Reg No: 261230955)

Under the guidance of

Dr.S.VADIVELAN, M.Pharm., Ph.D.,

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

APRIL- 2014

Dr.S.Vadivelan, M.Pharm., Ph.D.,

Associate Professor,

Department of Pharmaceutical Analysis,

Edayathangudy.G.S.Pillay College of Pharmacy,

Nagapattinam – 611 002.

CERTIFICATE

This is to certify that the dissertation entitled **"DEVELOPMENT AND VALIDATION OF ASENAPINE AND ITS METABOLITE BY BIOANALYTICAL METHODS USING LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROSCOPY(LC-MS/MS)"** submitted by **V.Mohanam** (Reg No: 261230955) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in the Department of Pharmaceutics, Edayathangudy.G.S Pillay College of Pharmacy during the academic year 2013-2014.

Place: Nagapattinam **Dr.S.Vadivelan, M.Pharm., Ph.D.,**

Date:

Prof.Dr.D.Babu Ananth, M.Pharm., Ph.D.,

Principal,

Edayathangudy.G.S.Pillay College of Pharmacy,

Nagapattinam – 611 002.

CERTIFICATE

This is to certify that the dissertation **"DEVELOPMENT AND VALIDATION OF ASENAPINE AND ITS METABOLITE BY BIOANALYTICAL METHODS USING LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROSCOPY(LC-MS/MS)"** submitted by **V.Mohanam** (Reg No: 261230955) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under the guidance of **Dr.S.Vadivelan, M.Pharm., Ph.D.,** Associate Professor. Department of Pharmaceutical Analysis, Edayathangudy.G.S Pillay College of Pharmacy during the academic year 2013-2014.

Place: Nagapattinam **Prof.Dr.D.Babu Ananth, M.Pharm., Ph.D.,**

Date:

ACKNOWLEDGEMENT

I would like to express profound gratitude to **Chevalier Thiru.G.S.Pillay,** Chairman, E.G.S.Pillay College of Pharmacy, and **Thiru. S.Paramesvaran, M.Com., FCCA.,** Secretary, E.G.S.Pillay College of Pharmacy.

I express my sincere and deep sense of gratitude to my guide **Dr.S.Vadivelan, M.Pharm., Ph.D.,** Associate Professor Department of Pharmaceutical Analysis, E.G.S.Pillay College of Pharmacy, for his invaluable and extreme support, encouragement, and co-operation throughout the course of my work.

It is my privilege to express my heartfelt thanks to **Prof. Dr.D.Babu Ananth, M.Pharm, Ph.D.,** Principal, E.G.S.Pillay College of Pharmacy, for providing me all facilities and encouragement throughout the research work.

I express my sincere gratitude to **Prof. Dr.M.Murugan, M.Pharm., Ph.D.**, Director cum Professor, Head, Department of Pharmaceutics. E.G.S.Pillay College of Pharmacy, for his encouragement throughout the course of my work.

I wish to express my great thanks to **Prof.K.Shahul Hameed Maraicar, M.Pharm., (Ph.D),** Director cum Professor , Department of Pharmaceutics, E.G.S.Pillay College of Pharmacy, for his support and valuable guidance during my project work.

I would like to extend my thanks to all the **Teaching Staff** and **Non Teaching Staff**, who are all supported me for the successful completion of my project work.

Last but not least, I express my deep sense of gratitude to my parents, family members and friends for their constant valuable blessings and kindness.

INDEX

ABSTRACT

The objective of this study was to validate a simple and precise Ultra Performance liquid chromatographic method with Tandem Mass Spectrometry-(AB SCIEX) method for the determination of Asenapine and N-Desmethyl Asenapine (metabolite) in human plasma using Asenapine Maleate 13C D3 as Internal Standard (IS). The precision and accuracy data have to fulfill the requirements for quantification of the analytes in biological matrices to generate data for bioequivalence, bioavailability, pharmacokinetic or toxicology investigations. A Hypersil GOLD C18, 5µ column having 4.6 x 50 mm internal diameter in binary gradient mode with flow rate was 0.6 mL/min of mobile phase containing acetonitrile and ammonium formate (90:10) were used. The experiments were performed by loading in UPLC with a triple quadruple mass spectrometer, operating in the multiple reaction monitoring (MRM) modes. The method was validated over the concentration range of 0.1080 – 35.314 ng/mL(ANALYTE) and 0.1060 – 34.47 ng/mL (METABOLITE), by using 500 µL plasma samples.The mean recovery of Asenapine (81%) and N-Desmethyl Asenapine (80%) from spiked plasma samples was consistent and reproducible. The method was validated for linearity, accuracy, precision, specificity, and robustness. The intra- and inter-day precision and accuracy values were found to be within the assay variability limits as per the FDA guidelines. The developed assay method was applied to a clinical pharmacokinetic study in human volunteers.

Keywords: Asenapine, N-Desmethyl Asenapine , Asenapine Maleate 13C D3, LC-MS/MS, Linearity, Validation.

LIST OF SYMBOLS

°C	-	Degree Celsius
µg	-	micro gram
µL	-	micro litre
ng	-	nano gram
mg	-	milli gram
mL	-	milli litre
min	-	minute(s)
psi	-	per square inch
rpm	-	rotations per minute
≥	-	greater than or equal to
≤	-	less than or equal to
±	-	plus or minus
=	-	equal to

LIST OF ABBREVIATIONS

CC	:	Calibration Curve
QC	:	Quality Control
CV	:	Coefficient of Variation
LC-MS/MS	:	Liquid Spectroscopy Tandem Mass Spectroscopy
UPLC	:	Ultra Performance Liquid Chromatography
HPLC	:	High Performance Liquid Chromatography
RP-HPLC	:	Reverse Phase HPLC
GC	:	Gas Chromatography
API	:	Atmospheric Pressure Ion spray MS
CAD	:	Collision Activated Dissosciation
CUR	:	Curtain gas
CXP	:	Collision Exit Potential
DP	:	Declustering Potential
IS	:	Internal Standard
SPE	:	Solid Phase Extraction
LLE	:	Liquid – Liquid Extraction
ICH	:	International Conference on Harmonization
USP	:	United States Pharmacopeia
FDA	:	Food and Drug Assosciation
LOD	:	Limits Of Detection
LOQ	:	Limits Of Quantification
LLOQ	:	Lower Limit Of Quantification
ULOQ	:	Upper Limit Of Quantification
LQC	:	Lower Quality Control

LOQQC	:	Lowest Of Quantification Quality Control
MQC	:	Middle Quality Control
INTQC	:	Intermediate Quality Control
HQC	:	Highest Quality Control
LV	:	Liquid Vaporizer
MD	:	Method Development
MV	:	Method Validation
P&A	:	Precision and Accuracy
PMV	:	Pre-Method Validation
ME	:	Matrix Effect
MF	:	Matrix Factor
MRM	:	Multiple Reaction Monitoring
ACN	:	Acetonitrile
RT	:	Retention Time
SD	:	Standard Deviation
ASEN	:	Asenapine
DES ASE	:	N- Desmethyl Asenapine
ASEN 13C D3	:	Asenapine 13C D3
RIR	:	Reinjection Reproducibility
ASCOT	:	Auto Sampler Carry Over Test

1. INTRODUCTION

Analytical sciences are useful in the Qualitative and Quantitative analysis of pharmaceuticals and other compounds. Analytical methods are developed according to Regulatory guidelines.

1.1. Bio analysis

Bioanalysis is a sub-discipline of analytical chemistry covering the quantitative measurement of biological molecules, proteins, DNA, drugs and their metabolites in the biological systems. Bio analysis also applies to drugs used for illicit purposes, forensic investigations, anti-doping testing in sports, and environmental concerns.

1.2. Modern Bio analytical Chemistry

Many scientific endeavours are dependent upon accurate quantification of drugs and endogenous substances in biological samples. The focus of bio analysis in the pharmaceutical industry is to provide a quantitative measure of the active drug and/or its metabolite(s) for the purpose of pharmacokinetics, toxicokinetics, bioequivalence and exposure–response (pharmacokinetics /pharmacodynamics studies). Bio analysis was traditionally thought of in terms of measuring small molecule drugs. However, the past twenty years has seen an increase in biopharmaceuticals (e.g. proteins and peptides), which have been developed to address many of the same diseases as small molecules. Modern drugs are more potent, which has required more sensitive bio analytical assays to accurately and reliably determine these drugs at lower concentrations. This has driven improvements in technology and analytical methods.

1.3. Diseases and Drug:

Psychosis is defined as a serious mental disorder as characterized by defective or lost contact with reality often with hallucinations or delusions. Bipolar mood disorder, in which the patient cycles from severe depression to feelings of extreme excitation.

Anti-psychotic drugs control the symptoms of psychosis, and in many cases are effective in controlling the symptoms of Bipolar Disorder which causes unusual shifts in mood , energy, activity levels, ability to perform daily task and also Schizophrenia with the symptoms of Hallucination, delusions, disorders in thinking, flat effect, social withdrawal, cognitive deficits. Atypical Antipsychotic drugs are helpful in controlling mental disorders with less side effects. Some drugs include:

1. Chlorpromazine
2. Risperidone
3. Haloperidol
4. Olanzapine
5. **Asenapine**

The efficacy of asenapine is through a combination of potent antagonist activity at D2 and 5:HT2A receptors with the affinity to 5:HT2A receptors 19 times higher than that of D2 receptors. Pre clinical test have shown a low tendency for EPS, whereas other drugs that target dopamine D2receptors. This is the major reason to develop this drug, in order to cure the mental disorders.

Literature survey reveals that Asenapine and three metabolites were estimated in human plasma by LC/MS method. One is N-Desmethyl Asenapine obtained from the demethylation of Asenapine.

1.4. Bioanalytical techniques

Some techniques commonly used in bio analytical studies include:

1. **Hyphenated techniques**

   LC–MS (liquid chromatography–mass spectrometry)

   GC–MS (gas chromatography–mass spectrometry)

   LC–DAD (liquid chromatography–diode array detection)

   CE–MS (capillary electrophoresis–mass spectrometry)

2. **Chromatographic methods**

   HPLC (high performance liquid chromatography)

   GC (gas chromatography)

   UPLC (ultra performance liquid chromatography)

   Supercritical fluid chromatography

3. **Electrophoresis**
4. **Ligand binding assays**

   Dual polarization interferometer

   ELISA (Enzyme-linked immunosorbent assay)

   MIA (magnetic immunoassay)

   RIA (radioimmunoassay)

5. **Mass spectrometry**
6. **Nuclear magnetic resonance**

The most frequently used techniques are: liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) for 'small' molecules and enzyme-linked immunosorbent assay (ELISA) for macromolecules

1.5. LC – MS

It is an analytical instrument that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. It is used for analyzing high sensitive compounds.

1.5.1. Application

1. Qualitative and quantitative analysis
2. Impurity profiling
3. Metabolite studies
4. Pharmacokinetics and bio medical studies.

Fig – 1: UPLC- MS/MS (Aquity – AB SCIEX)

1.5.2. Advantages of LC–MS/MS:

1. Here we can select our interested ion from the chromatograph and accumulate as fragmentation for further MS study.
2. Label free analysis (reduced protein loading, increased sequence coverage for protein and proteome coverage).
3. Easy to fractionate the complex mixtures.

In this work RP-HPLC with Multiple Quadrupole tandem Mass Spectroscopy (API 4000) has been used to develop and validate a method for the determination of the drug Asenapine in human plasma in order to achieve a minimum Retention time, good precision and accuracy within a lowest Range of Concentration.

1.6. ASENAPINE

Drug Name: Asenapine

Iupacname:(3a*R*,12b*R*)-rel-5-chloro-2,3,3a,12b-tetrahydro-2-methyl-1*H*-dibenz(2,3:6,7)oxepino[4,5-c]pyrrole (2*Z*)-2-butenedioate (1:1)

Molecular Formula: $C_{17}H_{16}ClNO \cdot C_4H_4O_4$ (and enantiomer)

Molecular weight: 401.84(285.8 as free base.)

Category: Antipsychotic Agents

Description: SAPHRIS is a novel antipsychotic, belonging to the dibenzo-oxepino pyrroles. It has antagonist activity on the dopamine 2 (D2) and serotonin (5-HT)-2A receptors.

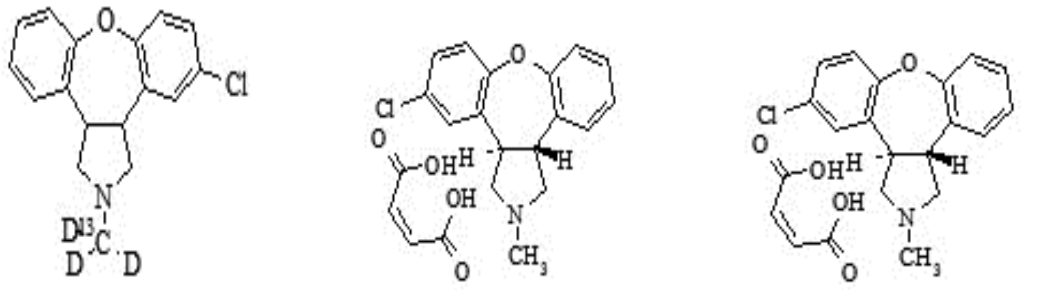
Solubility: The solubility of asenapine (active entity) in water is 3.7 mg/mL, in 0.1M HCl is 13 mg/mL and in aqueous buffers of pH 4.0 and 7.0 the solubility is 3.8 mg/mL and 3.0 mg/mL, respectively.

pKa: is 8.6 (determined in water/methanol). Asenapine has a log P (n-octanol/water) of 4.9 for the neutral species and 1.4 for the cationic species.

1.7. N- DESMETHYL ASENAPINE:

- Molecular Formula: **$C_{16}H_{14}ClNO.HCl$,**
- Molecular Weight: **271.75**
- Cmax = 0.39ng/ml

Structure



Asenapine 13C-D3 **Asenapine Maleate** **N-Desmethyl Asenapine**

Fig – 2: Structure of Analyte, Metabolite and IS

1.8. AIM & OBJECTIVE OF PRESENT WORK:

To develop a sensitive, precise and accurate method for determination of **Asenapine and its metabolite** by Bioanalytical Method in human plasma using LC-MS/MS.

The main objectives of the RP-HPLC-MS/MS method development to rapidly assay and determine the related substances of Asenapine in the pharmaceutical formulation.

- To develop an efficient method for analysis of Asenapine and its metabolite using LC-MS/MS.
- To perform pre- method validation experiments.
- To fully validate the developed method by studying various parameters like accuracy, specificity, matrix effect etc.

2. LITERATURE REVIEW

2.1. Analytical sciences and Bio analysis:

Qualitative and quantitative chromatographic analyses are used extensively in all areas of analytical sciences. Due to the high sensitivities of the detection instruments available today the techniques are invaluable in the analysis of environmental samples , in chemical ecology studies, for forensic purposes, in pharmaceutical and clinical studies, in chemical biology and in virtually any situation where they might find an application. (***Anthony D. Wright et al-2012)***[3]

The safety and efficacy of biopharmaceuticals is controlled by measurements of their quality attributes. To measure these attributes for protein pharmaceuticals, a set of analytical methods are developed that have to meet the requirements specified by the International Conference on Harmonization (ICH) guideline. (***Izydor Aposto et al, 2012***)[14]

LC-MS based method that utilized both RPLC and HILIC separations was carried out, followed by multivariate data analysis to discriminate the global urine profiles of BC patients and healthy controls. The purpose of this study was to identify a potential biomarker pattern in urine using metabonomics to aid non invasive BC detection using complementary chromatographic techniques. ***(Wei Hang et al., - 2011)***[31]

Metabolomics involves the unbiased quantitative and qualitative analysis of the complete set of metabolites present in cells, body fluids and tissues, which is done by development of a method using Gas Chromatography – Mass Spectroscopy.(***Maud M. Koek et al., 2010***).[24]

2.2. Diseases and Drug:

The development of sublingual asenapine began in 1996 for the treatment of schizophrenia, and in 2004 for the treatment of bipolar disorder. Asenapine is classified as a dibenzo-oxepino pyrrole and has properties that are most similar to those of quetiapine, olanzapine, and clozapine. ***(Maurizio Pompili et al., 2011)*** [25]

As with other antipsychotic agents, asenapine exhibits a higher binding affinity for the 5HT2A receptor compared with D2 receptors. Moreover, asenapine has a broad range of effects on other neurotransmitter systems. One major difference between asenapine and most other atypical antipsychotics is that it exhibits little muscarinic receptor antagonist effects, which may produce a less cognitively deleterious profile, but it may result in weight gain. ***(Maurizio Pompili et al., 2011).***[25]

Asenapine is a new atypical antipsychotic agent currently under development for the treatment of schizophrenia and bipolar disorder. It has high affinity for various receptors including antagonism at 5HT2A, 5HT2B, 5HT2C, 5HT6 and 5HT7 serotonergic receptor subtypes, α1A, α2A, α2B and α2C adrenergic and D3 and D4 dopaminergic receptors.(***David Taylor et al., - 2009***)[7].

Asenapine was initially approved by the US Food and Drug Administration (FDA) in 2009 for the treatment of acute schizophrenia and acute manic or mixed episodes associated with bipolar I disorder in adults, and subsequently received approval for the maintenance phase of schizophrenia and for adjunctive use with lithium or valproate for acute manic or mixed episodes associated with bipolar I disorder. (***Leslie Citrome – 2011***)[21].

Amisulpride is a highly selective benzamide with 10 × higher affinities to D2 and D3 receptors than sulpiride and with little activity at serotonergic, histaminergic, or muscarinic receptors. While the agonistic effects on presynaptic D2/D3 receptors prevail at lower doses (increased dopamine transmission), at higher doses, amisulpride acts preferentially on postsynaptic D2/D3 receptors, reducing dopaminergic transmission. Asenapine will be evaluated for whether it: a) causes a reduction in symptoms of schizophrenia. (***Daniel Huys et al.,- 2012***)[6].

2.3. Chemistry:

Asenapine (trans-5-chloro-2-methyl-2, 3, 3a, 12b-tetrahydro-1H dibenz [2,3:6,7] oxepino[4,5- c]pyrrolidine) maleate (Org 5222) was developed by altering the structure of mianserin. The molecular formula of asenapine maleate is C17H16CINO.C4H4O4 with a molecular weight of 401.84. Asenapine is quite stable in crystalline form although excessive light can induce degradation. Clinical studies have used fast-dissolving highly porous asenapine tablets. (**Arpi MinassianJared W Young – 2012)[4].**

Asenapine maleate is chemically (3aRS,12bRS)—chloro-2-methyl-2,3,3a,12b-tetrahydro-1*H*-dibenzo[2,3:6,7]oxepino[4,5-c]pyrrole(2Z)-2 butenediate is a atypical antipsychotic drug. (***Aneesh T.P. et al., 2012***)**[1].**

The solubility of asenapine (active entity) in water is 3.7 mg/mL, in 0.1M HCl is 13 mg/mL and in aqueous buffers of pH 4.0 and 7.0 the solubility is 3.8 mg/mL and 3.0 mg/mL, respectively. pKa is 8.6 (determined in water/methanol). Asenapine has a log P (n-octanol/water) of 4.9 for the neutral species and 1.4 for the cationic species. (***EMEA/H/C/001177***)[10].

Major metabolic routes were direct Glucuronidation and N Desmethylation. The principal circulating metabolites were Asenapine N+glucuronide,N-desmethylasenapine-N-carbamoyl-glucuronide,N-desmethylasenapine and Asenapine 11-O-sulfate.(***Jacobs et al., 2010***) [15].

N-desmethyl Asenapine is not intended for the for the diagnostic and therapeutic uses. (***EMEA)*** [10].

The peak plasma concentration of Asenapine and its metabolite N-Desmethyl Asenapine in plasma for 5mg/10mg dose are 4ng/ml and 0.39ng/ml at tmax approximately 1 hr. (***EMEA/H/C/001177)*** [10].

2.4. Mechanism of action of Asenapine:

Consistent with other atypical antipsychotics asenapine exhibits a higher binding affinity for the 5HT2A receptor compared to D2 receptors. Moreover, asenapine exhibits a broad range of effects on other neurotransmitter systems including 5-HT2C, 5-HT7, 5-HT2B, 5-HT6, α2B, D3, H1, D4, α1A, α2A, α2C, D2L, D1, D2S, 5-HT1A, 5-HT1B, and H2 receptors.(**Arpi MinassianJared W Young – 2012**) [4].

It is an antagonist at 5-HT , dopamine and α-adrenergic receptors and has high affinity for dopamine(D2) and serotonin(5-HT2A) receptors and its efficacy is mainly mediated through the combination of antagonist activity at D2 and 5-HT2A receptors. It is indicated for treatment of various psychotic conditions like schizophrenia and bipolar disorders in adults4,5,6 and mainly works by controlling the psychotic symptoms through antagonism of selected dopamine and serotonin receptors in the CNS. (***Aneesh T.P. et al., 2012***) [1].

The **mechanism of action** of asenapine, as with other drugs having efficacy in schizophrenia and bipolar disorder, is not fully understood.

However, based on its receptor pharmacology, it is proposed that the efficacy of asenapine is mediated through a combination of antagonist activity at D2 and 5-HT2A receptors. Actions at other receptors e.g., 5-HT1A, 5-HT1B, 5 HT2C, 5-HT6, 5-HT7, D3, and α2-adrenergic receptors, may also contribute to the clinical effects of asenapine. (**Jacobs et al., 2010)** [15].

2.5. Pharmacokinetics:

Absorption of asenapine is rapid with peak plasma concentrations occurring within 0.5 to 1.5 hours. The average peak plasma concentrations at steady state of 5 and 10 mg twice daily were 3.6ng/mL and 7.0ng/mL respectively. The absolute bioavailability of sublingual asenapine at 5 mg is 35%. Increasing the dose from 5 to 10 mg twice daily (a two-fold increase) results in less than linear (1.7 times) increases in both the extent of exposure and maximum concentration. The absolute bioavailability of asenapine when swallowed is low (< 2% with an oral tablet formulation).

Asenapine is rapidly **distributed** and has a large volume of distribution (approximately 1700L), indicating extensive extravascular distribution. Asenapine is highly bound (95-97% at 1-500ng/mL) to plasma proteins *in vitro*, including albumin and α1-acid glycoprotein.

Asenapine is extensively **metabolised**. Oxidative metabolism by cytochrome P450 isoenzymes (predominantly CYP 1A2) and direct glucuronidation by UGT1A4 are the primary metabolic pathways for asenapine. In an *in vivo* study in humans with radio-labelled asenapine, the predominant drug-related entity in plasma was asenapine N+-glucuronide; others included N-desmethylasenapine, N-desmethylasenapine N-carbamoyl glucuronide, and unchanged asenapine in smaller amounts.

Asenapine is a high **clearance** drug, with a clearance after intravenous administration of 52 L/h. In a mass balance study, the majority of the radioactive dose was recovered in urine (about 50%) and faeces (about 40%), with only a small amount excreted in faeces (5-16%) as unchanged drug. Following an initial more rapid distribution phase, the terminal half-life of asenapine is approximately 24 hours. (***EMEA***) [10].

Sublingual administration of asenapine results in a rapid absorption with peak plasma concentrations within 0.5–1.5 hours and moderate (35%) bioavailability. This is in the lower to mid range of other antipsychotics which exhibit 20–70% bioavailability at appropriate doses. (***Arpi MinassianJared W Young – 2012***) [4].

2.6. METHOD DEVELOPMENT

It is a step wise procedure or formulating the materials, conditions, and protocol for measuring an analyte. Laboratories may make *minor modifications to methods to improve performance*, in which case, the modified methods should be subject to more rigorous testing and evaluation by the laboratory.

2.6.1. Instrumentation:

The LC-MS system consisting of Shimadzu HPLC System consisting of RP- C18 column, variable wavelength programmable UV Visible Detector SPD-20A and rheodyne injector with 20µl fixed loop. ***(Aneesh T.P., et al. IJPPS, 2012)*** [1].

Shimadzu HPLC System with 10- at detector and rheodyne injector with 20µl fixed loop was used. ***(Kiran Aarelly et al. JCPR, 2012)*** [23].

HPLC system (1200 series model, Agilent Technologies, Waldbronn, Germany), Mass spectrometry API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. **(*Chhalotiya et al., -2011*)** [30].

2.6.2. Chromatographic conditions of the Instrument:

The mobile phase consisting of methanol, n-butanol, and glacial acetic acid were degassed and filtered using a 0.45µm membrane filter. The eluent were monitored at 270 nm, flow rate was 1.0 mL/min, with ambient temperature, and runtime was 6 min. The volume of injection loop was 20µl. ***(Aneesh.t.p. et al. 2012)*** [1].

Hypersil ODS C18 Column - 250 X 4.6 mm (particle size of 5µ) and constant flow pump. Rheodyne injector with 20 µl loop. The mobile phase methanol was delivered at flow rate 1.0 ml/min. The mobile phase was filtered through a 0.45 µ membrane filter and sonicated for 15min. Analysis was performed at ambient temperature. ***(Kiran Aarelly et al., 2012)*** [23].

Various mixtures containing aqueous buffer, methanol and acetonitrile were tried as mobile phases in the initial stage of method development, but satisfactory resolution of drug and degradation peaks were not achieved. The mobile phase 0.02M potassium dihydrogen phosphate: acetonitrile (95:05, v/v, pH 3.5 adjusted with O - phosphoric acid) was found to be satisfactory and gave symmetric peak for ASP. **(*Chhalotiya et al., -2011*)** [30].

Zorbax Bonus-RP C18, 4.6 x 75 mm, 3.5µm was selected as the analytical column at a temperature of 30°C. The mobile phase composition was 0.2% formic acid: methanol (35:65 v/v) at a flow rate of 0.5 mL/min. Amisulpride-*d*5 was found to be an appropriate internal standard in terms of

chromatography and extractability. The retention time of Amisulpride, Amisulpride-*d*5 was found to be approximately 1.1 ± 0.2 min. (***Mogili et al., -2011***) [28].

2.6.3. Tuning:

Turbo ion spray (API) positive mode with Unit Resolution, MRM was used for the detection of Amisulpride and Amisulpride-*d*5. The [MH] + was monitored at m/z: 370.1, for Amisulpride and m/z: 375.2 for Amisulpride-*d*5. Fragments of m/z: 242.0 for Amisulpride and m/z: 242.1 Amisulpride-*d*5 formed from the respective precursor ions. Mass parameters were optimized as source temperature 500 °C, nebulizer source gas 30 (nitrogen) psi, heater gas 45 (nitrogen) psi, curtain gas 20 (nitrogen) psi, CAD gas 7 (nitrogen) psi, Ion Spray (IS) voltage 5500 volts, source flow rate 500µL/min without split, entrance potential 10 V, collision cell exit potential (CXP) 12 V, declustering potential (DP) 70 V, Collision energy 38 V for Amisulpride and Amisulpride-*d*5. ***(Mogili et al., SP-2011)*** [28].

2.6.4. Extraction Procedure Optimization:

To optimize sample preparation methods, SPE and LLE with different conditions were tested. Combination of protein precipitation and SPE was also used for preparation of plasma sample ASP, but it is expensive and labor intensive. LLE is a superior method for sample preparation. It gives cleaner samples compared with protein precipitation and, in some cases, better samples than SPE. ***(Liusheng Huang et al., 2012)*** [22].

The SPE extraction mirrors that used previously in the laboratory, and demonstrated increased process efficiency as compared to protein precipitation methods during assay development.***(Rower et al.,.- 2012)*** [28]

Liquid-liquid extraction was used to isolate Amisulpride and Amisulpride-*d*5 from human plasma and it gave good results without interference. ***(Mogili et al., 2011)*** [28].

A 0.5 ml of plasma was mixed with 0.1 ml of internal standard solution and 1 ml of borate buffer of pH 9.0. The solution was vortexed and extracted with ethyl acetate. The upper organic layer was separated, evaporated and reconstituted with mobile phase.LLE method was found to be more precise than SPE. ***(Muralidharan et al., - 2011)*** [26]

2.7. METHOD VALIDATION:

Method validation. The process of testing a measurement procedure to assess its performance and to validate that performance is acceptable. It helps in finding maximum acceptable error by analysing the acceptability of the experiment to defined requirements.

Once the HPLC method development was over, the method was validated in terms of parameters like, precision, accuracy, linearity and range, LOD, LOQ, recovery studies, system suitability parameters etc. For all the parameters percentage relative standard deviation values were calculated. The proposed HPLC method was validated as per ICH guidelines.

Aneesh et al[1] describes about the various parameters of Validation. **Linearity** was obeyed in the concentration range of 10-100µg/ml and the correlation 0.998. The regression equation of Asenapine maleate concentration over its peak area ratio was found to be Y=7727x-6525, where Y is the peak area ratio and X is the concentration of Asenapine maleate (µg/ml). The intraday and interday **precision** studies were carried out and the percent relative standard deviation (% RSD) was calculated and it was found to be 0.53 and 0.98

respectively, which is within the acceptable criteria of not more than 2.0. The **System suitability parameters** like number of theoretical plates (N) was found to be 3601, tailing factor 1.1 and asymmetry factor 0.95, which indicates efficient performance of column. The drug solution was subjected to small, deliberate changes like flow rate, wavelength and pH. The results obtained were not affected by varying the conditions and were in accordance with the results in original conditions. This shows the method was **robust.**

The **limit of detection and limit of quantification** was found to be 5.46µg/ml and 16.57µg/ml respectively, indicates the sensitivity of the method. Satisfactory recovery in the range of 98.9-100% is obtained by the proposed method. A good percentage of **recovery** indicates that the proposed method is accurate. Estimation of Asenapine maleate in pharmaceutical dosage form was carried out and got 98.7% as **assay** value. (IJPPS, 2012)

***Kiran Aarelly et al*[23],** found that, from the optical characteristics of the proposed method it was found that the drug obeys **linearity** range within the concentration of 2-10µg/ml. From the results shown **precision** it was found that the percent RSD is less than 2%, which indicates that the method has good reproducibility. From the results shown in accuracy it was found that the percent **recovery** values of pure drug from the pre analyzed solutions of formulations were in between 98.07- 101.28%, which indicates that the method is **accurate**.

The **system suitability parameters** are within the specified limits and which refers the commonly used excipients and additives present in the pharmaceutical formulations did not interfere in the proposed method. The proposed method was found to be simple, precise, accurate and rapid for

determination of Asenapine from pure form. The mobile phase is simple to prepare and economical. (JCPR, 2012)

Accuracy of the proposed method was determined by the recovery studies, and good %recovery (98- 101.2%) of the drug obtained indicate that the method is accurate. The method was found to be precise as %RSD values for interday and intraday was found to be less than 2. The method was also found to be rugged and robust as the % RSD values were found to be less than 2. The limit of detection and limit of quantification of the proposed method was found to be 1.40and 4.26 µg/ml indicating that the method developed is sensitive. The results of assay obtained were found to be in good agreement with the labeled claim, indicating the absence of interference of the excipients. ***(Liusheng Huang, Der Pharma Chemica, 2012)*** [22]

Stability (Freeze - thaw, Auto sampler, Bench top, Long term). The concentrations ranged from 93.16 to 103.3% for amisulpride of the theoretical values. These results confirmed the stability of Amisulpride in human plasma for at least 55 days at −30°C ***(Mogili et al)*** [28]**.**

Short Term Stock Solution **Stability** *Short term stock solution stability at room temperature* for acyclovir and internal standard the % change was -2.56 and -6.18 respectively. *Short term stock solution stability at refrigerator (2-8 0C)* : For acyclovir and internal standard the % change was -1.16 and -0.84 respectively. Short Term Working Solution Stability *Short term working solution stability at room temperature:* One for internal standard the % change found is -4.62. *Short term working solution stability at refrigerator (2-8 0C)* for internal standard the % change found was 0.75. Long Term Tock and Working Solution Stability The long-term stock and working solution stability experiment were completed after completion of the study sample analysis. *Long*

term stock solution stability in refrigerator between 2-8 0C :For acyclovir and internal standard, the % change was 2.23 and 1.62 respectively. *Long term working solution stability in refrigerator between 2-8 0C* For acyclovir and internal standard, the % change found is 2.30 and -0.14 respectively.

Bench Top Stability The bench top stability samples each of low and high QC (stability samples) was kept on bench at room temperature was found stable at approximately 14 Hrs and 30 Min. The % change for LQC and HQC were 2.27 and 5.97 respectively.

Freeze and Thaw stability (at -20 ± 5 0C) The freeze and thaw stability samples each of LQC and HQC were found to be stable in human plasma after four freeze and thaw cycles (at -20 ± 5 0C). The % change for LQC and HQC were 1.85 and 1.05 respectively.

Auto sampler Stability The stability samples each of LQC and HQC was found to be stable for approximately 70 Hrs and 00 Min in auto sampler (at 5 ± 3 0C). The % change for LQC and HQC were -0.78 and 0.29 respectively. Long term stability of Acyclovir and Internal Standard in Biological Matrix (Human Plasma) (**P. Susantakumar et al**) [29]

Separation, Specificity/Selectivity and Sensitivity Selected blank human plasma from six different sources and were carried through the extraction procedure and chromatographed to determine the extent to which endogenous human plasma components may contribute to chromatographic interference with the acyclovir or IS. (**P. Susantakumar et al)** [29]

The **Specificity and selectivity** analysis of Amisulpride and Amisulpride-*d*5 using MRM function was highly selective with no interfering compounds. Calibration was found to be linear over the concentration range of 2.0–2500.0ng/mL for Amisulpride (Fig. 4). The CV% for Amisulpride was less than 3.9%. The accuracy ranged from 96.5 to 101.5% for Amisulpride. The determination coefficient (r2) for Amisulpride was greater than 0.9998 for all curves. (***Chhalotiya et al., -2011***) [30]

Matrix Factor Samples of the relevant biological matrix from six different sources were collected. The lower calibration standard samples from each source were prepared and injected along with the six replicates of aqueous lower calibration standard level concentrations. The %CV of matrix factor for acyclovir and internal standard were 4.69 and 1.39 and % CV of matrix factor for internal standard normalized was 4.89, respectively (**P. Susantakumar et al)** [29]

Matrix Effect The CV % of ion suppression/enhancement in the signal was found to be 1.2% at MQC level for Amisulpride, indicating that the matrix effect on the ionization of the analyte is within the acceptable range under these conditions. (***Chhalotiya et al., -2011***) [30]

Ruggedness Different analyst with different column defines ruggedness. The run consisted of a calibration curve and a total of 18 spiked samples, including 6 replicate each of the low, medium and high quality control samples. The % coefficient of variation ranged from 1.03 to 12.12 and the percentage of nominal values ranged from 96.24 to 107.27. (**P. Susantakumar et al)** [29]

3. MATERIALS & METHODOLOGY

3.1. CHEMICALS AND REAGENTS USED:

Table-1: List of chemicals and reagents used

S.No	Name Of The Chemical/Reagents	Manufactures	Grade
01.	Asenapine Maleate (IS)	Clearsynth Inspiring Chemistry Pvt Ltd	NA
02.	N- Desmethyl Asenapine Maleate	Clearsynth Inspiring Chemistry Pvt Ltd	NA
03.	Asenapine 13C D3 Maleate	Clearsynth Inspiring Chemistry Pvt Ltd	NA
04.	Methanol	Finar	HPLC
05.	Acetonitrile	Fisher Scientific	HPLC
06.	Ammonium Formate	Loba chemie	
07.	Potassium Di hydrogen ortho phosphate	Rankem	
08.	Ethyl Acetate	Fisher Scientific	
09.	Tertiary Butyl Methyl Ester	Merck	
10.	Formic acid	Rankem	GR
11.	Milli-Q-water	Inhouse	HPLC
12.	K_2EDTA human plasma	Inhouse (Volunteers)	NA
13.	RIA vial	Abdos	NA
14.	Injector vials	Agilent	NA

3.2. INSTRUMENTS USED:

Table-2: List of instruments used

S no	List of instruments used	Instrument manufacturer	Range
01.	Micro pipette	Eppendorf	2-20µL, 20-200µL, 100-1000µL.
02.	Multipipette	Handy step	20µL-25000µL
03.	Electronic balance	Satrorius	0.001gm-220gm
04.	Vortex mixer	Cyclo mixcer	0-2500 rpm Touch and continuous
05.	Vibramax	Heidolph	rpm=0-3000rpm Time=0-30min
06.	Sonicator	Power sonic 420	40 kHz
07.	Refrigerated centrifuge	Kendro	rpm=0-5500rpm Time=0-30min
08.	Evaporator	Zymark turbovap	Pressure=0-30psi No. of sample=50
09.	LC-MS/MS	API 4000	ANALYST – 2.5.6

3.3. METHOD DEVELOPMENT

It refers to the process of formulating the materials, conditions, and protocol for measuring an analyte. It is the process of developing a method to determine the bioavailability and bioequivalence of administered drug in plasma. Method development is a trial and error process. It consists of various steps.

- Preparation of stock & tuning solutions (refer appendix II)
- Tuning of LC-MS/MS
- Chromatographic condition optimization
- Serial dilution
- Aqueous linearity
- Extraction procedure optimization

3.3.1. TUNING

Tuning is the adjustment of working parameters of LC-MS/MS to enable an operator to get the best signal possible for the analyte by optimizing the Q1 mass, Q3 mass of analyte, metabolite and Internal Standard based on molecular weights. The molecular weight of Asenapine and N- Desmethyl Asenapine are 285.8 and 271.75. Manual, semiautomatic, and automatic tuning procedures require the introduction of a tuning solution of the analyte of interest into the MS at a steady rate. It can be done either by directly injecting through syringe pump or by injecting the sample into the effluent of the LC by using a loop injection valve or tee union.

3.3.1.1. Procedure:

Stock solution of Asenapine was prepared as in Appendix-II and it was diluted to 100ng/ml to be used as Tuning Solution. Tuning solution was infused in full scan mode. From result, m/z of parent ion was selected. (Molecular weight).

Fragmentation of parent ion was determined by infusing the stock dilution in product ion mode and checked for m/z of various daughter ions obtained. Prominent and suitable daughter ion was selected by altering various parameters in Multiple Reaction Monitoring (MRM)

3.3.1.2. OPTIMIZED CONDITIONS:

Table - 3: Optimized Tuning Parameters

TUNING	Q1 MASS	Q3 MASS	DWELL (msec)	PARAMETERS		
				DP	CE	CXP
Analyte (ASEN)	286.20	229.20	200	85	30	18
Metabolite (DESASEN)	272.10	229.20	200	70	25	18
IS(ASEN 13CD3)	290.10	229.20	200	85	30	20

CAD GAS : 10.00

CUR GAS : 14.00

GS1 : 50.00

GS2 : 55.00

TEM : 400.00

IS : 5500.00

EP : 10.00

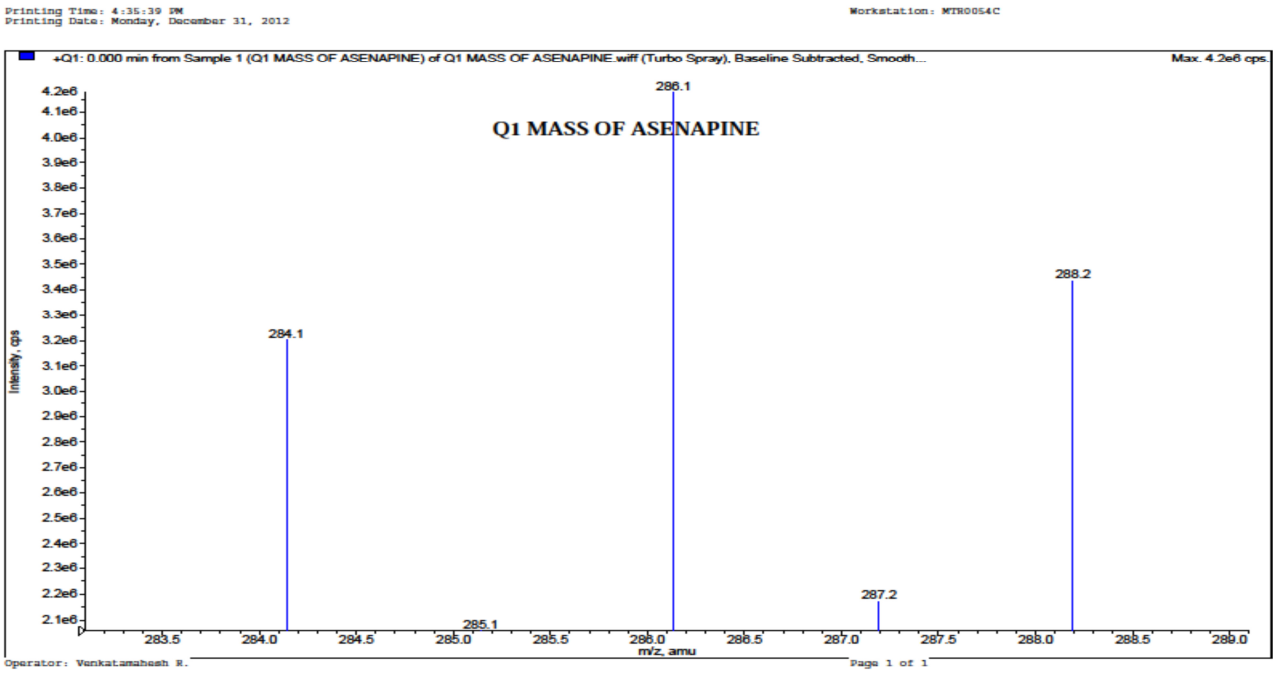


Fig- 3 : Q1 scan of Asenapine

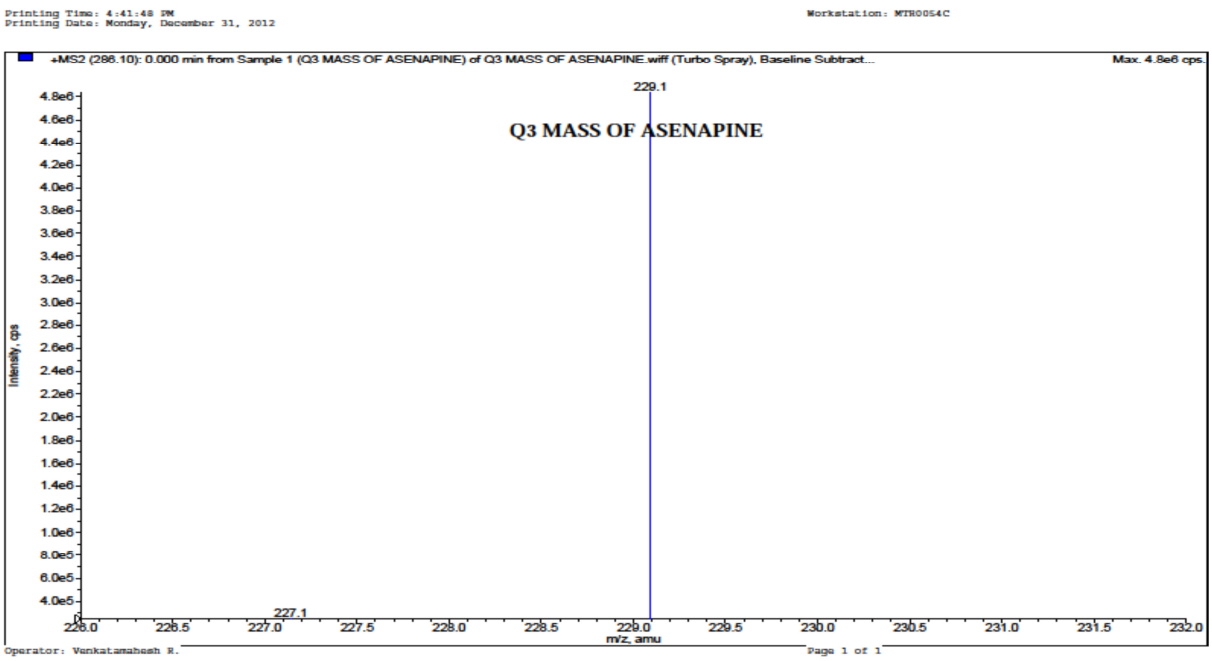


Fig- 4 : Q3 scan of Asenapine

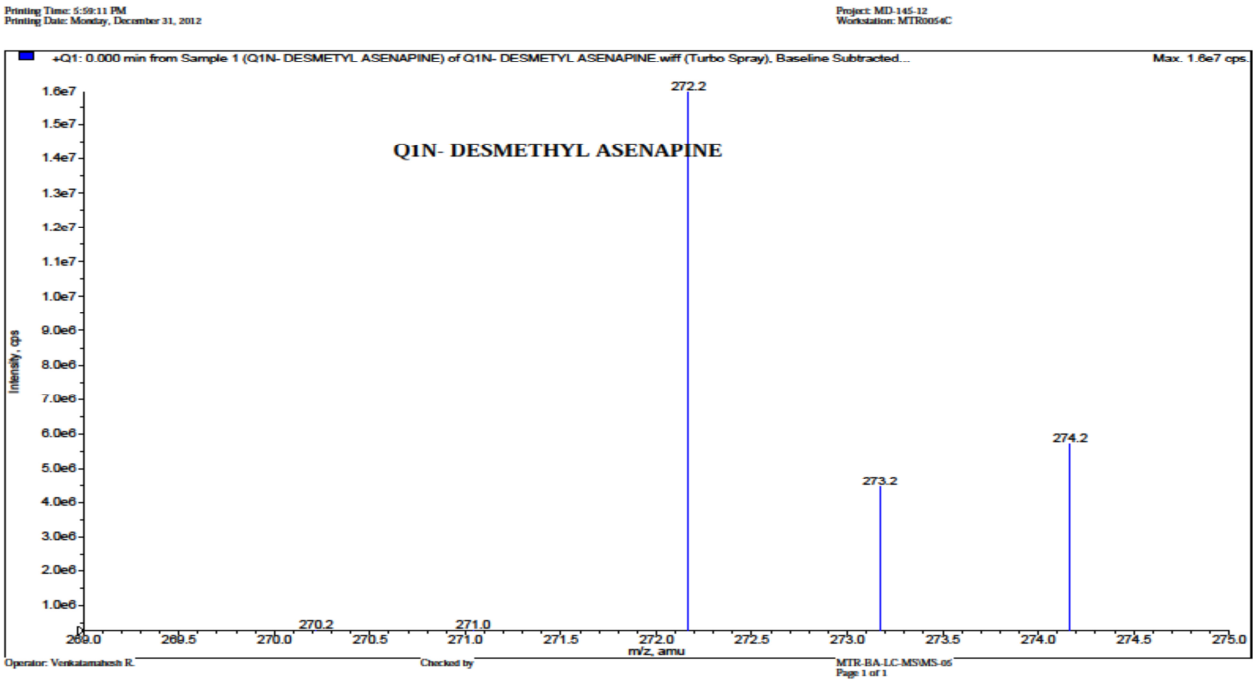


Fig-5: Q1 scan of N- Desmethyl Asenapine

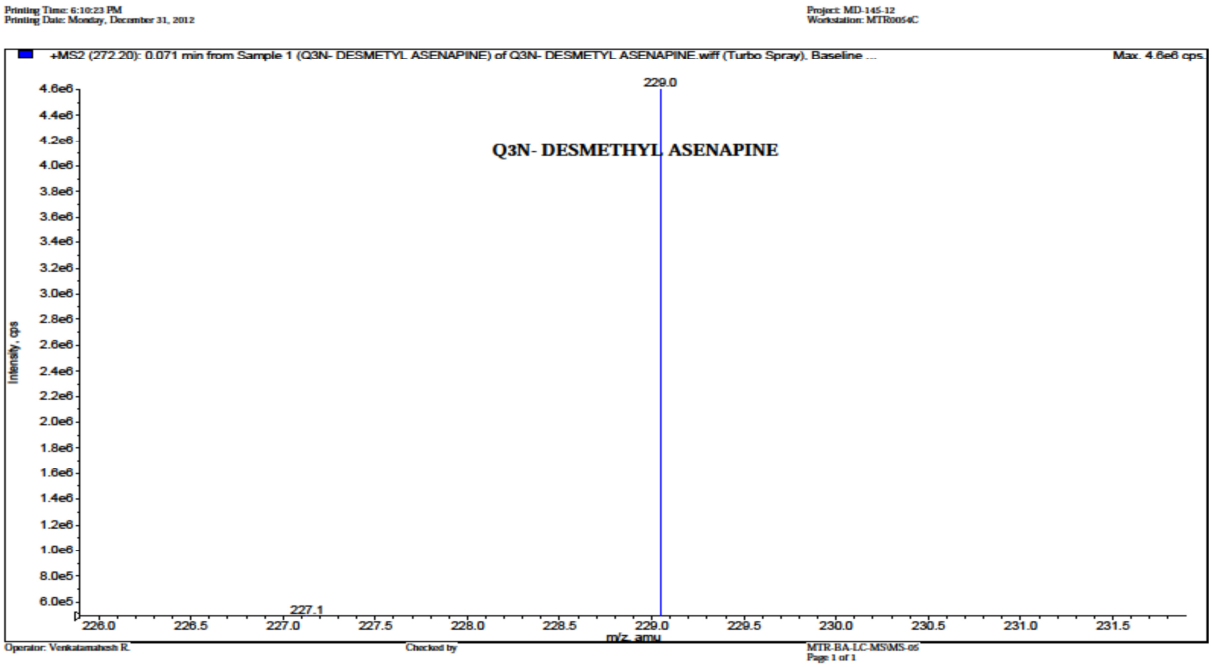


Fig-6: Q3 scan of N- Desmethyl Asenapine

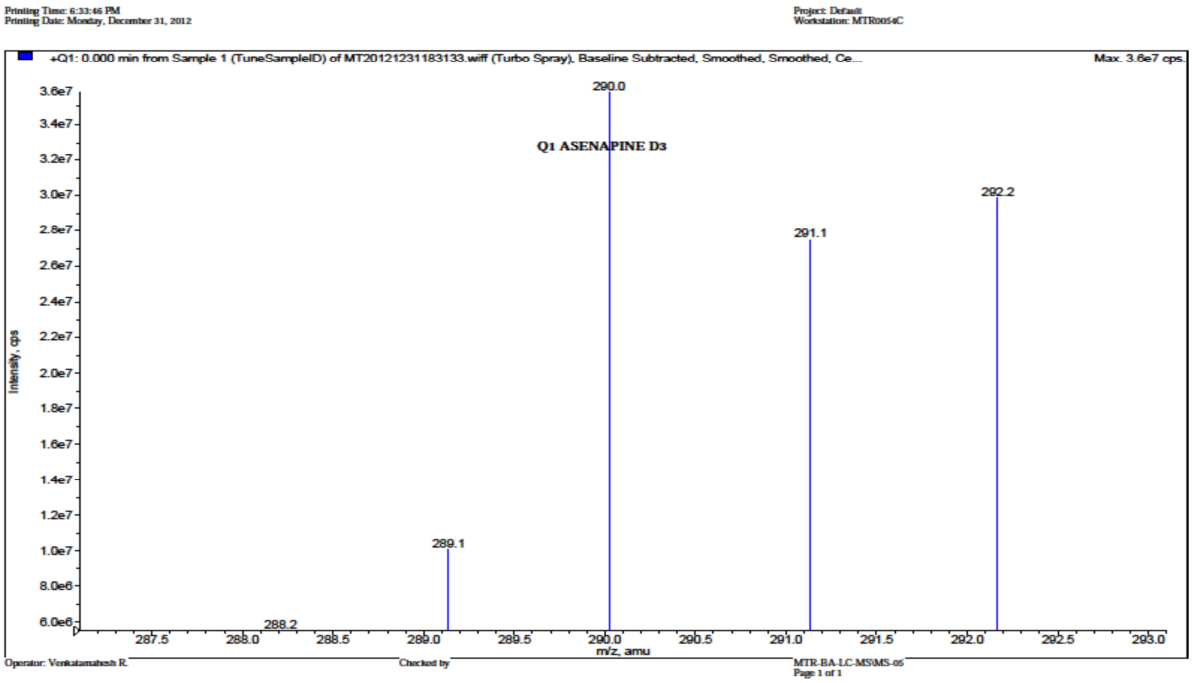


Fig-7: Q1 scan of Asenapine 13C D3

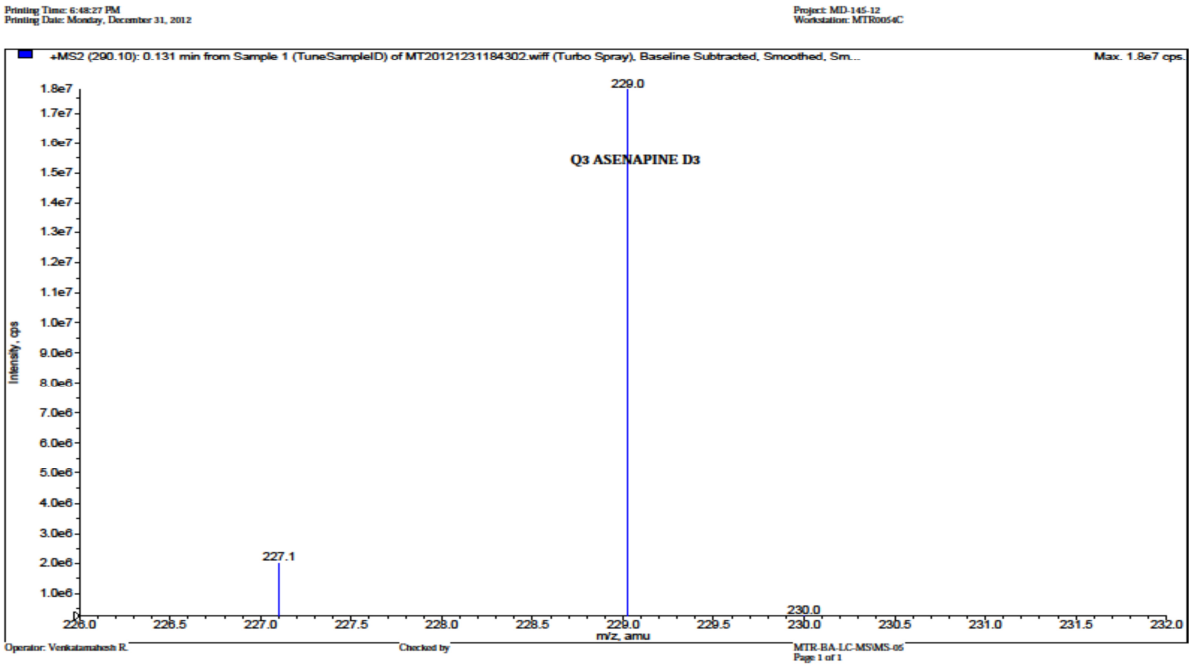


Fig-8: Q3 scan of Asenapine 13C D3

The response graph depicts the response in the Y-axis and mass of the ions in the X-axis. The determined parent ion mass (Q1 mass) is equal to the molecular weight of the species plus one. This is because during the electrospray ionization, the ions are protonated, resulting in an increase in the charge and consequently, the sensitivity. Hence the mass increases by one. The cleaved ions (or daughter ions) are screened and the mass of the daughter ion species giving the highest response is chosen (Q3 mass).

In the fig (3- 8), the response obtained for ions of various masses is shown. The ion with the mass which gives highest response was selected in each case for the parent and daughter ion scan of Asenapine, N- Desmethyl Asenapine, Asenapine 13C D3.

3.3.2. CHROMATOGRAPHIC CONDITION OPTIMIZATION:

The suitable column, mobile phase and flow rate etc had to be selected and optimized to develop an efficient method.

3.3.2.1. Column and Mobile Phase

Table - 4: Trails for column and mobile Phase

Trail	Column	Mobile Phase	Response
1	XTERRA- C8	Acetonitrile: 5mM Ammonium Acetate (80:20)	Poor peak shape, More tailing, Poor Baseline Stability Very less response
2	XTERRA- C8	Acetonitrile: 10mM Ammonium Acetate (90:10)	Poor peak shape Less tailing Relatively better response
3	HYPERSIL GOLD – C18	Acetonitrile: 5mM Ammonium Acetate (80:20)	Good peak shape, Very less tailing, Better response
4	HYPERSIL GOLD – C18	Acetonitrile: 10mM Ammonium Acetate (90:10)	Good peak shape, least tailing, good baseline Stability, Good response.

Retention Time, Run time of the drug were determined.

3.3.2.2. Flow rate and Temperature:

- Less Flow rate improves the elution efficiency. So usually Flow rate must be between 0.5 and 1.0 mL/min.
- Various temperatures for column oven and auto sampler were set to obtain a proper good chromatograph shape and height.

3.3.2.3. OPTIMIZED CONDITIONS:

Trail 4: Hypersil Gold - C18, (5µm.4.6x50mm) with Acetonitrile: 10mM Ammonium Acetate (90:10) as mobile phase

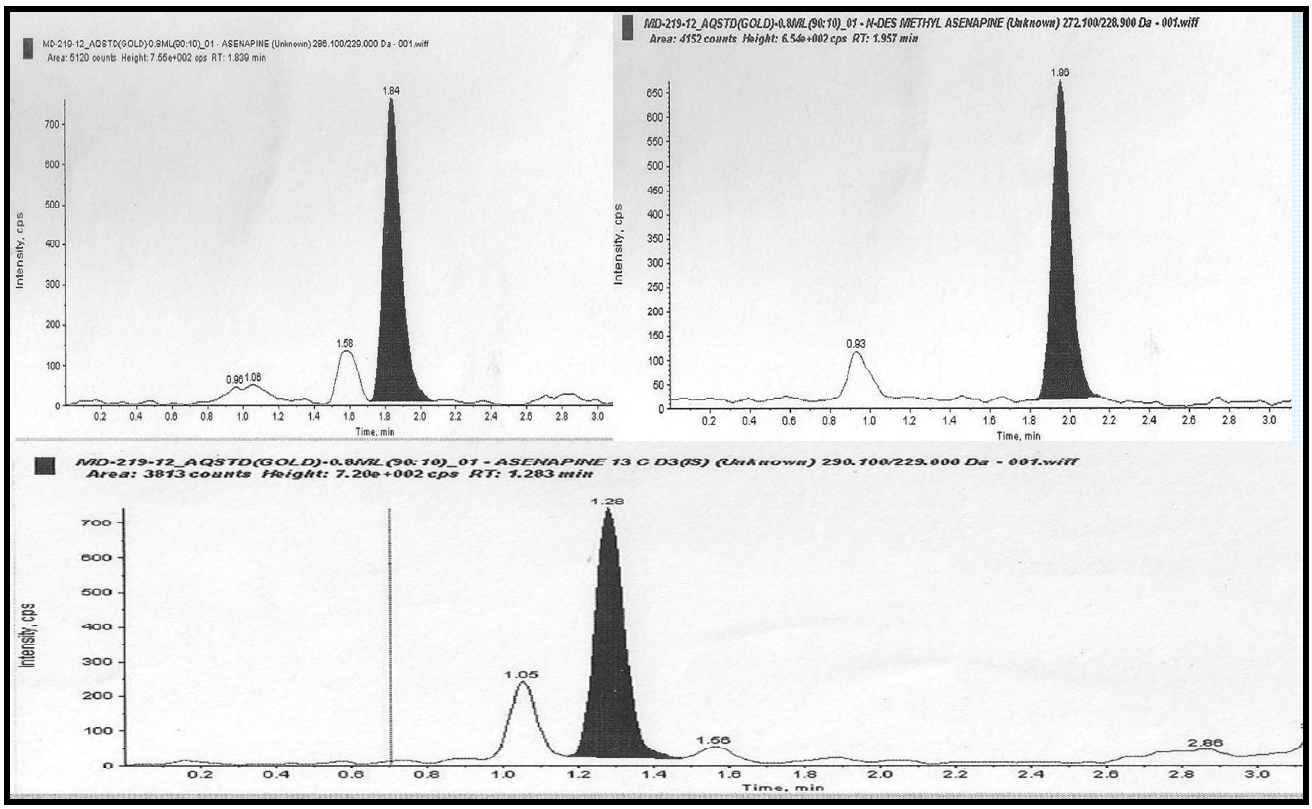


Fig – 9: Analyte, Metabolite and IS chromatograms showing maximum response for optimized column and mobile phase

Table - 5: Optimized Chromatographic Conditions

PARAMETERS	OPTIMIZED CONDITIONS
Column	Hypersil Gold (50mmx4.6mm, 5µm)
Mobile Phase	Acetonitrile : 10mM Ammonium Acetate (90:10,v/v)
Injection Volume	10µL
Flow Rate	0.600 mL/min
Column oven Temperature	40°C
Auto sampler Temperature	10°C
Total Run Time	3.5 mins
Retention Time	Analyte : 2.20±0.3 min Metabolite : 2.10±0.3 min IS : 2.20±0.3 min

3.3.3. AQUEOUS LINEARITY

3.3.3.1. Serial Dilution for CC and Aqueous QC:

Standards A to H have to be prepared based on the Cmax value:

[H] = Cmax x 2- Highest concn ;

[A] = Cmax/64- lowest concn

ULOQ = [H]

LLOQ ~ [A] and LOQQC

CC

LLOQ	=	atleast 100- 105% of Cmax
1st STD after LLOQ	=	2xLLOQ
STD before ULOQ	=	70-85% ULOQ
ULOQ	=	atleast 2x Cmax

QC

LOQQC	=	LLOQ/STD A(100 and 105%)
INTQC	=	5-30% of [H]
MQC	=	30-45% of [H]
HQC	=	70-85% of [H]

Aqueous samples were prepared from the serial dilution on recovery basis.2% drug content was fixed for all standards and the amount of each dilution to be added was determined so as to obtain this content. Diluent used was 90:10 methanol: water. Aqueous samples were prepared from each of the serial dilutions to obtain calibration curve standards (A-H) and quality control standards (LOQQC-HQC).

3.3.4. EXTRACTION PROCEDURE OPTIMIZATION:

The drug has to be extracted from the biological matrix (plasma) before injecting into the LC-MS/MS. Extraction procedure refers to the method used to separate the drug from the plasma to obtain at least a 50% recovery. To optimize the extraction procedure, the drug solution is spiked in matrix and extracted with the extraction procedure. The method giving the maximum

recovery of drug from the biological matrix is chosen. This is called extraction procedure optimization.

Three methods of extraction procedures in the order of increasing cost for performing is as follows:

- Protein precipitation,
- Liquid-Liquid Extraction
- Solid Phase Extraction.

3.3.4.1. Extraction-1 by Precipitation:

The spiked plasma samples from the deep freezer were allowed to thaw at room temperature. 0.5ml was aliquoted into a clean RIA vial and 50 µl of Internal Standard (10µg/ml) was added. Vortexed and mixed well. 1.5ml of **Acetonitrile** was added and vibramaxed for 10 minutes. The sample was centrifuged at 4500rpm for 10 minutes at 4ºC. 1ml supernatant was collected and evaporated till dryness. The residue was reconstituted with 0.5ml of mobile phase and injected 10 µl into LC-MS/MS.

3.3.4.2. Extraction- 2 by Liquid-Liquid Extraction:

Trail 1 - Ethyl Acetate: n- Hexane as Extraction Solvent:

Spiked plasma Samples were vortexed. 500µL plasma, 50 µL IS and 300µL of Buffer -2(A were added in RIA vials and vortexed. 2.5mL of **Ethyl Acetate** was added as extraction solvent and vibramaxed at 2000 rpm for 10minutes. This was then centrifuged at 4000rpm for 5minutes. 2mL of supernatant was transferred into new vials and dried in nitrogen evaporator at 40° C and 15psi. After drying it was reconstituted with 250µL of mobile phase Vortexed and loaded into LC-MS/MS by transferring into injector vials.

Fig - 10: LV Evaporator for drying samples

Trial 2 – Tertiary Butyl Methyl Ether : n-Hexane as extraction solvent:

Spiked plasma Samples were vortexed.500µL plasma, 50 µL IS and 300µL of Buffer -2 were added in RIA vials and vortexed. 2.5mL of **TBME** was added as extraction solvent and vibramaxed at 2000 rpm for 10minutes. This was then centrifuged at 4000rpm for 5minutes. 2mL of supernatant was transferred into new vials and dried in nitrogen evaporator at 40° C and 15psi. After drying it was reconstituted with 250µL of mobile phase Vortexed and loaded into LC-MS/MS by transferring into injector vials.

3.3.4.3. Extraction- 3 by Solid Phase Extraction:

Spiked plasma Samples were vortexed.500µL plasma, 50 µL IS were added in RIA vials and vortexed. **Strata X- C cartridge (60mg/3cc)** was conditioned using 2ml methanol and 2ml water. Sample was loaded and passed through the cartridge. It was then washed with 2ml water and 2ml 10% methanol.

Fig-11: 48 position positive pressure equipment for Solid Phase Extraction.

Based on the pKa value of asenapine, Elution was done with acid and base. The pKa value of Asenapine is 8.6, so it was eluted with Acetic acid in new vials.The eluted sample was loaded on to injector vials and injected into the LC-MS/MS to obtain the chromatogram.

Table – 6: Extraction Trails with Different mobile phase and column

TRAIL	EXTRACTING METHOD		RESPONSE (Area)			CONCLUSION
			IS	ASEN	DES ASE	
1	Precipitation	Acetonitrile	23765	765	924	Poor Peak Shape, recovery is very less. Matrix Effect is more
2	Liquid-Liquid Extraction	Ethyl Acetate : n-Hexane	57940	1722	2233	peak shape and peak response or recovery of IS was satisfactory. Metabolite and analyte recovery was relatively less
		Tertiary Butyl Methyl Ether: n-Hexane	64456	2212	2058	peak shape and peak response or recovery of IS, analyte and metabolite were good and satisfactory
3	Solid Phase Extraction	Strata X-C Cartridge	59783	1993	2117	Peak area response of was less than LLE and greater than precipitation method

3.3.4.4. OPTIMIZED CONDITIONS:

Trial 2 – Tertiary Butyl Methyl Ether: n-Hexane as extraction solvent:

- Spiked plasma Samples were vortexed.
- 500µL plasma, 50 µL IS and 300µL of Buffer -2 were added in RIA vials and vortexed.
- 2.5mL of **TBME** was added as extraction solvent and vibramaxed at 2000 rpm for 10minutes.
- This was then centrifuged at 4000rpm for 5minutes.
- 2mL of supernatant was transferred into new vials and dried in nitrogen evaporator at 40° C and 15psi.
- After dried it was reconstituted with 250µL of mobile phase Vortexed and loaded into LC-MS/MS by transferring into injector vials.

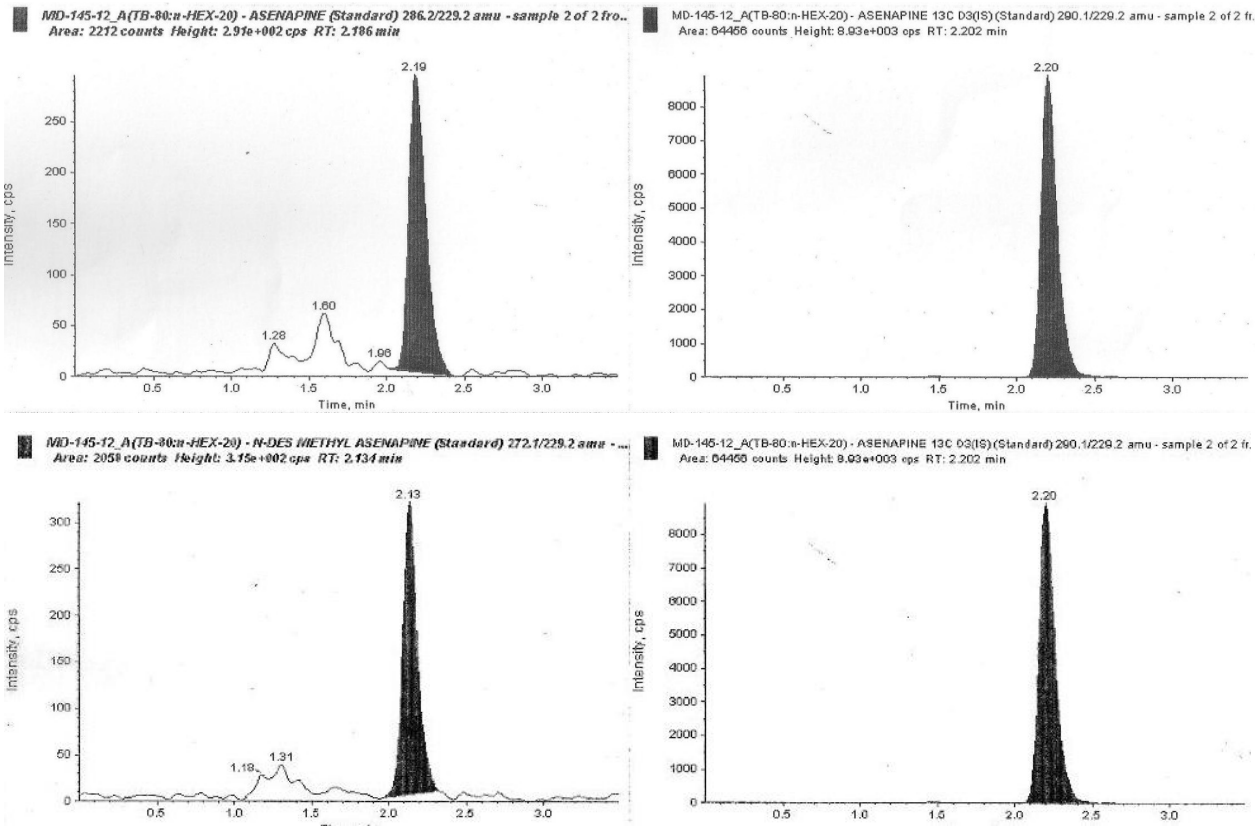


Fig - 12: Optimized Extraction Conditions - LLE

3.4. PRE-METHOD VALIDATION:

After developing a method for the drug, the following pre- method validation tests were carried out :

- Aqueous Linearity test
- Specificity/Selectivity
- MD Precision & Accuracy batch
- Recovery
- Auto sampler Carryover Test (ASCOT)

3.4.1. AQUEOUS LINEARITY TEST:

This is used to determine the linearity of different concentrations of aqueous samples.

Procedure: Different serial dilution was repeated and fresh aqueous standards (for CCs) were prepared. An appropriate regression model with minimal or no weighing (1/x or $1/x^2$) was used. Weighing factor is 1/x =1/ (concentration) and $1/x^2=1/(conc)^2$. The regression model / weighing factor were selected based on the least deviation of the linearity curve. The standards were run in the LC-MS/MS and linearity was evaluated.

Acceptance Criteria:

1. The standard curve should consist of a minimum of six standard points excluding blanks.
2. The standard curve should cover the entire range of expected concentrations.
3. LLOQ and ULOQ values should not be excluded.
4. Two consecutive standards should not be excluded from the calibration curve.

5. All the CCs except the lowest should be ±15 % of the nominal value. The lowest values may be ±20% of nominal value.
6. The correlation coefficient r^2 should be ≥ 0.98
7. At least 75% of non- zero standards should meet the acceptance criteria.

3.4.2. SELECTIVITY/SPECIFICITY:

This is to check the presence or absence of interference in the analyte or metabolite or IS peak because of other molecules in the plasma and to ensure that the method is selective towards the particular analyte.

Procedure: Two sets of six normal lots of plasma, and one haemolysed were taken. One set of blank normal and blank haemolysed lots with buffer were processed. The aqueous LLOQ dilution were prepared and were spiked in another set of six normal lots of plasma and one haemolysed lot to achieve LLOQ concentration for analyte. After extraction both were reconstituted with mobile phase. Both the sets of samples were injected into the LC-MS/MS and the peak areas of the blank samples and respective LLOQ samples were compared to check the interference due to the plasma. Selectivity samples were prepared in the presence of both analyte and internal standard using six normal blank plasma and one haemolysed.

Acceptance criteria:

1. Peak area obtained in blank samples at retention time of the drug should be ≤ 20% of that obtained in LLOQ samples.
2. Peak area obtained in blank samples at retention time of the IS should be ≤5% of that obtained in LLOQ samples.
3. %CV of the area ratio in the extracted LLOQ samples should be ≤ 20.
4. S/N ratio of each LLOQ sample should be ≥5.

3.4.3. MATRIX EFFECT:

This is to determine whether the biological matrix has any effect on the analyte that will interfere with the response peak.

Procedure: An aqueous standard of lower and higher concentration was taken and injected 6times at the same vial position. Two sets of six blank normal lots of plasma, and one blank haemolysed were taken. To Both the sets, buffer was added and processed through optimized extraction procedure. After drying, one set was reconstituted with AQ LQC and another set with AQHQC. These were run in the instrument against AQ LQC + IS and AQHQC + IS (Only Analyte with IS) to compare response. The peak areas were compared to study the effect of the biological matrix on the drug molecule.

Formulae:

Matrix factor:

$$M.F.= \frac{Peak\ area\ ratio\ of\ drug\ \&\ IS\ of\ post\ extracted\ spiked\ sample}{Mean\ peak\ area\ ratio\ \ 獦f\ drug\ \&\ IS\ of\ aqueous\ sample}$$

Matrix effect:

$$M.E.= \frac{Mean\ area\ ratio\ of\ drug\ \&\ IS\ of\ post-extracted\ spikede\ sample}{Mean\ peak\ area\ ratio\ of\ drug\ \&\ IS\ of\ the\ aqueous\ sample} \times 100$$

Acceptance Criteria:

1. Matrix factor should be within 0.85 to 1.15.
2. %CV of matrix factor should be ≤15%.
3. Matrix effect should be within ±15%.

3.4.4. AUTOSAMPLER CARRYOVER TEST:

This test is used to check if the response of a high concentration sample is carried to the next injection by injecting a blank sample after a high concentration sample.

Procedure: blank (i), LLOQ (ii) and ULOQ(iii) concentrations were injected consecutively and the same blank was again re-injected (iv) after ULOQ. The peak area ratio of blank was compared between the both injections.

Formulae:

For analyte:

$$Carry\ over = \frac{(area\ of analyte\ in\ blank\ B2\ \ - area\ of\ analyte\ in\ blank\ B1)}{Area\ of\ analy 檐e\ in\ LLOQ} \times 100$$

For IS:

$$Carryover = \frac{(area\ of\ IS\ in\ blank\ B2 - area\ of\ IS\ in\ blank\ B1)}{Area\ of\ IS\ in\ ULOQ} \times 100$$

Acceptance Criteria:

1. For analyte, % carry over should be ≤ 20% of LLOQ area.
2. For IS it should be ≤ 5% of IS area of ULOQ.

3.4.5. PRECISION AND ACCURACY BATCHES:

This test is to ensure the correctness of the value as well as the reproducibility of the value in subsequent injections of different samples of same concentration.

Procedure: One set of CCs and six replicates of each of the QCs were spiked in plasma and processed according to the extraction procedure. The peak area of

each sample was compared with its replicates and the % deviation of each set was checked.

Formulae:

The precision (%CVs) is obtained by dividing the standard deviation with the mean concentration and multiplying by 100.

$$\%CV = \frac{\sigma}{\mu} \times 100$$

Accuracy is obtained by dividing the calculated concentration of a QC with its nominal concentration and multiplying by 100.

$$\%CV = \frac{Calculated\ concentration}{nominal\ concentration} \times 100$$

Acceptance Criteria:

Precision:

1. The precision should be ≤ 15% for all and ≤20% for LQC.

Accuracy:

1. %CV should be 80-120% for LLOQ and 85-115% for others.

3.4.6. RECOVERY:

To determine the amount of drug that can be extracted from the plasma, using the optimized extraction procedure. The area obtained in the extracted samples is compared with that of the respective aqueous samples.

Procedure: One set extracted samples were run in P&A batch, from which the mean value of area of all the LQCs, MQCs, and HQCs were individually divided by the mean area of respective aqueous samples with analyte prepared

by serial dilution that were run against extracted samples. The divided value gives the recovery.

Formulae:

%recovery can be calculated as follows

$$\%R = \frac{Average\ area\ response\ of\ extracted\ samples}{Average\ area\ response\ of\ aqueous\ samples} \times 100$$

Acceptance Criteria:

1. According to regulatory guidelines, the maximum recovery allowed is 115%. Although there is no minimum recovery limit, for practical purposes, the lower limit was fixed at 60%.
2. %CV should be ≤ 15%.

3.5. METHOD VALIDATION:

Method validation refers to establishing through documented evidence, a high degree of assurance that an analytical method will consistently yield results that accurately reflect quality characteristics of the product testing. It involves the following experiments:

- System Suitability test
- Specificity/Selectivity
- Matrix Effect
- Auto sampler Carryover Test (ASCOT)
- P & A batch
  - Intra-day precision & accuracy
  - Inter-day precision & accuracy
  - Ruggedness
- Recovery
- Reinjection Reproducibility
- Stability tests

3.5.1. SYSTEM SUITABILITY TEST:

It is timely determination of instrument performance by analysis of a standard prior to running an analytical batch. This is to ensure that the complete testing system is suitable for the intended application. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done.

Procedure: Aqueous standard equivalent to middle level of CC standard concentration with internal standard was prepared. Six replicate from the same vial was injected into the chromatographic device. Mean, Standard Deviation

and percentage coefficient of variation for the retention time and area/area ratio were calculated.

Acceptance Criteria:

- The %CV of peak area ratio and retention time should not be more than 5 and 15, respectively during system suitability.

3.5.2. SELECTIVITY/SPECIFICITY:

Same procedure and acceptance criteria as mentioned above in PRE METHOD VALIDATION – 3.4.2.

3.5.3. MATRIX EFFECT:

Same procedure, formulae and acceptance criteria as mentioned above in PRE METHOD VALIDATION- 3.4.3.

3.5.4. AUTOSAMPLER CARRYOVER TEST:

Same procedure, formulae and acceptance criteria as mentioned above in PRE METHOD VALIDATION – 3.4.4.

3.5.5. PRECISION AND ACCURACY BATCHES:

Validation is carried out using a minimum of three acceptable batches. The precision was determined by calculating percentage %CV at each concentration level of QC sample and the accuracy was determined by calculating the percentage of nominal value at each concentration level of QC samples.

Procedure: The procedure is same as mentioned above in PRE-METHOD VALIDATION – 3.4.5.

Formulae:

The **precision** (%CV's) is obtained by:

$$\%CV = \frac{\sigma}{\mu} \times 100$$

Accuracy (%nominal) is obtained by

$$\%CV = \frac{Mean\ concentration}{nominal\ concentration} \times 100$$

Intra batch:

To obtain **within-batch data precision**, the mean, standard deviation and %CV of each concentration in the same P&A was calculated.

Inter batch:

To obtain **between-batch data precision**, the global mean, standard deviation and %CV from all acceptable batches for each QC concentration were calculated.

To obtain **within-batch data accuracy**, the mean of respective QC was calculated by dividing with its nominal concentration and multiplied by 100.

To obtain **between-batch data accuracy**, the global mean of respective QC was calculated and divided with its nominal concentration and multiplied by 100.

Ruggedness:

One P&A batch was performed by employing the same instrument with different analyst and alternatively performed on different instrument of same make.

Acceptance criteria:

- **Precision**

  The within-batch and between-batch precision for the QC's (LQC, INTQC, and MQC & HQC) and LOQQC should be ≤15% and ≤20% using a minimum of three P&A batches.

- **Accuracy**

  The mean of within and between-batch accuracy should be 85-115% of the nominal value for QCs (80–120% of the nominal value for LOQQC). 67% of total QCs per level must be 85–115% of the nominal value except for LOQQC, where it should be 80-120% of the nominal value.

3.5.6. RE-INJECTION REPRODUCIBILITY:

Re-injection reproducibility is a test to ensure consistency in the values obtained during repeated injection of same samples.

Procedure: After analysis of any acceptable P&A batch, the CC and QC samples were re-injected at least 3hours from the completion of the corresponding P&A batch. The back-calculated concentrations of the high and low re-injected QC samples using calibration curve standards of the originally run P&A batch were calculated.

Acceptance Criteria:

1. The mean of back calculated values should be between 85 to 115% of the nominal concentration and the %CV should be ≤ 15%. 67% of total QCs per level must be 100±15% nominal value.

2. Ratio of means should be within 1.0±0.15 while comparing the mean of back calculated values against the mean of values obtained from an original injection.

3.5.7. RECOVERY:

Same procedure, formulae and acceptance criteria as mentioned above in PRE METHOD VALIDATION – 3.4.6.

3.5.8. STABILITY:

Evaluation of stability should be carried out to ensure that every step taken during sample preparation and sample analysis, as well as the storage conditions used do not affect the concentration of the analyte. The following are the common stability tests conducted in method validation:

1. Stock Solution stability
    - Short Term stock solution stability
    - Long Term stock solution stability
2. Stability in biological matrix
    - Bench Top stability
    - Freeze Thaw stability
    - Long Term stability
    - Blood stability
3. In process/Post processing stability
    - Autosampler/Wet Extract stability
    - Dry Extract stability

3.5.8.1. Stock solution stability

The stability test for the standard stock solution must be done at the same temperature, container and solvent as that to be used for the study. The time period should be at least 6 hours

3.5.8.2. Short term matrix stability

This must be evaluated following the storage under laboratory conditions used for sample work-up for a period of e.g. 6 h to 24 h, and compared with data from the same samples prepared and analyzed without delay.

3.5.8.3. Long term stability

This is done to assess whether the analyte is stable in the plasma matrix under the sample storage conditions for the time period required for the samples generated in a clinical study to be analyzed.

3.5.8.4. Freeze -thaw stability

This stability test is done to ensure that the sample remains stable after it is subjected to multiple freeze-thaw cycles in the process of the study.

3.5.8.5. Bench Top Stability:

This is evaluated to confirm that the analyte should not degrade its processing (preparation, extraction) period to the analysis.

Procedure for analyte: Replicate stability samples and 6 sets of LQC and HQC samples from intended storage temperature at each concentration were allowed to remain on the bench-top for the period of 4-24 hours for which stability is to be assessed. It is left at room temperature/ ice cold water bath for a minimum of 6 hours and processed. These samples were injected into the instrument. The accuracy and precision of QC samples against freshly spiked CC standards were calculated and compared with freshly spiked comparison QC samples.

Internal Standard: Six replicate of screened pooled plasma were aliquoted and IS was added. The samples were kept on bench for intended duration. Six replicate of the screened pooled plasma were freshly aliquoted, internal standard was added and were mixed by vortexing. Both IS stability and comparison samples were processed.

Calculation:

$$\%\ \text{nominal against CS} = \frac{Peak\ area\ of\ incubated\ sample}{Peak\ area\ of\ normal\ sample} \times 100$$

Acceptance Criteria:

The % stability should be between 85 to 115%.

4. RESULTS AND DISCUSSION:

4.1. CHROMATOGRAMS

Chromatograms are obtained as a result of the detection of the relevant molecule by the LC-MS/MS instrument. It shows a plot of the response of the instrument against the time. The response is proportional to the concentration of the sample used and the peak forms at the retention time which is characteristic of a molecule. In the given chromatograms, the retention time and peak area are given below the molecule name.

4.1.1. SYSTEM SUITABILITY:

Aqueous standard refers to a solution of the analyte in solvent and not plasma. These standards are used to set an expected concentration for the extracted samples. A middle concentration of the analyte was injected as aqueous standard in order to check whether the system is suitable for the selected analyte. Sharp and singular peaks were obtained at the expected retention times of each molecule, which confirmed the stability and suitability of the instrument to be used.

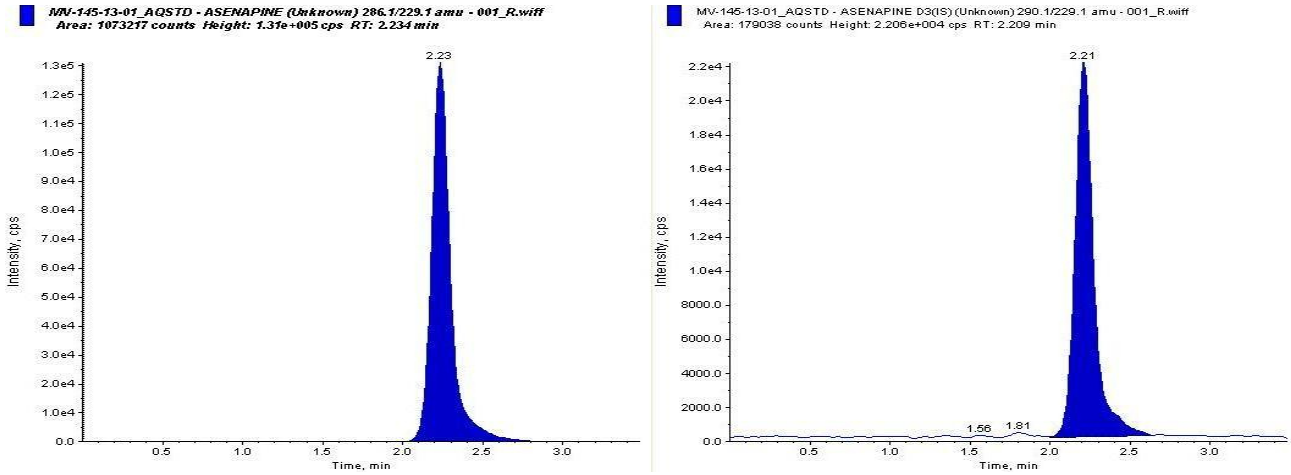


Fig - 13: Chromatogram of SS (ASEN & IS)

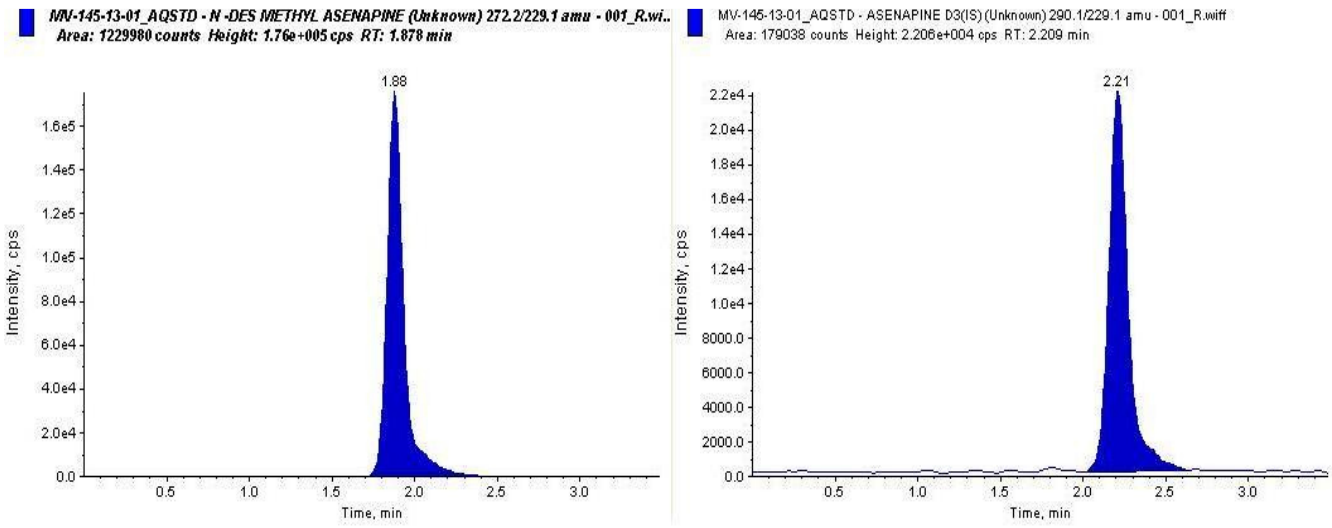


Fig - 14: Chromatogram of SS (DES ASE & IS)

4.1.2. LINEARITY

Aqueous standards with increasing concentrations were prepared in the range of 0.1080 – 35.314 ng/mL(ANALYTE) and 0.1060 – 34.47 ng/mL (METABOLITE) (A - H). These concentrations, called the calibration curve standards or CCs must give a linear curve when plotted for response against the concentration. Only if this linearity is passed, the method can be developed in that concentration range. Based on the criteria mentioned above, the aqueous standards were prepared and injected. The response obtained was plotted against the concentration of the analyte. The linearity passed for both Asenapine and Metabolite calibration curves by excluding two non-consecutive points out of the eight. The curve was fit to the equation:

$$\mathbf{y = mx + c,}$$

with a weighting factor of $1/x^2$. The slope and intercept of ASE and DES ASE were found to be 0.374, 0.0114 and 0.378, 0.00858 respectively. The correlation

coefficient r was 0.9992 and 0.9958 for Asenapine and N-Desmethyl Asenapine respectively. This curve serves as the standard graph to determine the expected concentration of extracted samples of the analyte.

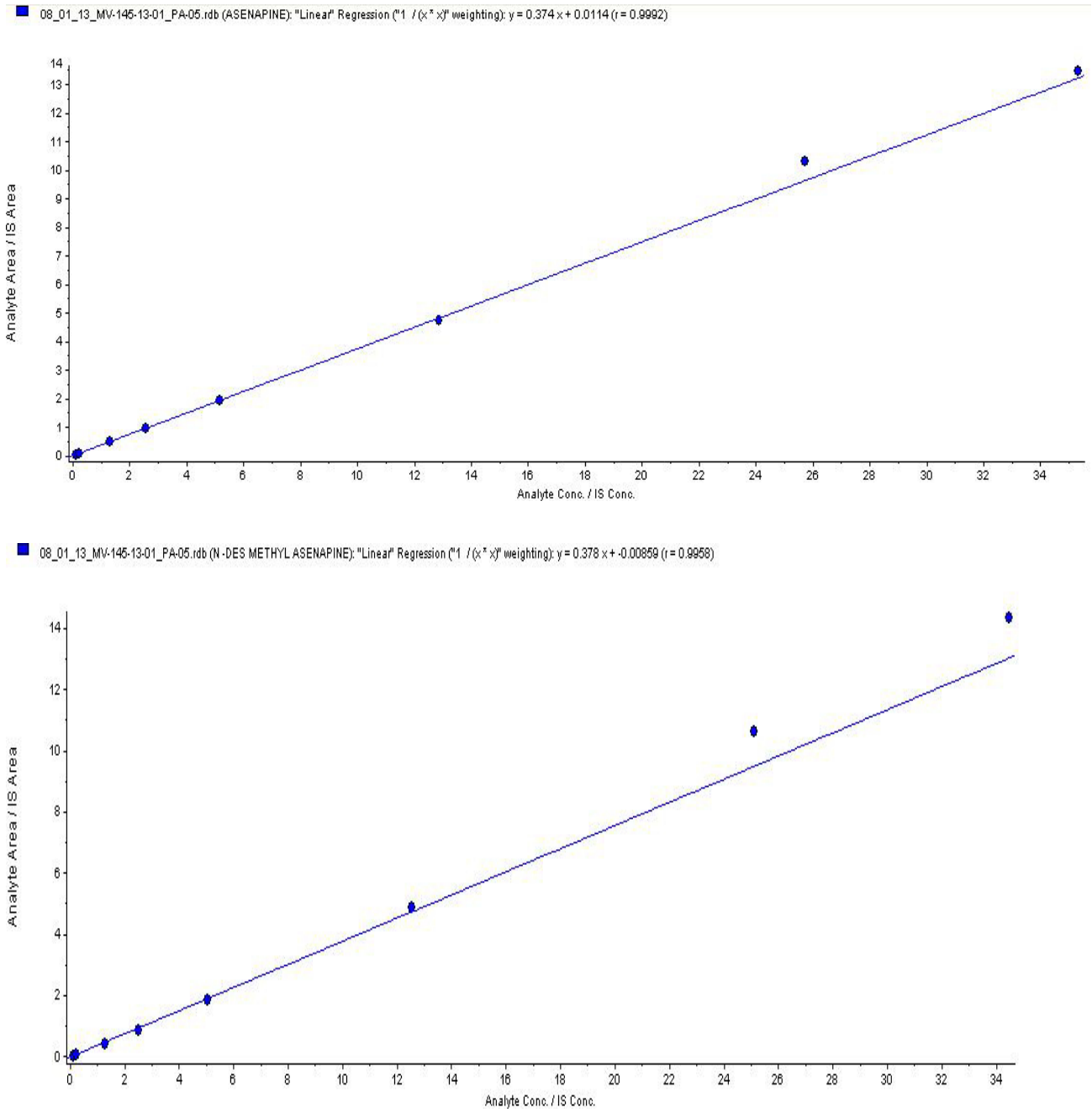


Fig- 15: Linearity curve for ASEN and DES ASE

4.1.3. STANDARD BLANK:

Blank refers to either the solvent/ mobile phase alone (aqueous blank) or plasma alone (extracted blank), without any analyte spiked in it. A blank sample has to be run in order to check the baseline stability and ensure that there is no contamination in the solvent or plasma. In order to expect minimum noise, the response should be less than e^4.The blank samples injected gave no peak, indicating the absence of any contamination. The baseline was found to be stable. The responses obtained were e^4, e^2, e^4 and e^3 for various blank injections.

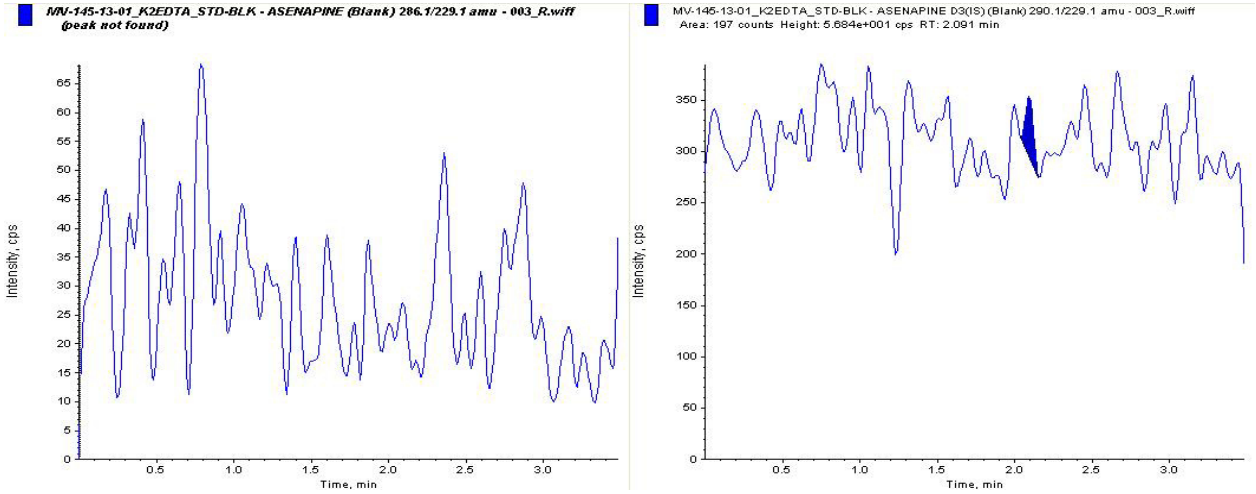


Fig- 16: Chromatogram for Blank (ASEN & IS)

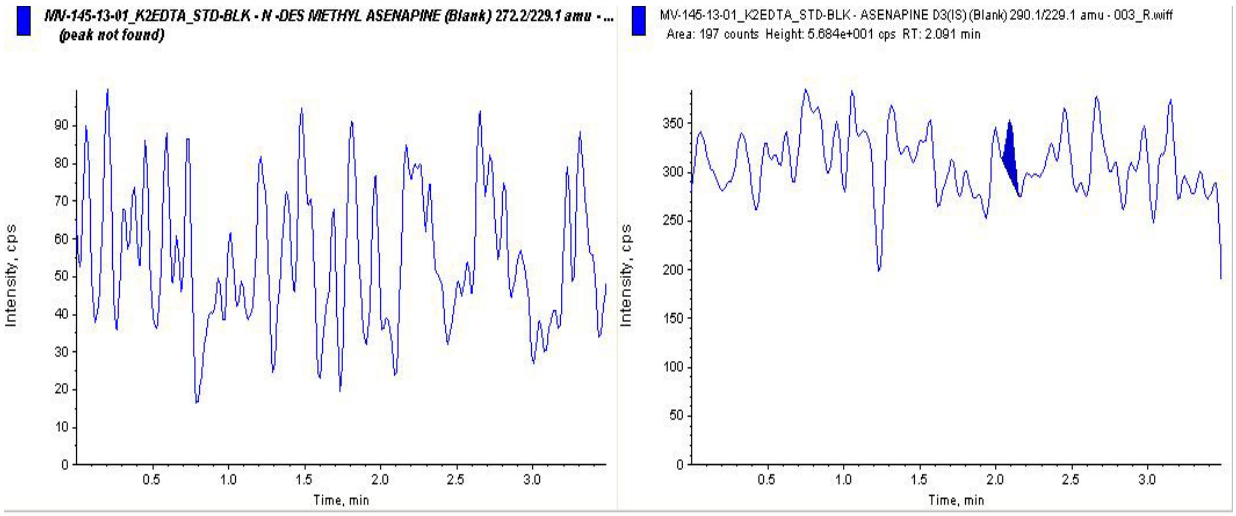


Fig- 17: Chromatogram for Blank (DES ASE & IS)

4.1.4. STANDARD ZERO:

Zero refers to the blank solution along with the internal standard alone. This is used to check for any IS contamination and also to determine the IS area obtained. The zero samples gave no peak for the analyte molecules at their retention times. It produced a sharp peak at the IS retention times 2.24 for IS.

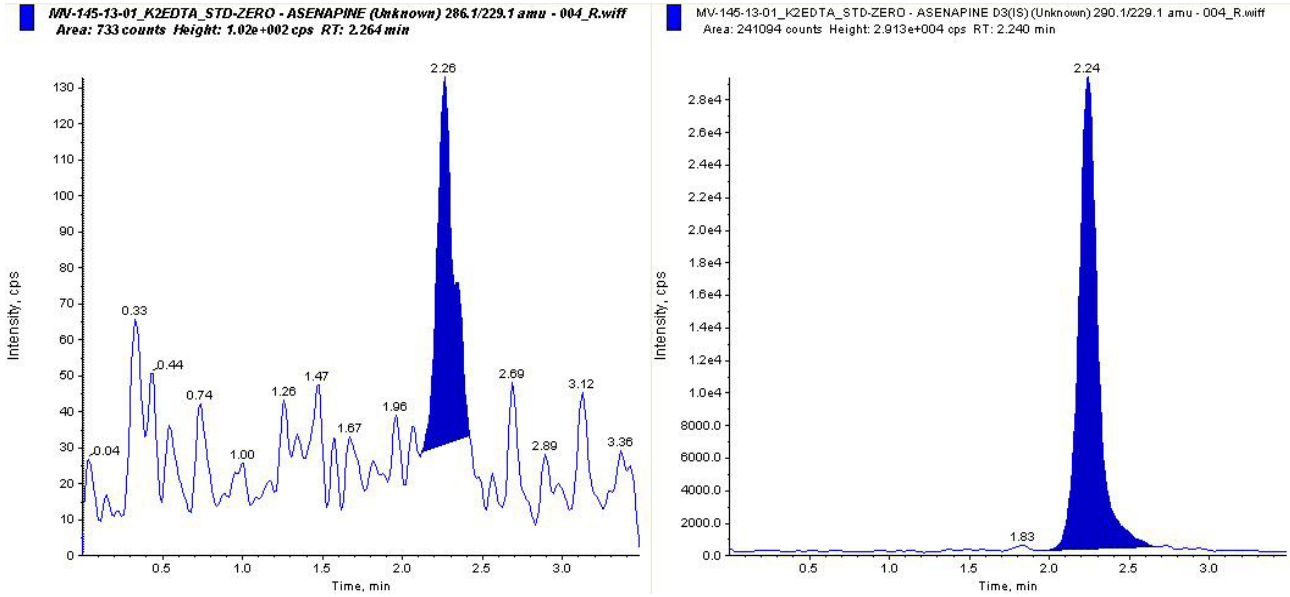


Fig- 18: Chromatogram for Zero (ASEN & IS)

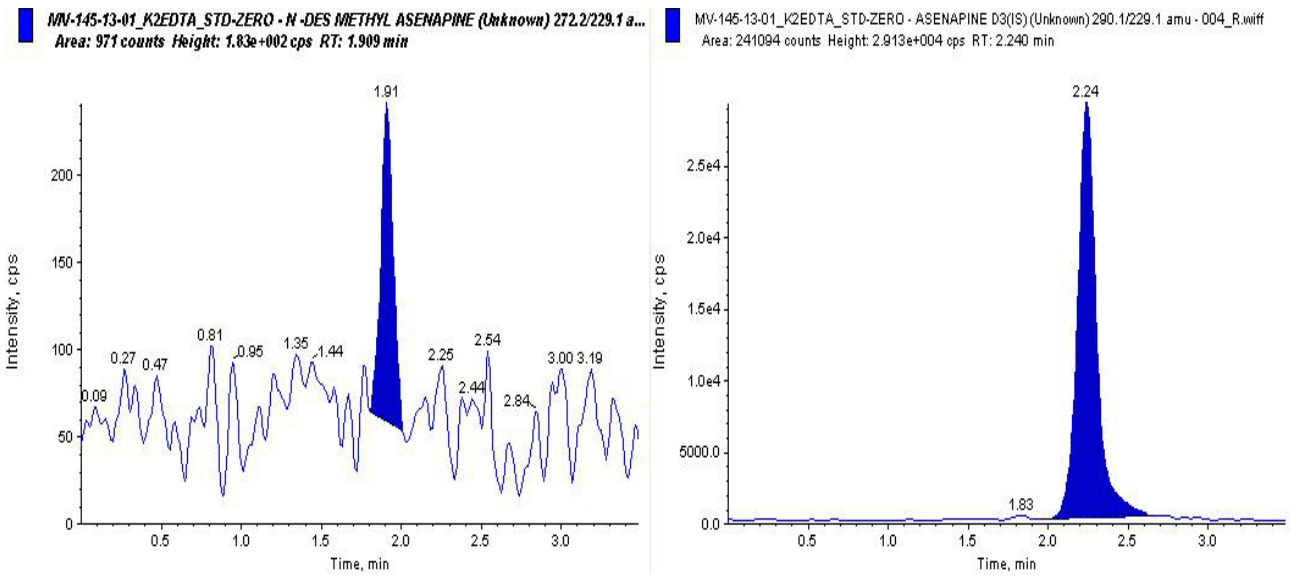


Fig- 19: Chromatogram for Blank (DES ASEN & IS)

4.1.5. QUALITY CONTROL CURVES:

Quality controls are standards of lower to higher concentrations within the selected range. The responses of these aqueous standards provide the expected concentration in extracted / plasma samples. The chromatograms are obtained in which the peak forms at the retention time of the molecule and the peak area was found to increase with the concentration. Five concentrations were used as the quality controls (LOQQC, LQC, INTQC, MQC, HQC).

4.1.5.1. LOWEST OF QUANTIFICATION QUALITY CONTROL(LOQQC)

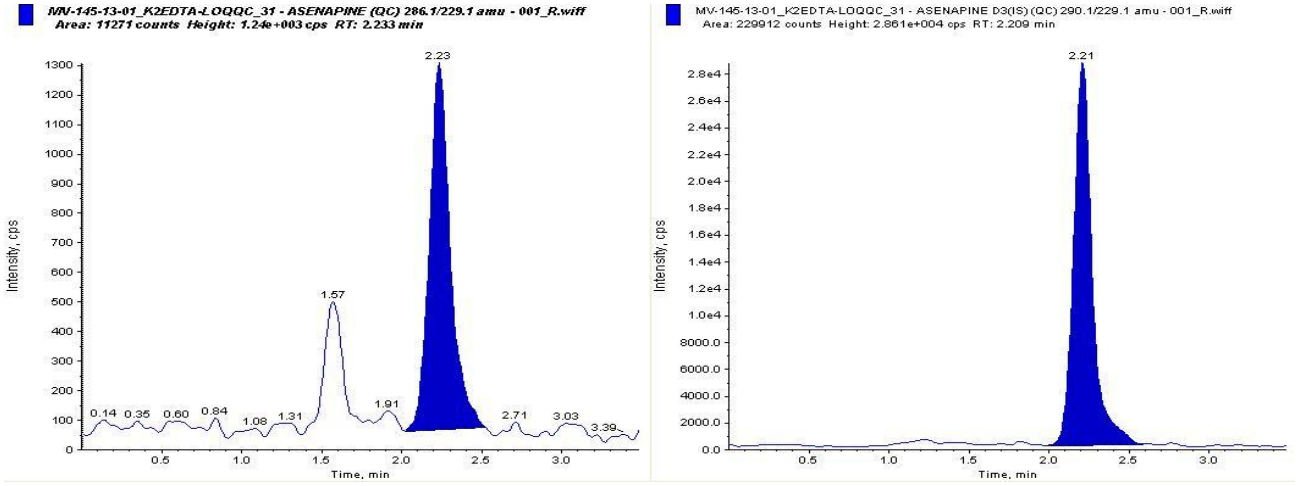


Fig- 20: Chromatogram for LOQQC (ASEN & IS)

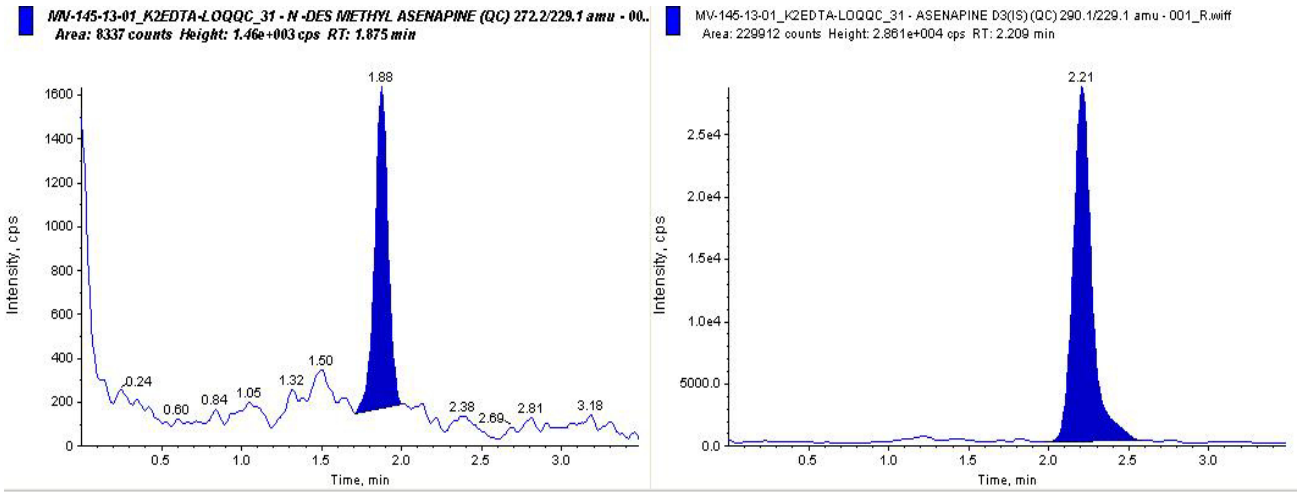


Fig- 21: Chromatogram for LOQQC (DES ASE & IS)

4.1.5.2. LOWER QUALITY CONTROL (LQC):

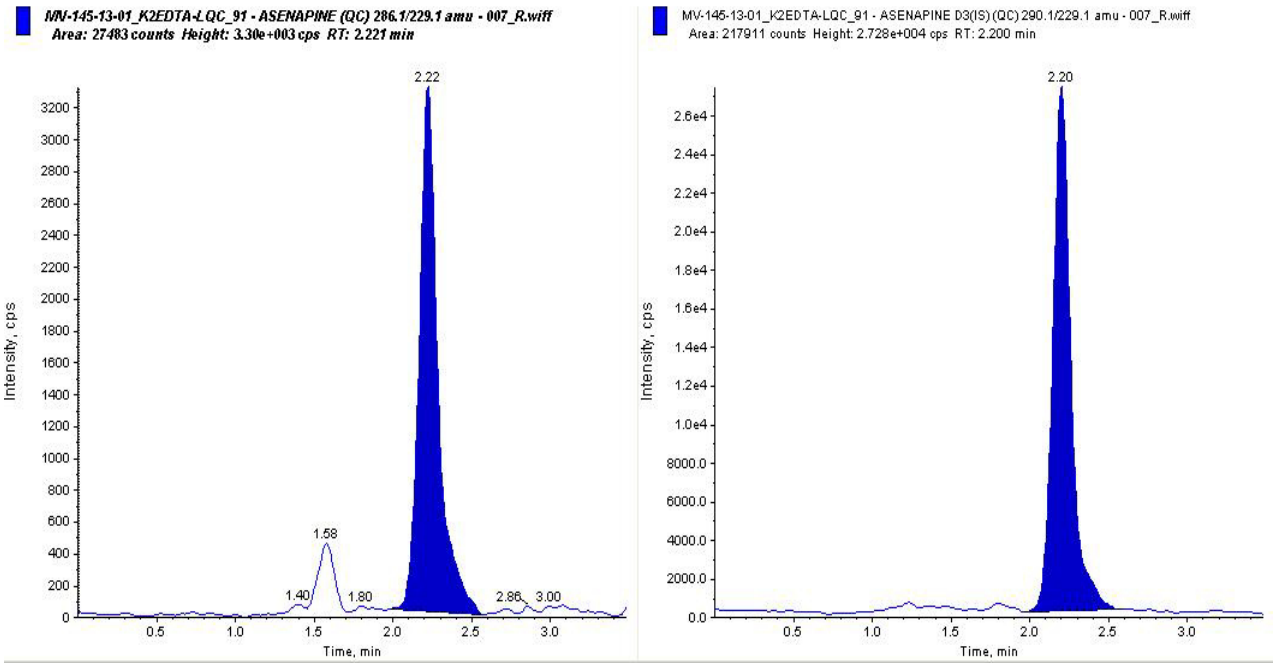


Fig- 22: Chromatogram for LQC (ASEN & IS)

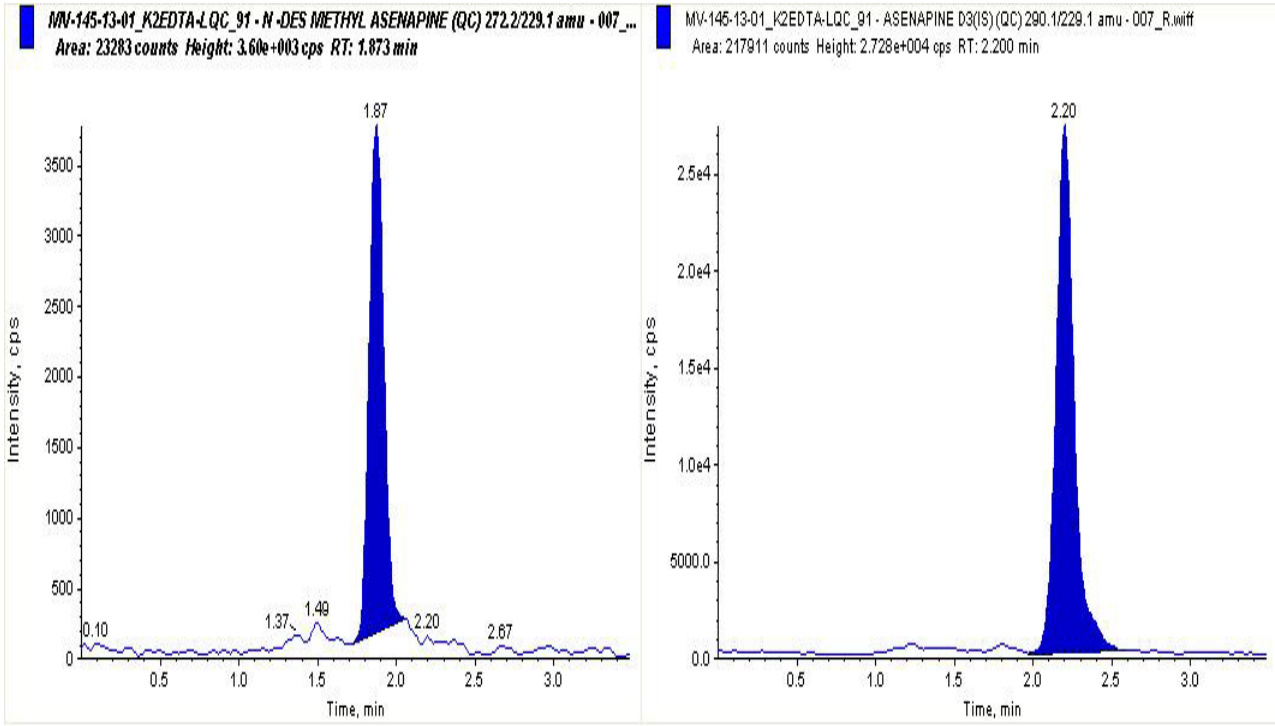


Fig- 23: Chromatogram for LQC (DES ASE & IS)

4.1.5.3. INTERMEDIATE QUALITY CONTROL (INTQC):

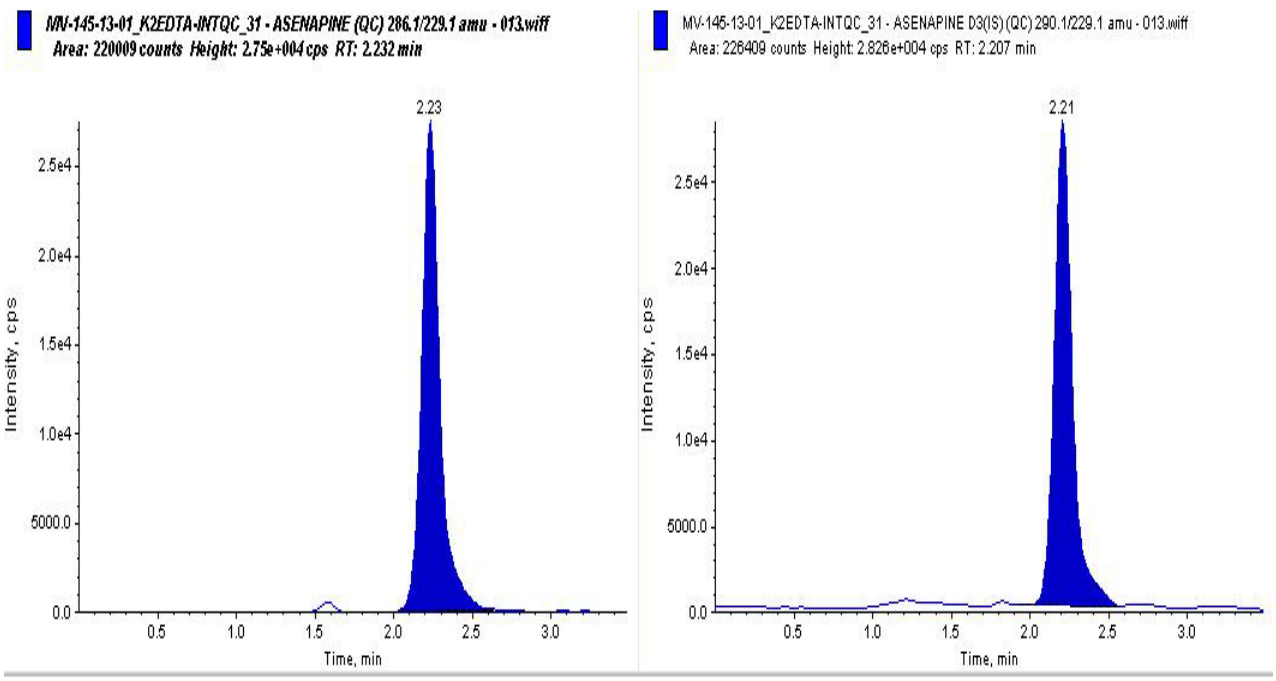


Fig- 24: Chromatogram for INTQC (ASEN & IS)

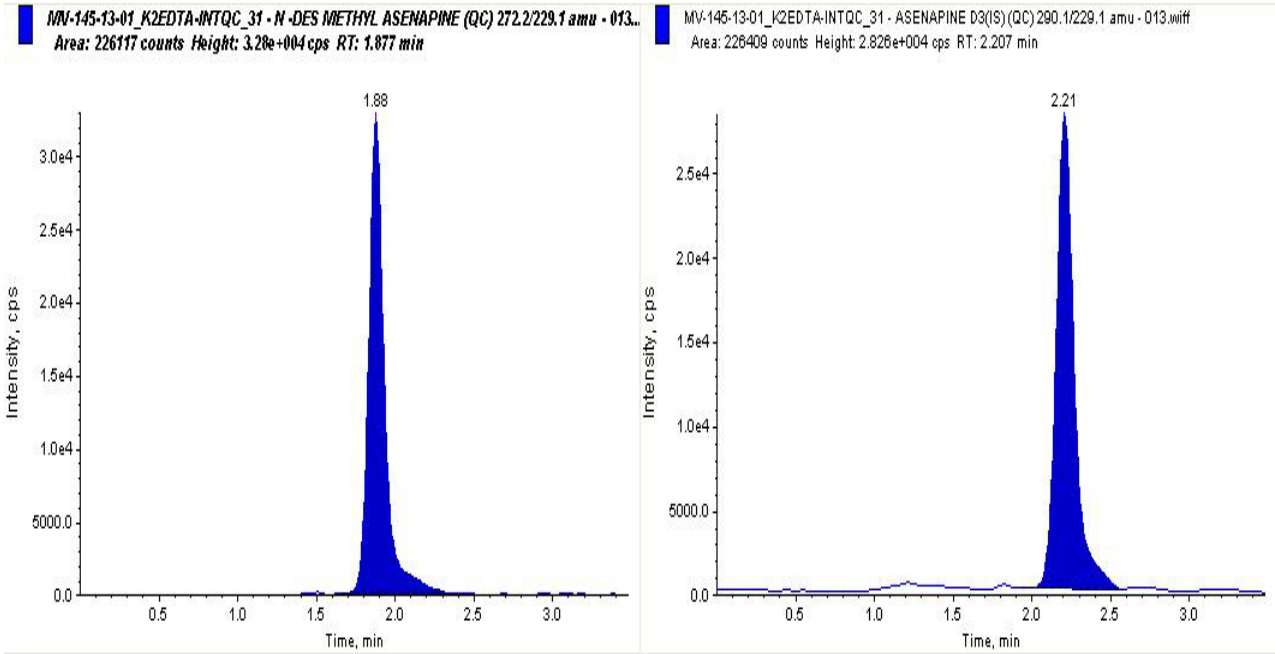


Fig- 25: Chromatogram for INTQC (DES ASE & IS)

4.1.5.4. MIDDLE CONCENTRATION:

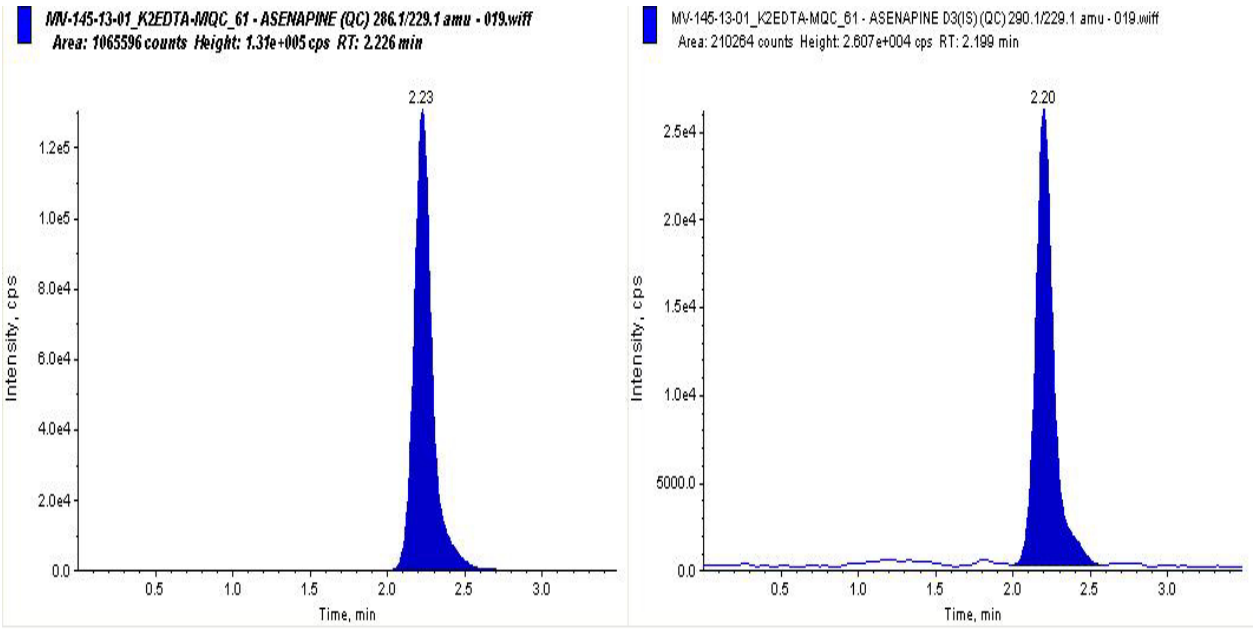


Fig- 26: Chromatogram for MQC (DES ASE & IS)

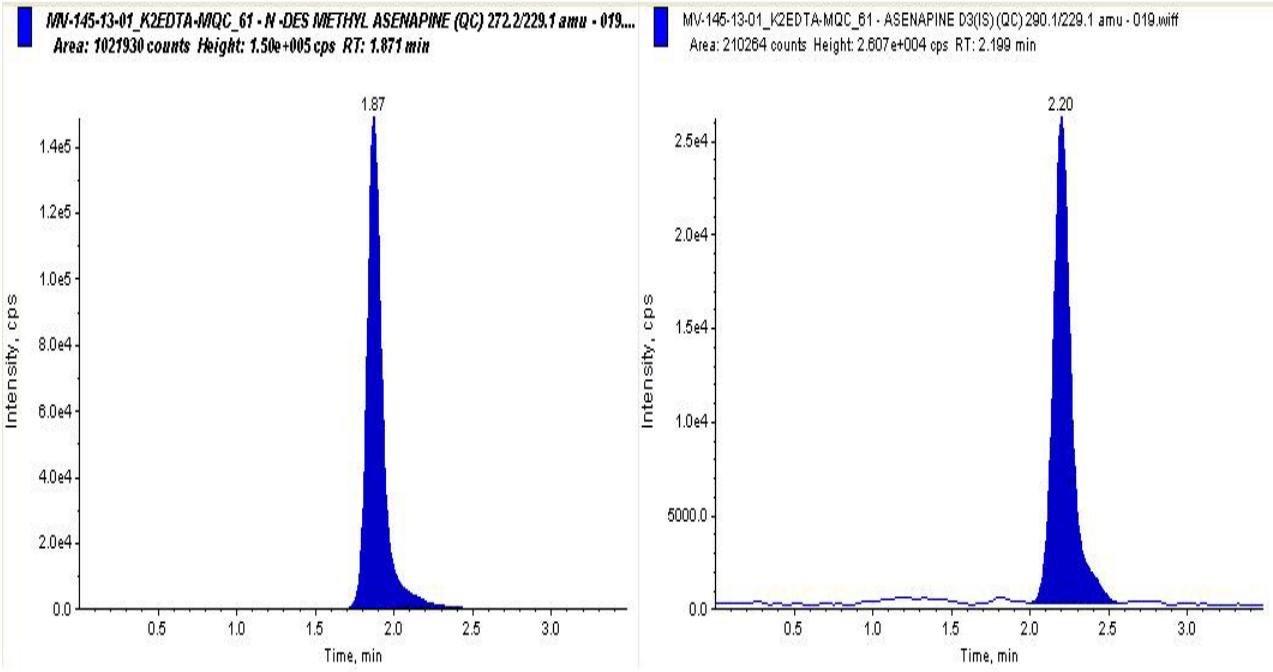


Fig- 27: Chromatogram for MQC (DES ASE & IS)

4.1.5.5. HIGHEST QUALITY CONTROL (HQC)

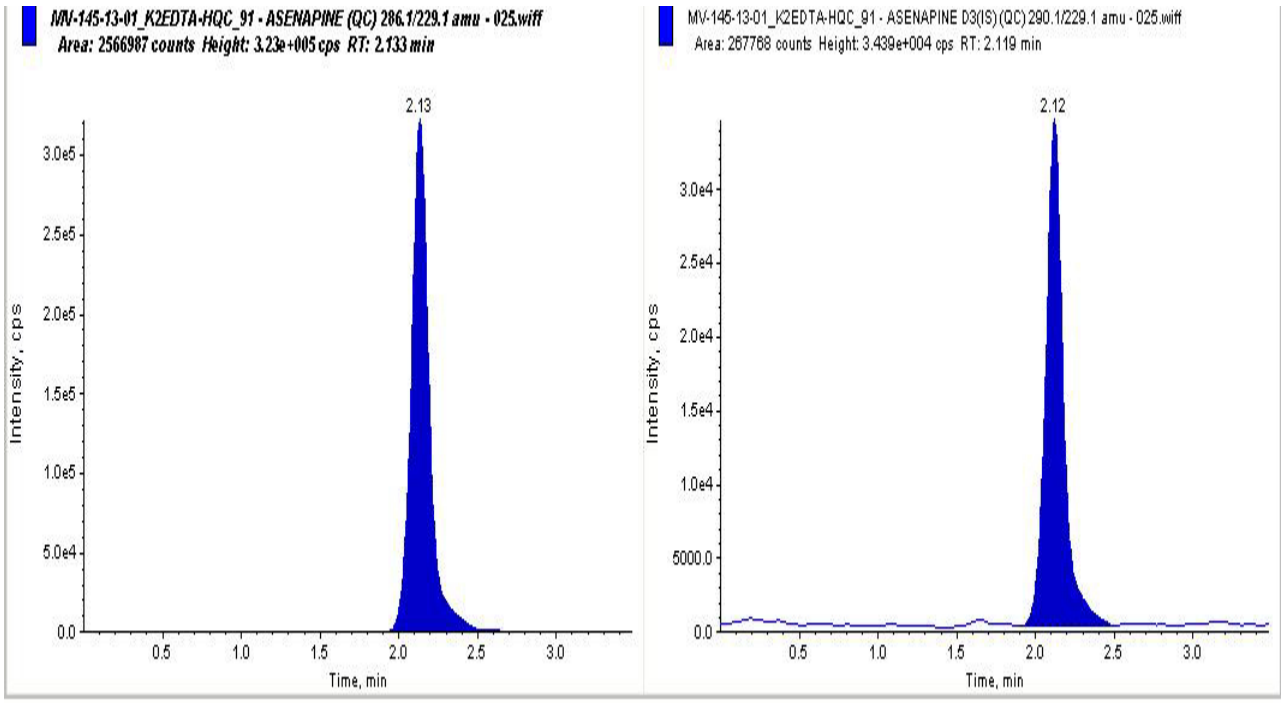


Fig- 28: Chromatogram for MQC (ASEN & IS)

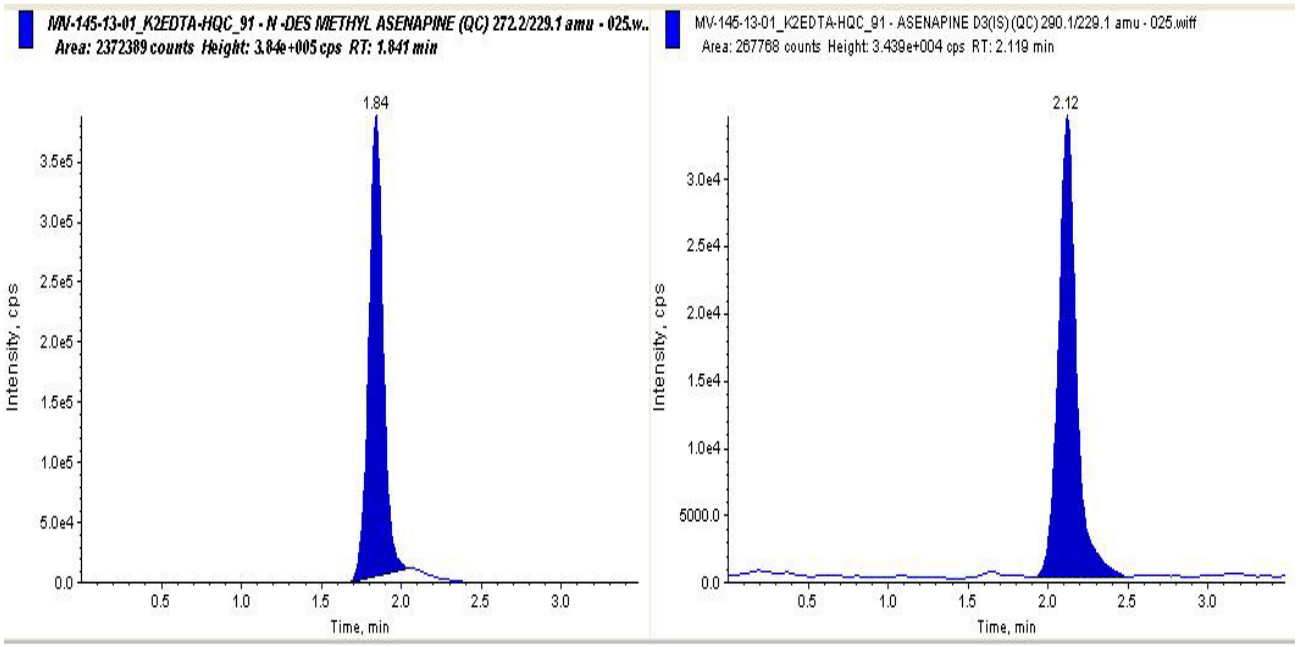


Fig- 29: Chromatogram for MQC (DES ASE & IS)

4.2. RESULT TABLES

4.2.2. SPECIFICITY/ SELECTIVITY TEST:

Specificity and selectivity test ensures that only the relevant molecule is detected and there is no interference from any other molecule in the plasma. In table (7) and (8) first column indicates plasma lot number used. The specificity is tested with the blank plasma, under which all lots show no peak area for analyte and metabolite as Asenapine is an exogeneous drug. Selectivity is given under the samples containing LLOQ concentration of the analyte, where the obtained peak areas are given for the analyte , metabolite and IS. % interference in blank is a comparison of the blank and LLOQ areas for the analyte/ metabolite and IS.

Table-7: Result of Specificity/ selectivity (ASEN)

Plasma lot ID	Specificity (blank)		Selectivity (Spiked LLOQ)		% Interference in Blank		Area Ratio	S/N Ratio (≥5)
	Analyte	IS peak	Analyte	IS peak	Analyte (≤20%)	IS (≤5%)	Analyte/ IS	Analyte
1	0	0	3167	110590	0.0000	0.0000	0.0286	217.764
2	0	0	3305	109492	0.0000	0.0000	0.0302	242.884
3	0	0	3390	109914	0.0000	0.0000	0.0308	289.048
4	0	0	3479	107712	0.0000	0.0000	0.0323	205.072
5	0	0	3360	111424	0.0000	0.0000	0.0302	337.115
6	0	0	3515	105480	0.0000	0.0000	0.0333	217.603
7	0	0	3811	112549	0.0000	0.0000	0.0339	387.889
8	0	0	3590	120868	0.0000	0.0000	0.0297	207.186
9	0	0	3255	108073	0.0000	0.0000	0.0301	321.977
10	0	0	3616	112664	0.0000	0.0000	0.0321	222.191
11	0	0	3575	110119	0.0000	0.0000	0.0325	199.544
12	0	0	3677	105788	0.0000	0.0000	0.0348	280.325
13	0	0	3147	105127	0.0000	0.0000	0.0299	248.143
14	0	0	3039	109003	0.0000	0.0000	0.0279	275.348
15	0	0	3506	103712	0.0000	0.0000	0.0338	321.074
16 (H)	0	0	3234	106429	0.0000	0.0000	0.0304	249.751
				MEAN	**0.00000**	**0.00000**	**0.03128**	
						SD	**0.002010**	
						%CV	**6.43**	

Table-8: Result of Specificity/ selectivity (DES ASE)

Plasma lot ID	Specificity (blank)		Selectivity (Spiked LLOQ)		% Interference in Blank		Area Ratio	S/N Ratio (≥5)
	Metabolite	IS peak	Metabolite	IS peak	Metabolite (≤20%)	IS (≤5%)	Metabolite/ IS	Metabolite
1	0	0	2979	110599	0.0000	0.0000	0.0269	217.477
2	0	0	3009	109492	0.0000	0.0000	0.0275	226.019
3	0	0	2809	109914	0.0000	0.0000	0.0256	239.894
4	0	0	3310	107712	0.0000	0.0000	0.0307	295.967
5	0	0	3224	111424	0.0000	0.0000	0.0289	182.073
6	0	0	2874	105480	0.0000	0.0000	0.0272	223.470
7	0	0	3352	112549	0.0000	0.0000	0.0298	246.484
8	0	0	3429	120868	0.0000	0.0000	0.0284	169.512
9	0	0	3053	108073	0.0000	0.0000	0.0283	209.646
10	0	0	3428	112664	0.0000	0.0000	0.0304	230.863
11	0	0	3337	110119	0.0000	0.0000	0.0303	235.131
12	0	0	3069	105788	0.0000	0.0000	0.0290	249.241
13	0	0	3139	105127	0.0000	0.0000	0.0299	300.303
14	0	0	3056	109003	0.0000	0.0000	0.0280	303.736
15	0	0	2958	103712	0.0000	0.0000	0.0285	211.800
16(H)	0	0	3067	106429	0.0000	0.0000	0.0288	255.619
				MEAN	**0.00000**	**0.00000**	**0.02864**	
						SD	**0.001402**	
						%CV	**4.90**	

The analyte and metabolite interference were ≤ 20% and IS interference was ≤ 5%. The area ratio was determined as the ratio of analyte area to IS area. The S/N ratio was obtained by comparing the original peak (signal) to the baseline interferences (noise). It was found to be ≥ 5.

The specificity and selectivity were found to be satisfactory with all the values within the expected acceptance criteria.

Model Calculation:

$$\%\ interference\ in\ blank = \frac{Blank\ area}{LLOQ\ area} \times 100$$

$$Area\ ratio = \frac{Analyte(or)Metabolite\ area}{IS\ area}$$

For lot no. 1:

$$\%\ interference\ in\ blank = \frac{0}{2979} \times 100 = 0$$

$$Area\ ratio = \frac{2979}{110599} = 0.0269$$

$$Standard\ deviation, \sigma = \sqrt{\frac{1}{7}(0.0269 - 0.02864)^2} = 0.001402$$

$$\%\ coefficient\ of\ variation = \frac{0.001402}{0.02864} \times 100 = 4.90$$

4.2.3. MATRIX EFFECT:

Matrix effect is tested in order to ensure that the plasma has no effect on the analyte and metabolite. It compares the spiked sample area to the aqueous sample area and the area ratio is calculated for both aqueous and spiked samples. The calculations were done individually for the LQC and the HQC concentrations. The matrix factor was determined by the given formula, which gives the extent to which the plasma affects the peak area obtained.

Table – 9: Result of Matrix Effect (ASEN- LQC)

Plasma Lot No.	Aqueous Sample		Spiked Sample		Area Ratio		Matrix Factor
	Analyte area	IS Area	Analyte Area	IS Area	Aqueous Sample	Spiked Sample	
1	12707	144637	12618	145645	0.0879	0.0866	0.98
2	11927	141006	12073	144099	0.0846	0.0838	0.95
3	12252	141279	11944	143886	0.0867	0.0830	0.94
4	12614	144040	11909	145977	0.0876	0.0816	0.93
5	12939	143733	12485	139666	0.0900	0.0894	1.02
6	12832	141418	11588	141619	0.0907	0.0818	0.93
7 (H)*			11765	136007		0.0865	0.98
				MEAN	**0.08792**	**0.084673**	**0.9614**
					SD		**0.033381**
					%CV		**3.47**

Table – 10: Result of Matrix Effect (ASEN- HQC)

Plasma Lot No.	Aqueous Sample		Spiked Sample		Area Ratio		Matrix Factor
	Analyte area	IS Area	Analyte Area	IS Area	Aqueous Sample	Spiked Sample	
1	900681	136577	909231	134898	6.5947	6.7401	1.02
2	907043	137813	905281	137409	6.5817	6.5882	0.99
3	905590	133659	917562	135630	6.7754	6.7652	1.02
4	893690	136727	901117	136366	6.5363	6.6081	1.00
5	915618	137210	900857	138650	6.6731	6.4973	0.98
6	905342	136088	893063	137730	6.6526	6.4842	0.98
7 (H)*			917182	138626		6.6162	1.00
				MEAN	**6.63563**	**6.61419**	**0.99857**
					SD		**0.016762**
					%CV		**1.68**

Table – 11: Result of Matrix Effect (DES ASE- LQC)

Plasma Lot No.	Aqueous Sample		Spiked Sample		Area Ratio		Matrix Factor
	Analyte area	IS Area	Analyte Area	IS Area	Aqueous Sample	Spiked Sample	
1	10002	144637	9901	145645	0.0692	0.0680	0.99
2	9863	141006	9644	144099	0.0669	0.0669	0.98
3	9317	141279	9158	143886	0.0659	0.0636	0.93
4	9927	144040	9499	145977	0.0689	0.0651	0.95
5	10018	143733	9568	139666	0.0697	0.0685	1.00
6	9466	141418	9424	141619	0.0669	0.0665	0.97
7 (H)*			9928	136007		0.0730	1.07
				MEAN	**0.06842**	**0.06737**	**0.98429**
				SD			**0.044668**
				%CV			**4.54**

Table- 12: Result of Matrix Effect (DES ASE- HQC)

Plasma Lot No.	Aqueous Sample		Spiked Sample		Area Ratio		Matrix Factor
	Analyte area	IS Area	Analyte Area	IS Area	Aqueous Sample	Spiked Sample	
1	731686	136577	726008	134898	5.3573	5.3819	0.99
2	756329	137813	728727	137409	5.4881	5.3034	0.98
3	724736	133659	706483	135630	5.4223	5.2089	0.96
4	749198	136727	707059	136366	5.4795	5.1850	0.96
5	729702	137210	723883	138650	5.3181	5.2209	0.96
6	745773	136088	712845	137730	5.4801	5.1757	0.95
7 (H)*			713542	138626		5.1473	0.95
				MEAN	**5.42423**	**5.23187**	**0.96429**
				SD			**0.015119**
				%CV			**1.57**

The standard deviation and coefficient of variation were calculated for the matrix factor. It was found that the matrix factor was between 0.85 and 1.15 for all samples. The %CV was 3.47 AND 4.54 for analyte and metabolite, which is ≤ 15. Hence the acceptance criteria (Page-38) have been satisfied and the matrix effect is negligible.

Model Calculation:

$$Area\ ratio = \frac{Analyte\ area}{IS\ area}$$

$$Matrix\ factor = \frac{peak\ area\ ratio\ of\ spiked\ samples}{peak\ area\ ratio\ of\ aqueous\ samples}$$

$$Standard\ deviation, \sigma = \sqrt{\frac{1}{N}(x-\mu)^2}$$

$$\%\ coefficient\ of\ variation = \frac{\sigma}{\mu} \times 100$$

For lot no. 1:

$$Area\ ratio = \frac{12707}{144637} = 0.0879$$

$$Matrix\ factor = \frac{0.0866}{0.0879} = 0.98$$

$$Standard\ deviation, \sigma = \sqrt{\frac{1}{7}(0.98-0.9614)^2} = 0.033381$$

$$\%\ coefficient\ of\ variation = \frac{0.033381}{0.9614} \times 100 = 3.47$$

4.2.4.ALL SAMPLE CARRY OVER TEST:

Auto sampler carry over test is done to ensure that the injection of a high concentration sample does not affect the consecutive injection of blank samples.A blank, LLOQ, ULOQ were injected consecutively and the blank was then re-injected.

Table – 13: Result of ASCOT (ASEN)

Sample ID	Analyte Peak Area	IS Peak Area
Extracted Blank	0	0
Extracted LLOQ+IS	4419	114743
Extracted ULOQ+IS	1845907	134401
Extracted Blank	0	0
% Carry Over	**0.00**	**0.0**

Table – 14: Result of ASCOT (DES ASE)

Sample ID	Analyte Peak Area	IS Peak Area
Extracted Blank	0	0
Extracted LLOQ+IS	3286	114743
Extracted ULOQ+IS	1312376	134401
Extracted Blank	0	0
% Carry Over	**0.00**	**0.0**

The re-injected blank was found to show a peak area ≤ 20 % of the LLOQ for the analyte and metabolite ≤ 5% of the ULOQ for the IS. [Table (13&14)]. Hence the carry over in the instrument was found negligible.

Model Calculation:

$$\%\ Carry\ over = \frac{Extracted\ blank\ injected\ 2nd\ time}{LLOQ\ sample} \times 100$$

For Asenapine analyte peak area:

$$\%\ Carry\ over = \frac{0}{4419} \times 100 = 0$$

4.2.4. PRECISION AND ACCURACY BATCH:

The precision and accuracy test is done to determine how exact and correct the values obtained are. It is calculated separately for the CCs and QCs. The actual concentration in table (15) and (16) refers to the value of the aqueous standards. The calculated concentration is the concentration obtained for spiked samples. Table shows only single values for the CCs for which only a single set of spiked samples were used. The six calculated concentrations for the extracted samples in table below were taken on average and compared to the actual concentration by calculating the % nominal which indicates the accuracy. The coefficient of variation indicates the precision of the values.

Table – 15: Result of P&A Batch (ASEN)

Std Units	A	B	C	D	E	F	G	H	Slope	Intercept	R^2
Actual Conc. (ng/mL)	0.1060	0.2120	1.282	2.562	5.1220	12.8040	25.6080	35.3200	0.00214	0.004226	0.99
Cal.Conc. (ng/mL)	0.1014	0.2317	1.263	2.498	4.8294	13.1801	25.6990	35.8090			
%Nominal	**95.66**	**109.29**	**98.58**	**97.50**	**94.29**	**102.94**	**100.36**	**101.38**			
Result	**Pass**	**Pas**	**Pass**	**Pass**	**Pass**	**Pass**	**Pass**	**Pass**			

QC ID	LOQQC	LQC	INTQC	MQC	HQC
ACT CONC.	**0.1080**	**0.2960**	**2.5580**	**12.7860**	**25.5720**
CAL. CONC.	0.0978	0.2769	2.5523	12.8988	25.7184
	0.0928	0.2625	2.4986	13.7751	26.9084
	0.0725	0.2953	2.5364	13.7566	27.6304
	0.0826	0.3204	2.4058	13.3284	25.8956
	0.0875	0.3246	2.4063	13.2787	26.8808
	0.1134	0.2713	2.5459	13.3717	26.3680
MEAN	**0.09110**	**0.29183**	**2.49088**	**13.40155**	**26.56693**
S.D	**0.013973**	**0.026103**	**0.068299**	**0.328540**	**0.71503**
%CV	**15.34**	**8.94**	**2.74**	**2.45**	**2.69**
%NOMINAL	**84.35**	**98.59**	**97.38**	**104.81**	**103.89**

Table – 16: Result of P&A Batch (DES ASE)

Std UNITS	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**SLOPE**	**INT**	**r^2**
actual conc. (pg/ml)	**0.1060**	**0.2120**	**1.2860**	**2.5700**	**5.1400**	**12.8500**	**25.7000**	**35.4480**	0.00193 5	0.001 5	0.997
calculated conc. (pg/ml)	0.1125	0.1878	1.2192	2.6211	4.8563	13.0836	25.6743	39.8102			
%Nominal	**106.13**	**88.58**	**94.81**	**101.99**	**94.48**	**101.82**	**99.90**	**112.31**			
Result	**PASS**	**PASS**	**PASS**	**PASS**	**PASS**	**PASS**	**PASS**	**PASS**			

QC ID	**LOQQC**	**LQC**	**INTQC**	**MQC**	**HQC**
ACTUAL CONC.	**0.1100**	**0.3000**	**2.5920**	**12.9620**	**25.9240**
CALCULATED CONC.	0.1194	0.2876	2.3366	12.0451	24.1130
	0.1241	0.2852	2.2373	13.0870	25.4401
	0.1306	0.2742	2.4093	11.5188	25.2139
	0.1106	0.2982	2.2301	12.4097	24.3470
	0.0945	0.2624	2.1488	12.6614	24.7720
	0.1337	0.2408	2.2388	12.4613	23.5695
MEAN	**0.11882**	**0.27473**	**2.26682**	**12.36388**	**24.57592**
S.D	**0.014474**	**0.020638**	**0.091764**	**0.536431**	**0.703115**
%CV	**12.18**	**7.51**	**4.05**	**4.34**	**2.86**
%NOMINAL	**108.02**	**91.58**	**87.45**	**95.39**	**94.80**

% nominal for all the samples was found to be between 85 and 115%. %CV for all the samples was found to be ≤ 15% as per the acceptance criteria (Page-40). Hence the PA batch was passed for the given acceptance criteria.

Model Calculation:

$$Standard\ deviation, \sigma = \sqrt{\frac{1}{N}(x-\mu)^2}$$

$$\%\ coefficient\ of\ variation = \frac{\sigma}{\mu} \times 100$$

$$\%\ nominal = \frac{mean\ of\ calculated\ values}{actual\ values} \times 100$$

INTER AND INTRA DAY PRECISION:

Similarly inter and intra day P&A batch were also performed inorder to find the Precision, Accuracy and Ruggedness.

Table – 17: Result of Inter and Intra P&A Batch (ASEN)

QC ID	LOQQC	LQC	INTQC	MQC	HQC
Act Conc (ng/mL)	**0.1100**	**0.2980**	**2.5520**	**12.7640**	**25.5280**
Calculated Concentration (ng/mL) PA – 02 07 Mar 13	0.1306	0.3146	2.6204	12.7453	26.4243
	0.1150	0.3066	2.5636	13.0620	26.8256
	0.1215	0.2927	2.5248	13.1402	25.7862
	0.1250	0.2790	2.5839	13.2403	25.9268
	0.1251	0.2870	2.5874	13.1796	26.1853
	0.1262	0.2971	2.5970	12.7074	26.1746
Mean	**0.12390**	**0.29617**	**2.57952**	**13.01247**	**26.22047**
SD	**0.005250**	**0.012963**	**0.032575**	**0.229371**	**0.370379**
%CV	**4.24**	**4.38**	**1.26**	**1.76**	**1.41**
%Nominal	**112.64**	**99.38**	**101.08**	**101.95**	**102.71**
Calculated	0.1136	0.3149	2.5407	13.1206	25.2332
	0.1224	0.3055	2.4366	13.1042	25.4600

Concentration (ng/mL)	0.1188	0.3007	2.5912	12.7639	25.7128
PA – 03	0.1179	0.3072	2.4733	12.7392	26.0920
07 Mar 13	0.1200	0.3045	2.5011	12.9900	25.8031
	0.1111	0.3004	2.5329	13.3425	26.7973
Mean	**0.11730**	**0.30553**	**2.51263**	**13.01007**	**25.84973**
SD	**0.004196**	**0.005316**	**0.054465**	**0.230634**	**0.549455**
%CV	**3.58**	**1.74**	**2.17**	**1.77**	**2.13**
%Nominal	**106.64**	**102.53**	**98.46**	**101.93**	**101.26**
Calculated Concentration (ng/mL)	0.4229*	0.2888	2.5262	12.6272	23.1539
	0.1007	0.4217*	2.5225	12.3875	25.1890
PA – 04	0.1097	0.2823	2.5356	12.4267	27.5285
	0.1038	0.2889	2.5629	12.4556	24.8686
08 Mar 13	0.1094	0.2635	2.6519	12.5785	24.3997
	0.0927	0.3083	2.5752	12.6713	26.6160
Mean	**0.10326**	**0.28636**	**2.56238**	**12.52447**	**25.29262**
SD	**0.007027**	**0.016069**	**0.048570**	**0.116700**	**1.569233**
%CV	**6.81**	**5.61**	**1.90**	**0.93**	**6.20**
%Nominal	**93.87**	**96.09**	**100.41**	**98.12**	**99.08**
Calculated Concentration (ng/mL)	0.1005	0.3064	2.5650	13.5058	25.5754
	0.1232	0.3231	2.6428	12.8194	26.5014
PA – 05	0.0964	0.2844	2.6140	13.4811	24.9675
	0.0816	0.3038	2.5029	13.0308	25.8194
09 Mar 13	0.1028	0.3198	2.5479	13.4079	25.1698
	0.1176	0.2981	2.4359	12.9267	25.4783
Mean	**0.10368**	**0.30593**	**2.55142**	**13.19528**	**25.58530**
SD	**0.015008**	**0.014260**	**0.075050**	**0.304564**	**0.540353**
%CV	**14.47**	**4.66**	**2.94**	**2.31**	**2.11**
%Nominal	**94.26**	**102.66**	**99.98**	**103.38**	**100.22**

Note*: LOQQC-25 & LQC-26 were not included in the calculation due to increase in analyte peak area and decrease in internal standard area observed.

Table – 18: Result of Inter and Intra P&A Batch (DES ASE)

QC ID	LOQQC	LQC	INTQC	MQC	HQC
Act Conc (ng/mL)	**0.1080**	**0.2920**	**2.5080**	**12.5440**	**25.0860**
Cal. Conc (ng/mL) PA – 01 06 Mar 13	0.1275	0.2883	2.4527	12.6913	25.0637
	0.1126	0.3299	2.6032	12.9009	26.1374
	0.1208	0.3279	2.6023	12.1371	24.5664
	0.1133	0.3060	2.3402	12.1978	23.9863
	0.1220	0.3094	2.3163	12.1279	24.5801
	0.1224	0.3039	2.5131	11.9441	23.5757
Mean	**0.11977**	**0.31090**	**2.47130**	**12.33318**	**24.65160**
SD	**0.005761**	**0.15723**	**0.124791**	**0.374394**	**0.893307**
%CV	**4.81**	**5.06**	**5.05**	**3.04**	**3.62**
%Nominal	**110.90**	**106.47**	**98.54**	**98.32**	**98.27**
Calculated Concentration (ng/mL) PA – 02 07 Mar 13	0.1192	0.2716	2.3016	11.3075	23.6310
	0.1077	0.2774	2.2975	11.4470	25.3775
	0.1061	0.2304	2.1778	11.3394	22.7298
	0.0940	0.2338	2.2235	11.0628	22.8670
	0.1204	0.2500	2.3368	10.6648	22.2770
	0.1057	0.2505	2.1376	11.0467	25.0590
Mean	**0.10885**	**0.25228**	**2.24580**	**11.14470**	**23.65688**
SD	**0.009796**	**0.019146**	**0.078608**	**0.283694**	**1.289543**
%CV	**9.00**	**7.59**	**3.50**	**2.55**	**5.45**
%Nominal	**100.79**	**86.40**	**89.55**	**88.84**	**94.30**
Calculated Concentration (ng/mL) PA – 03 07 Mar 13	0.1308	0.2856	2.4967	12.8099	24.8484
	0.1070	0.3087	2.4203	12.7620	24.6048
	0.1091	0.2856	2.5655	11.6574	23.7209
	0.1030	0.2851	2.4067	12.1180	23.7001
	0.1083	0.2809	2.3713	11.9956	22.9256
	0.1094	0.2728	2.4367	11.7287	23.8418
Mean	**0.11127**	**0.28645**	**2.44953**	**12.17860**	**23.94027**
SD	**0.009850**	**0.011962**	**0.070210**	**0.500023**	**0.694458**
%CV	**8.85**	**4.18**	**2.87**	**4.11**	**2.90**
%Nominal	**103.02**	**98.10**	**97.67**	**97.09**	**95.43**
Calculated Concentration (ng/mL) PA – 04 08 Mar 13	0.4141	0.2637	2.5264	11.3724	21.2046
	0.0911	0.3844	2.4378	11.3625	24.0112
	0.1039	0.2690	2.5632	11.5323	27.3929
	0.0940	0.2569	2.3825	12.0130	24.2429
	0.0835	0.2917	2.6219	11.9468	22.9111
	0.0966	0.2401	2.4872	11.9982	26.6316
Mean	**0.14720**	**0.28430**	**2.50317**	**11.70420**	**24.39905**
SD	**0.130924**	**0.051841**	**0.086406**	**0.315293**	**2.303942**
%CV	**88.94**	**18.23**	**3.45**	**2.69**	**9.44**
%Nominal	**136.30**	**97.36**	**99.81**	**93.31**	**97.26**
Calculated Concentration (ng/mL) PA – 05	0.1186	0.3052	2.6631	12.8722	23.4465
	0.1278	0.3836	2.8007	12.4424	23.5755
	0.1075	0.2810	2.6746	13.4837	23.1479
	0.1275	0.3035	2.4097	12.6551	22.6159
	0.1224	0.2769	2.4680	11.9816	22.4846

09 Mar 13	0.1265	0.3357	2.5480	11.4625	22.3437
Mean	**0.12172**	**0.31432**	**2.59402**	**12.48292**	**22.93568**
SD	**0.007818**	**0.039919**	**0.154471**	**0.703842**	**0.523740**
%CV	**6.42**	**12.70**	**5.61**	**5.64**	**2.28**
%Nominal	**112.70**	**107.64**	**103.43**	**99.51**	**91.43**

% nominal for all the samples was found to be between 85 and 115%. %CV for all the samples was found to be ≤ 15% as per the acceptance criteria (Page-46). Hence the inter and intra day PA batch was passed for the given acceptance criteria.

4.2.5. REINJECTION REPRODUCIBILITY:

The RIR test is conducted to ensure repeatability of the experiment after a period of time. A set of six samples each of LQC and HQC concentrations used for a PA batch were injected after 31.75hours of completion of a PA batch. The values of actual and calculated concentrations obtained for the two injections were compared and the mean, SD, %CV, %nominal and ratio of means were calculated in Table(19&20).

Table – 19: Result of RIR (ASEN)

Batch ID	**Reinjection Reproducibility**			
	PA-03 Samples		**Reinjected Samples (31.75 hrs)**	
	LQC	**HQC**	**LQC**	**HQC**
Actual Conc(mg/mL)	**0.2980**	**25.5280**	**0.2980**	**25.5280**
Calc. Conc(ng/mL)	0.3149	25.2332	0.3114	24.9602
	0.3055	25.4600	0.2952	24.7545
	0.3007	25.7128	0.3079	25.4905
	0.3072	26.0920	0.2908	25.4115
	0.3045	25.8031	0.2987	25.4004
	0.3004	26.7973	0.3049	25.2369
Mean	**0.30553**	**25.84973**	**0.30148**	**25.20900**
SD	**0.005316**	**0.549455**	**0.007906**	**0.291936**
%CV	**1.74**	**2.13**	**2.62**	**1.16**
%Nominal	**102.53**	**101.26**	**101.17**	**98.75**
Ratio of Means			**0.99**	**0.98**

Table – 20: Result of RIR (DES ASE)

Batch ID	Reinjection Reproducibility			
	PA-03 Samples		Reinjected Samples (31.75 hrs)	
	LQC	HQC	LQC	HQC
Act. Conc(mg/mL)	**0.2980**	**25.5280**	**0.2980**	**25.5280**
Calc. Conc.(ng/mL)	0.2856	24.8484	0.2862	24.9608
	0.3087	24.6048	0.3053	24.7556
	0.2856	23.7209	0.2912	25.4912
	0.2851	23.7001	0.2935	25.4123
	0.2809	22.9256	0.2987	25.4012
	0.2728	23.8418	0.3056	25.2429
Mean	**0.28645**	**23.94027**	**0.30163**	**25.21000**
SD	**0.011962**	**0.694458**	**0.007913**	**0.292136**
%CV	**4.18**	**2.90**	**2.74**	**1.36**
%Nominal	**98.10**	**95.43**	**102.12**	**99.65**
Ratio of Means			**0.99**	**0.99**

The mean values were found to be between 85 and 115% of the actual concentrations for all the samples. The %CV was ≤ 15% for all samples. The ratio of means was within 1.0 ± 0.15. Hence the RIR test was passed.

Model Calculation:

$$Standard\ deviation, \sigma = \sqrt{\frac{1}{N}(x-\mu)^2}$$

$$\%\ coefficient\ of\ variation = \frac{\sigma}{\mu} \times 100$$

$$\%\ nominal = \frac{mean\ of\ calculated\ concentration}{actual\ concentration} \times 100$$

$$Ratio\ of\ means = \frac{mean\ of\ extracted\ sample}{mean\ of\ aqueous\ sample}$$

For ASENAPINE aqueous LQC sample:

$$Standard\ deviation, \sigma = \sqrt{\frac{1}{6}(0.3149 - 0.30553)^2} = 0.005316$$

$$\%\ coefficient\ of\ variation = \frac{0.005316}{0.30553} \times 100 = 1.74$$

$$\%\ nominal = \frac{0.30553}{0.2980} \times 100 = 102.53$$

$$Ratio\ of\ means = \frac{0.30148}{0.30553} = 0.99$$

4.2.6. RECOVERY:

Recovery refers to the response from the amount of drug that can be separated from the spiked plasma as compared to the aqueous sample of the drug. It is the deciding factor in selection of a method of extraction.

Table – 21: Result of Recovery (ASEN)

Quality Control Samples ID	Aqueous Analyte Area	Extracted Analyte Area
LQC	16894	15333
	17438	14793
	18034	15646
	17777	14983
	16901	14569
	17691	14181
Mean	**17456**	**14918**
SD	**473**	**527**
%CV	**2.71**	**3.53**
%Recovery	**85.46**	
MQC	747561	626312
	780634	612935
	748360	590844
	750097	631328
	769826	568301
	781737	601957
Mean	**783036**	**605280**
SD	**16296**	**23522**
%CV	**2.14**	**3.89**
%Recovery	**79.33**	
HQC	1608663	1199514
	1650385	1228869
	1650090	1230270
	1671796	1375831
	1612254	1310097
	1654997	1268525
Mean	**1641364**	**1268851**
SD	**25244**	**64870**
%CV	**1.54**	**5.11**
%Recovery	**77.30**	

Recovery Result	
LQC	85.46
MQC	79.33
HQC	77.30
MEAN	**80.70**
SD	**4.25**
%CV	**5.26**

Table – 22: Recovery of Recovery (DES ASE)

Quality Control Samples ID	Aqueous Analyte Area	Extracted Analyte Area
LQC	12107	10652
	12095	11548
	12513	11983
	12275	10731
	12328	10993
	12645	10428
Mean	**123270**	**11056**
SD	**219**	**595**
%CV	**1.78**	**5.38**
%Recovery	**89.69**	
MQC	561710	448976
	557012	442950
	548951	416296
	551383	432435
	564679	397408
	586655	407794
Mean	**561732**	**424310**
SD	**13584**	**20409**
%CV	**2.42**	**4.81**
%Recovery	**75.54**	
HQC	1162544	860922
	1162065	897717
	1163929	855382
	1162055	904968
	1166202	896729
	1171397	830364
Mean	**1164699**	**874347**
SD	**3641**	**29864**
%CV	**0.31**	**3.42**
%Recovery	**75.07**	

Recovery Result	
LQC	89.69
MQC	75.54
HQC	75.07
MEAN	**80.10**
SD	**8.31**
%CV	**10.37**

Table – 24: Result of Bench Top Stability (ASEN)

Standard units	A	B	C	D	E	F	G	H	SLOPE	Intercept
Actual Concentration	**0.1080**	**0.2160**	**1.2860**	**2.5720**	**5.1440**	**12.8620**	**25.7220**	**35.3140**		
Calculated Concentration	0.1031	0.2362	1.2978	2.4635	5.0218	12.7233	26.0581	35.5319	0.3710	0.0026
% nominal	**95.46**	**109.35**	**100.92**	**95.78**	**97.62**	**98.92**	**101.31**	**100.62**		

QC ID	0 HOUR		10.12 HOURS	
	LQC (CS)	HQC (CS)	LQC (Stability)	HQC (Stability)
Actual Concentration (ng/mL)	**0.2980**	**25.5280**	**0.2980**	**25.5280**
Calculated Concentration (ng/mL)	0.3000	24.6690	0.3043	25.6073
	0.2760	25.3686	0.2907	26.1759
	0.2931	25.9458	0.3028	25.5687
	0.2992	25.3441	0.2570	26.2222
	0.2952	26.2861	0.2920	25.8095
	0.2871	22.3737	0.2912	26.5278
Mean	**0.29177**	**24.99788**	**0.28967**	**25.98523**
SD	**0.009022**	**1.400703**	**0.017103**	**0.383171**
%CV	**3.09**	**5.60**	**5.90**	**1.47**
%Nominal	**97.91**	**97.92**	**97.20**	**101.79**
%Nominal against CS			**99.28**	**103.95**

Table – 23: Result of Recovery (ASEN 13 C D3)

Quality Control Samples ID	Aqueous Analyte Area	Extracted Analyte Area
LQC	149857	143381
	154406	135113
	152794	141122
	151945	135786
	159934	137495
	157881	132908
MQC	163389	131842
	175894	127957
	166611	127832
	170247	132126
	172586	122125
	175713	127247
HQC	164247	127955
	167392	127940
	171296	129706
	168886	140545
	165318	135900
	167788	131206
MEAN	**164232**	**132677**

The % recovery was greater than 60% and less than 115% for all samples. The % CV was less than 15%. Hence the acceptance criteria for recovery (Page-41) were satisfied.

Model Calculation:

$$Standard\ deviation, \sigma = \sqrt{\frac{1}{N}(x-\mu)^2}$$

$$\%\ coefficient\ of\ variation = \frac{\sigma}{\mu} \times 100$$

$$\%\ recovery = \frac{mean\ extracted\ peak\ area}{mean\ aqueous\ peak\ area} \times 100$$

For ASENAPINE aqueous sample LQC:

$$Standard\ deviation, \sigma = \sqrt{\frac{1}{6}(16894 - 17456)^2} = 473$$

$$\%\ coefficient\ of\ variation = \frac{473}{17456} \times 100 = 2.71$$

$$\%\ recovery = \frac{14918}{17456} \times 100 = \mathbf{85.46}\%$$

4.2.7. BENCH TOP STABILITY:

Stability tests are conducted to ensure that the sample is stable under various conditions and time period. Long term stability test was conducted by injecting one set of LQC and HQC concentrations immediately and two sets of LQC and HQC samples after 55days at -30ºC and -70ºC respectively. Aqueous samples were injected once and extracted were injected six times. The mean, SD, %CV, % nominal and % nominal against CS sample.

Table – 25: Result of Bench Top Stability (DES ASE)

Std Units	A	B	C	D	E	F	G	H	SLOPE	Intercept
Actual Concentration	**0.1060**	**0.2120**	**1.2560**	**2.5120**	**5.0220**	**12.5540**	**25.1080**	**34.4700**		
Calculated Concentration	0.1131	0.1859	1.1509	2.4950	4.9972	13.4773	26.9047	34.6513	0.4020	0.0032
% Nominal	**106.79**	**87.69**	**91.63**	**99.32**	**99.51**	**107.35**	**107.16**	**100.53**		

QC ID	0 HOUR		10.12 HOURS	
	LQC (CS)	HQC (CS)	LQC (Stability)	HQC (Stability)
Actual Conc(ng/mL)	**0.2920**	**25.0860**	**0.2920**	**25.0860**
Calculated Concentration (ng/mL)	0.2854	25.1165	0.2735	25.0735
	0.2825	25.7278	0.2961	26.6872
	0.2909	26.8409	0.2927	25.2600
	0.2968	25.8562	0.2826	26.1269
	0.3091	27.6989	0.2790	25.3350
	0.2543	26.2016	0.2934	26.0748
Mean	**0.28650**	**26.24032**	**0.28622**	**25.75957**
SD	**0.018386**	**0.912452**	**0.009145**	**0.631715**
%CV	**6.42**	**3.48**	**3.20**	**2.45**
%Nominal	**98.12**	**104.60**	**98.02**	**102.69**
%Nominal against CS			**99.90**	**98.17**

Table – 24: Result of Bench Top Stability (ASEN)

Standard units	A	B	C	D	E	F	G	H	SLOPE	Intercept
Actual Concentration	**0.1080**	**0.2160**	**1.2860**	**2.5720**	**5.1440**	**12.8620**	**25.7220**	**35.3140**		
Calculated Concentration	0.1031	0.2362	1.2978	2.4635	5.0218	12.7233	26.0581	35.5319	0.3710	0.0026
% nominal	**95.46**	**109.35**	**100.92**	**95.78**	**97.62**	**98.92**	**101.31**	**100.62**		

QC ID	0 HOUR		10.12 HOURS	
	LQC (CS)	HQC (CS)	LQC (Stability)	HQC (Stability)
Actual Concentration (ng/mL)	**0.2980**	**25.5280**	**0.2980**	**25.5280**
Calculated Concentration (ng/mL)	0.3000	24.6690	0.3043	25.6073
	0.2760	25.3686	0.2907	26.1759
	0.2931	25.9458	0.3028	25.5687
	0.2992	25.3441	0.2570	26.2222
	0.2952	26.2861	0.2920	25.8095
	0.2871	22.3737	0.2912	26.5278
Mean	**0.29177**	**24.99788**	**0.28967**	**25.98523**
SD	**0.009022**	**1.400703**	**0.017103**	**0.383171**
%CV	**3.09**	**5.60**	**5.90**	**1.47**
%Nominal	**97.91**	**97.92**	**97.20**	**101.79**
%Nominal against CS			**99.28**	**103.95**

%nominal was found to be between 85 and 15% for all cases. Thus the acceptance criteria (Page-48) were satisfied. Hence the drug passed the stability test and is considered suitable for injection even after long periods of incubation.

Model Calculation:

$$Standard\ deviation, \sigma = \sqrt{\frac{1}{N}(x-\mu)^2}$$

$$\%\ coefficient\ of\ variation = \frac{\sigma}{\mu} \times 100$$

$$\%\ nominal = \frac{mean\ of\ calculated\ concentration}{actual\ Concentration} \times 100$$

For ASENAPINE sample LQC:

$$Standard\ deviation, \sigma = \sqrt{\frac{1}{6}(0.3000 - 0.29177)^2} = 0.009022$$

$$\%\ coefficient\ of\ variation = \frac{0.009022}{0.29177} \times 100 = 3.09$$

$$\%\ nominal = \frac{0.29177}{0.2980} \times 100 = 97.91$$

SUMMARY

Internal Standard Used : the deuterated compound of the analyte – Asenapine 13C D3 to reduce the matrix effect.

The MS was tuned with the analyte solution and the parameters were set based on maximum response. Q1 mass of Analyte, Metabolite and IS were found to be 286.20, 272.10, 290.10 respectively.Q3 mass of them were same as 229.20. Dwell Time for analyte, metabolite and IS was 200 msec. At the condtions of certain parameters such as CAD GAS of 10.00, CURTAIN GAS 14.00, GAS1 and GAS 2 as 50 and 55, Ionization Potential and Exit Potential to be 5500 and 10.00 respectively. For analyte, metabolite and IS: Declustering Potential = 85, 70 and 85, Collision Energy = 30, 25 and 30 and Exit Potential = 18, 18 and 20 respectively.

Serial dilution within the range of 0.1080 – 35.314 ng/mL(ANALYTE) and 0.1060 – 34.47 ng/mL (METABOLITE) provided a linear calibration curve fit with a weighting factor $1/x^2$.

The optimum chromatographic conditions were found to be the use of Hypersil Gold C18 (50mmx4.6mm,5µm) column, having Acetonitrile : 10mM Ammonium Acetate (90:10) as mobile phase at 0.6 mL/min flow rate. Column oven temperature and auto sampler temperature were 40°C and 10°C. Injection volume was 10µL and Total run time was 3.5 minutes. The retention times were Analyte: 2.20±0.3 min, Metabolite: 2.10±0.3 min, IS : 2.20±0.3 min

The best extraction procedure of drug from plasma was found to be a Liquid- Liquid Extraction by using Extraction Solvent as Tertiary Butyl Methyl Ether: n- Hexane (90:10).

Under these conditions, the method was proved to be precise, accurate, devoid of matrix effect and specific and selective for the analyte. There was no carryover of analyte. The mean recovery of Asenapine (80.70%) and N-Desmethyl Asenapine (80.10%) from spiked plasma samples was consistent and reproducible.

The drug was tested for stability under different conditions and was found to be stable. The method was proved to be repeatable during re-injection after long periods of time (31.75 hours).

5. CONCLUSION

The main objective of the present work is to develop a new simple, rapid, accurate and robust quantitative procedure, to validate, to determine Asenapine and N-Desmethyl Asenapine in Human Plasma by High Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry

Literature review revealed that only few Analytical methods have been reported to determine the Asenapine and its metabolite in plasma samples. An attempt was made to develop a LC-MS/MS method for the determination of Asenapine and N-Desmethyl Asenapine in human plasma at picogram level concentration.

An efficient bio analytical method for analysis of asenapine and its metabolite have been developed using LC-MS/MS. Pre- method validation experiments have been performed. Also the developed method was fully validated by studying the various parameters like accuracy, precision, specificity etc.

6. APPENDICES:

APPENDIX – 1: PREPARATION OF SOLUTIONS:

1. STOCK SOLUTION:

Analyte:

2mg of Asenapine Maleate was weighed and transferred into a 2 mL volumetric flask. 2 mL of Methanol was added to dissolve and make up the volume. The final concentration of 1mg/mL of Asenapine Maleate can be calculated as follows:

$$\frac{\text{Weight of Asenapine Maleate taken in mg}}{2\text{ mL}} \times \frac{\text{Potency(as in basis)}}{100} \times \frac{M_1}{M_2} \times 1000$$

Where M_1 is molecular weight of Asenapine (free) and M_2 is the molecular weight of Asenapine Maleate(salt).

Metabolite:

2mg of N-Desmethyl Asenapine Maleate was weighed and transferred into a 2 mL volumetric flask. 2 mL of Methanol was added to dissolve and make up the volume. The final concentration of 1mg/mL of N-Desmethyl Asenapine Maleate can be calculated as follows:

$$\frac{\text{Weight of N-Desmethyl Asenapine Maleate}}{2\text{ mL}} \times \frac{\text{Potency}}{100} \times \frac{M_1}{M_2} \times 1000$$

Where M_1 is molecular weight of N-Desmethyl Asenapine (free) and M_2 is the molecular weight of N-Desmethyl Asenapine Maleate(salt).

INTERNAL STANDARD:

2mg of Asenapine 13C-D3 Maleate was weighed and transferred into a 2 mL volumetric flask. 2 mL of Methanol was added to dissolve and make up the volume to obtain 1µg/mL concentration. The final concentration of Asenapine 13C-D3 Maleate can be calculated as follows:

$$\frac{\text{Weight of Asenapine 13C-D3 Maleate}}{2\ \text{mL}} \times \frac{\text{Potency (as in basis)}}{100} \times \frac{M_1}{M_2} \times 1000$$

Where M_1 is molecular weight of Asenapine 13C-D3 (free) and M_2 is the molecular weight of Asenapine 13C-D3 Maleate (salt).

Table- 26 : Preparation of IS stock solution

Stock Conc (µg/mL)	Stock Aliquot(mL)	Diluent Added(mL)	Final Volume (mL)	Final Conc (ng/mL)
1000	0.250	24.750	25.000	10000.0000
10	0.025	24.975	25.000	100.000

2. **TUNING SOLUTION:**

20µl of each stock solutions were taken and made up to 2ml using Methanol. This resulted in 3 solutions of 100ng/ml concentration each, which were used **as tuning solutions.**

3. **BUFFER -1 SOLUTION:** (10mm ammonium Formate)

630.60mg of Ammonium formate was weighed and transferred into 1000mL volumetric flask containing about 500mL of HPLC water, dissolved and 500mL of water was added. It was mixed well and sonicated in Ultrasonic bath for few minutes and Filtered through 0.2µm nylon membrane filter.

4. **BUFFER-2SOLUTION:**(10mmPotassium Dihydrogen Orthophosphate)
   1.360gm of Potassium Dihydrogen Orthophosphate was weighed and transferred into 1000 mL volumetric flask containing about 500mL of HPLC water, dissolved and 500mL of water was added. It was mixed well and sonicated in Ultrasonic bath for few minutes and Filtered through 0.2µm nylon membrane filter.
5. **MOBILE PHASE: (**ACETONITRILE: BUFFFER-1 (90:10, V/V))
   900 mL of acetonitrile was transferred into 1000mL reagent bottle. 100mL of Buffer-1 was added. It was mixed well and sonicated in Ultrasonic bath for few minutes. Then it was filtered through 0.2µm nylon membrane filter.
6. **DILUENT:** ACETONITRILE: WATER (50:50%V/V)
   500mL of Acetonitrile was transferred into 1000 mL reagent bottle and 500mL of HPLC water was added. It was Mixed well and sonicated in Ultrasonic bath for few minutes. Then it was filtered through 0.2µm nylon membrane filter.
7. **WEAK WASH SOLUTION:** METHANOL: WATER (50:50,V/V)
   500mL of Methanol was transferred into 1000ml reagent bottle; 500mL of HPLC water was added. This was mixed well using the Sonicator for few minutes. Then it was filtered through 0.2µm nylon membrane filter.
8. **STRONG WASH SOLUTION:** (METHANOL: WATER (90:10, V/V))
   900 mL of Methanol was transferred into 1000 mL reagent bottle, and 100 mL of Water was added .It was mixed well by using Sonicator and filtered through 0.2µm nylon membrane filter.

Table – 27 : BULK SPIKING SHEET FOR CCs

MOLECULE		ASE	DES MET
AMOUNT WEIGHED (mg)		2.166	2.163
VOLUME (mL)*		2.0	2.0
ASSAY (%)		99.68	99.88
MOLECULAR WEIGHT (FREE)		285.77	271.74
MOLECULAR WEIGHT (SALT)		401.84	387.81
FINAL CONCENTRATION (ug/mL)		767.7149	756.9020
STOCK ID		ASEN-04/Jan	DES-ASE-04/Jan
STOCK VOLUME (mL)		0.100	0.099
DILUENT ADDED (mL)**		9.801	
FINAL VOLUME (mL)		10.000	
CONCENTRATION (ug/mL)		7.6771	7.4933
STOCK ID		CC-SD-04/Jan	

DRUG: Asenapine
Batch No.: CS-AA-227
Light Sensitive (Y/N):yes
EXPIRY date: Mar 14

DRUG: N-DesMethyl Asenapine
Batch No.: CS-DAM-382
Light Sensitive (Y/N):NO
Retest date: May 14

STOCK ID		CC-SD-04/Jan	STD SS H	STD SS G	STD SS F	STD SS E	STD SS D	STD SS C	STD SS B
STOCK CONCENTRATION (ug/mL)	ASE	7.6771	1.7657	1.2861	0.6431	0.2572	0.1286	0.0643	0.0108
	DES ASE	7.4933	1.7235	1.2554	0.6277	0.2511	0.1256	0.0628	0.0106
STOCK VOLUME (mL)		1.150	3.642	2.500	2.000	2.500	2.500	0.842	2.500
DILUENT ADDED (mL)**		3.850	1.358	2.500	3.000	2.500	2.500	4.158	2.500
FINAL VOLUME (mL)		5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
CONCENTRATION (ug/mL)	ASE	1.7657	1.2861	0.6431	0.2572	0.1286	0.0643	0.0108	0.0054
	DES ASE	1.7235	1.2554	0.6277	0.2511	0.1256	0.0628	0.0106	0.0053
STOCK ID		STD SS H	STD SS G	STD SS F	STD SS E	STD SS D	STD SS C	STD SS B	STD SS A
SPIKED VOLUME (µL)		1000	1000	1000	1000	1000	1000	1000	1000
BLANK MATRIX ADDED (µL)		49000	49000	49000	49000	49000	49000	49000	49000
TOTAL SPIKED VOLUME (mL)		50	50	50	50	50	50	50	50
CC ID		STD-H	STD-G	STD-F	STD-E	STD-D	STD-C	STD-B	STD-A
MATRIX SPIKED CONCENTRATION (ng/mL)	ASE	35.3140	25.7220	12.8620	5.1440	2.5720	1.2860	0.2160	0.1080
	DES ASE	34.4700	25.1080	12.5540	5.0220	2.5120	1.2560	0.2120	0.1060
CC RANGE %	ASE		72.84						
	DES ASE		72.84						
Suggested Range		2 x Cmax	70-85%					2 x A	

APPENDIX- 2 : TERMINOLOGY

Bio Analysis : is a sub-discipline of analytical chemistry covering the quantitative measurement of biological molecules, proteins, DNA, drugs and their metabolites in the biological systems

Bioavailability: Amount of a substance that becomes available to an organism's body for bioactivity when introduced through ingestion, inhalation, injection, or skin contact

Bioequivalence: The property wherein two drugs with identical active ingredients or two different dosage forms of the same drug possess similar bioavailability and produce the same effect at the site of physiological activity

Method Development: Refers to the process of formulating the materials, conditions, and protocol for measuring an analyte. Method development is mainly carried out by industry.

Method Validation: The process of testing a measurement procedure to assess its performance and to validate that performance is acceptable.

Tuning: Tuning is the adjustment of working parameters of LC-MS/MS to enable an operator to get the best signal possible for the analyte by optimizing the Q1 mass, Q3 mass of analyte, metabolite and Internal Standard based on molecular weights

Elution: The process of extracting a substance adsorbed to another by means of a suitable solvent or buffering agent as in column chromatography.

Mobile Phase: The mobile phase is the part of the chromatographic system which carries the solutes through the stationary phase. The mobile phases are either liquids or gases. The liquid mobile phases are used to adjust the chromatographic separation and retention in liquid chromatography and the

Table – 27 : BULK SPIKING SHEET FOR CCs

MOLECULE		ASE	DES MET
AMOUNT WEIGHED (mg)		2.166	2.163
VOLUME (mL)*		2.0	2.0
ASSAY (%)		99.68	99.88
MOLECULAR WEIGHT (FREE)		285.77	271.74
MOLECULAR WEIGHT (SALT)		401.84	387.81
FINAL CONCENTRATION (ug/mL)		767.7149	756.9020
STOCK ID		ASEN-04/Jan	DES-ASE-04/Jan
STOCK VOLUME (mL)		0.100	0.099
DILUENT ADDED (mL)**		9.801	
FINAL VOLUME (mL)		10.000	
CONCENTRATION (ug/mL)		7.6771	7.4933
STOCK ID		CC-SD-04/Jan	

DRUG: Asenapine
Batch No.: CS-AA-227
Light Sensitive (Y/N):yes
EXPIRY date: Mar 14

DRUG: N-DesMethyl Asenapine
Batch No.: CS-DAM-382
Light Sensitive (Y/N):NO
Retest date: May 14

STOCK ID		CC-SD-04/Jan	STD SS H	STD SS G	STD SS F	STD SS E	STD SS D	STD SS C	STD SS B
STOCK CONCENTRATION (ug/mL)	ASE	7.6771	1.7657	1.2861	0.6431	0.2572	0.1286	0.0643	0.0108
	DES ASE	7.4933	1.7235	1.2554	0.6277	0.2511	0.1256	0.0628	0.0106
STOCK VOLUME (mL)		1.150	3.642	2.500	2.000	2.500	2.500	0.842	2.500
DILUENT ADDED (mL)**		3.850	1.358	2.500	3.000	2.500	2.500	4.158	2.500
FINAL VOLUME (mL)		5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
CONCENTRATION (ug/mL)	ASE	1.7657	1.2861	0.6431	0.2572	0.1286	0.0643	0.0108	0.0054
	DES ASE	1.7235	1.2554	0.6277	0.2511	0.1256	0.0628	0.0106	0.0053
STOCK ID		STD SS H	STD SS G	STD SS F	STD SS E	STD SS D	STD SS C	STD SS B	STD SS A
SPIKED VOLUME (µL)		1000	1000	1000	1000	1000	1000	1000	1000
BLANK MATRIX ADDED (µL)		49000	49000	49000	49000	49000	49000	49000	49000
TOTAL SPIKED VOLUME (mL)		50	50	50	50	50	50	50	50
CC ID		STD-H	STD-G	STD-F	STD-E	STD-D	STD-C	STD-B	STD-A
MATRIX SPIKED CONCENTRATION (ng/mL)	ASE	35.3140	25.7220	12.8620	5.1440	2.5720	1.2860	0.2160	0.1080
	DES ASE	34.4700	25.1080	12.5540	5.0220	2.5120	1.2560	0.2120	0.1060
CC RANGE %	ASE		72.84						
	DES ASE		72.84						
Suggested Range		2 x Cmax	70-85%					2 x A	

Table – 28 : BULK SPIKING SHEET FOR QCs

MOLECULE		ASE	DES ASE
AMOUNT WEIGHED (mg)		2.159	2.149
VOLUME (mL)*		2.0	2.0
ASSAY (%)		99.68	99.88
MOLECULAR WEIGHT (FREE)		285.77	271.74
MOLECULAR WEIGHT (SALT)		401.84	387.81
FINAL CONCENTRATION (ug/mL)		765.2338	752.0029
STOCK ID		**ASEN-04/Jan**	**DES ASE-04/Jan**
STOCK VOLUME (mL)		0.050	0.050
DILUENT ADDED (mL)**		4.900	
FINAL VOLUME (mL)		5.000	
CONCENTRATION (ug/mL)		7.6523	7.5200
STOCK ID		QC-SD-04/Jan	

DRUG: Asenapine
Batch No.: CS-AA-227
Light Sensitive (Y/N):yes
EXPIRY date: Mar 14

DRUG: N-DesMethyl Asenapine
Batch No.: CS-DAM-382
Light Sensitive (Y/N):NO
Retest date: May 14

STOCK ID		QC-SD-04/Jan	SS HQC	SS MQC	SS INTQC	SS LQC
STOCK CONCENTRATION (ug/mL)	ASE	7.6523	1.2764	0.6382	0.1276	0.0149
	DES ASE	7.5200	1.2543	0.6272	0.1254	0.0146
STOCK VOLUME (mL)		4.170	5.000	2.000	1.165	3.680
DILUENT ADDED (mL)**		20.830	5.000	8.000	8.835	6.320
FINAL VOLUME (mL)		25.000	10.000	10.000	10.000	10.000
CONCENTRATION (ug/mL)	ASE	1.2764	0.6382	0.1276	0.0149	0.0055
	DES ASE	1.2543	0.6272	0.1254	0.0146	0.0054
STOCK ID		**SS HQC**	**SS MQC**	**SS INTQC**	**SS LQC**	**SS LOQQC**
SPIKED VOLUME (µL)		6000	5000	4000	6000	1500
BLANK MATRIX ADDED (µL)		294000	245000	196000	294000	73500
TOTAL SPIKED VOLUME (mL)		300	250	200	300	75
CC ID		**HQC**	**MQC**	**INTQC**	**LQC**	**LOQQC**
MATRIX SPIKED CONCENTRATION (ng/mL)	ASE	25.5280	12.7640	2.5520	0.2980	0.1100
	DES ASE	25.0860	12.5440	2.5080	0.2920	0.1080
CC RANGE %	ASE	72.29	36.14	7.23	2.76	101.85
	DES ASE	72.78	36.39	7.28	2.75	101.89
Suggested Range		70-85%	30-45%	5-30 %	2.5-3 TIMES of STD A	100-105%of STD A

temperature of the gas mobile phase is used to adjust the retention in gas chromatography.

CC: a calibration curve is a general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration.

QC: Quality control is a generic term that refers to the monitoring and assessment of laboratory testing processes to identify problems and maintain performance.

Linearity: The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analytes in samples within a given range or proportional by means of well-defined mathematical transformations

Specificity: The ability of an analytical method to measure only the sought-for analyte or measurand. Numerically characterized by determination of interferences and non-specific responses to other analytes or materials.

Selectivity: Selectivity refers to the extent to which a method can determine particular analyte in mixtures or matrices without interferences
from other components.

Matrix Effect: The combined effect of all components of the sample other than the analyte on the measurement of the quantity. If a specific component can be identified as causing an effect then this is referred to as interference.

ASCOT: All Samples Carry over Test is used to check if the response of a high concentration sample is carried to the next injection by injecting a blank sample after a high concentration sample.

Precision: Closeness of agreement between quantity values obtained by replicate measurements of a quantity, under specified conditions.

Accuracy: Closeness of the agreement between the result of a measurement and a true value of the measured

Recovery: The measurable increase in analyte concentration or activity in a sample after adding a known amount of that analyte to the sample. Characterizes the ability of an analytical method to correctly measure pure analyte when added to the matrix routinely analyzed.

Stability: The term system stability has been defined as the stability of the samples being analyzed in a sample solution.

System Suitability: System suitability is defined by ICH as "the checking of a system, before or during analysis of unknowns, to ensure system performance.

Haemolysed Plasma: the plasma with ruptured erythrocytes (red blood cells) and the release of their contents (cytoplasm) into surrounding fluid (*e.g.*, blood plasma).

Internal Standard: An internal standard is a known amount of a compound, different from analyte that is added to the unknown. Signal from analyte is compared with signal from the internal standard to find out how much analyte is present.

Coefficient Of Variation: The relative standard deviation, i.e., the standard deviation expressed as a percentage of the mean **[CV=100(s/x)]**.

Standard Deviation: A statistic that describes the dispersion or spread of a set of measurements about the mean value of a gaussian or normal distribution.

Regression Model: The equation for the line obtained in linear regression calculations (Y = a + bX). This equation is used to calculate the amount of systematic error from the comparison of methods experiment.

BIBLIOGRAPHY

1. Aneesh T.P. (2012) 'Stress degradation studies and development and validation of RPHPLC method for the estimation of asenapine maleate', Pramana – International Journal of Pharmacy and Pharmaceutical Sciences Vol.1, Issue IV.

2. Aneesh T.P. (2012) 'Development and validation of UV spectro photo metric method for the estimation of asenapine maleate in bulk in pharmaceutical formulation', Scholars Research Library, Der Pharma Chemica Vol. 2.

3. Anthony D. Wright (2012) 'The Importance of ^{1}H-Nuclear Magnetic Resonance Spectroscopy for Reference Standard Validation in Analytical Sciences', Plos one. Vol.7.

4. Arpi Minassian Jared W.Young (2010) 'Evaluation of the clinical efficacy of asenapine in schizophrenia', Bioclarity. Vol. 4.

5. Christy Sarmiento Daniel (2010) 'Development and application of quality standard procedures for an LC-MS systems', Erasmus Mundus Vol.6

6. Daniel Huys. Et al (2009) 'Update on the role of antisychotics in the treatment of Tourette Syndrone', Dovepress. Vol.2.

7. David Taylor(2009), ‘Asenapine monotherapy in the acute treatment of both schizophrenia and bipolar I disorder’, Dovepress. Vol.5

8. Dharmendra Patel (2011) ‘Matrix Effect in a view of LC-MS/MS: an overview’, International Journal of Pharma and Bio Sciences- Vol. 2.

9. Eduard Vieta (2011) ‘Acute and long term treatment of mania’, Pharmachological Aspects. Vol.5.

10. European Medicine Agencies(2012) ‘Assessment of Antipsychotic Drug Asenapine’.

11. Habton W Ressom (2012) ‘Using a spike in experiment to evaluate analysis of LC-MS data’, Proteome Science. Vol. 2.

12. Harshal Kanubhai Trivedi (2009) ‘Development of Stability indicating RPHPLC method for the determination of Rupatadine and its degradation products in solid oral dosage form’, Scientia Pharmaceutica. Vol.3.

13. Hugh M. Marston (2009) ‘Asenapine effects in animal models of Psychosis and cognative function’, Psycho pharmacology. Vol. 7.

14. Izydor Aposto (2012) ‘Uncertainty Estimates of Purity Measurements Based on Current Information: Toward a “Live Validation” of Purity Methods’, Springer. Vol. 3.

15. Jacobs. P.L. (2012) ‘Metabolisam and excretion of Asenapine in healthy male subjects’, Bioclarity. Vol.3.

16. James J Pitt (2010) ‘Principles and applications of LC-MS in clinical bio chemistry’, Bioclarity. Vol. 8.

17. Jodim Gonzalez (2009) ‘Review of the safety efficacy, side effect profile of asenapine in the treatment of bipolar disorder’, Dovepress. Vol. 2.

18. Jodim Gonzalez (2009) ‘Review of the safety efficacy, side effect profile of asenapine in the treatment of bipolar disorder’, Dovepress. Vol.3.

19. Jonathan R. Scarff (2012) ‘Newer oral atypical anticychotic agents : Review’, Bioclarity. Vol. 9.

20. Joseph E Rower (2010) ‘Plasma and its application to the pharmacokinetics studies’, Scientia Pharmaceutica. Vol. 3.

21. Leslie Citrome (2011) ‘A Review of New Atypical Antipsychotic Launches in the United States’, Psychiatry. Vol.7.

22. Liusheng Huang (2012) ‘Determinaion of lumefantrine in small-volume human plasma by LC-MS/MS: Using a deuterated lumefantrine to overcome matrix effect and ionization saturation’, Bioclarity. Vol. 4.

23. Kiran Aarelly (2012) ‘Method development and validation of asenapine in bulk by RPHPLC method’, Journal of chemical and pharmaceutical research. Vol. 5.

24. Maud M. Koek(2010) ‘Quantitative metabolomics based on gas chromatography mass spectrometry: status and perspectives’ Springer. Vol. 3.

25. Maurizio Pompili (2011) 'The role of asenapine in the treatment of manic or mixed states associated with bipolar I disorder', Dovepress.Vol.7.

26. Muralidharan Selvadurai(2011) 'Determination of deflazacort in human plasma by LC-MS after liquid-liquid extraction and its application in human pharmacokinetics studies', Dovepress. Vol. 5.

27. Nageshwara Rao Pilli (2010) 'Simultaneous determination of Atorvastatin and Aspirin in human plasma by LC-MS/MS: its pharmacokinetic application'. Vol. 4.

28. Ramakotaiah Mogili (2011) 'Development and validation Amisulpride in human plasma by HPLC coupled with Tandem MS and its application to a Pharmaco Kinetic Study', Scientia Pharmaceutica. Vol. 6.

29. Susanthakumar. P (2011) 'Development and validation of LC-MS/MS: Method for the estimation Acyclovir in pharmaceutical dosage form', Pharmacophor. Vol. 2.

30. Usmangani K. Chhalotiya (2009) 'Stability indicating liquid chromatography method for the quantification of the new anti pshychotic agent asenapine in bulk and in pharmaceutical formulation', Scientia Pharmaceutica. Vol. 3.

31. Wei Hang (2012) 'Discrimination of urine profiles of BC patients using Analytical Sciences', Pharmacophore. Vol.3.