CHAPTER – IV

STABILITY INDICATING LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF RELATED IMPURITIES OF DRUG PRODUCTS.
4.1. INTRODUCTION:

Impurities can be classified as Organic impurities, (process- and drug-related) Inorganic impurities, Residual solvents. Organic impurities can arise during the manufacturing process and/or storage of the new drug substance. They can be identified or unidentified, volatile or non-volatile, and include Starting materials, By-products, Intermediates, Degradation products, Reagents, ligands and catalysts Inorganic impurities can result from the manufacturing process. They are normally known and identified and include. reagents, ligands and catalysts, Heavy metals or other residual metals, Inorganic salts.

Stability testing forms an important part of the drug product testing, it provide evidence on how quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity, light and enables recommendation of storage conditions, retest period and shelf life to be established. The two main aspects of drug products that play an important role in shelf life determinations are assay of active drug and degradants generated during the stability study. There are several Stability-indicating methods have been reported for determination of assays and related impurities of drug product 1-6. The objective of the current study is to develop and validate stability indicating reversed-phase HPLC method for the determination related impurities of drug product. First HPLC method is developed for the determination of Voriconazole along with its degradation impurities. Whereas second HPLC method is developed for the determination of Eszopiclone along with its degradation impurities.

Voriconazole belongs to a class of antifungal medicine which is used to treat serious, invasive fungal infections, which is generally seen in immune compromised patients. Its chemical designation is (2R, 3S)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)-2-butanol. Voriconazole is commercially available in two strength 50 mg and 200 mg, with brand name Vorizol tablets (Natco Pharmaceuticals Ltd.). Diastereomers are compounds having the same chemical structure but differ in structural arrangement. Chemically sameness creates difficulty in separating diastereomers, but proposed method is capable of separating diastereomers in Voriconazole tablet dosage form. The base degradation of
Voriconazole may seriously affect the quality of products, and is usually associated with a reduction of the pharmacological activity and/or the occurrence of side effects. The stress conditions are useful for establishing degradation pathways, developing and validating suitable procedures. Hence there is need to develop stability indicating HPLC method for the determination of degradation impurities and diastereomers in Voriconazole tablet.

Eszopiclone is a nonbenzodiazepine hypnotic drug used to treat insomnia. Eszopiclone belongs to the class of drugs known as cyclopyrrolones. Eszopiclone is the (S)-enantiomer of zopiclone and its chemical designation is (S)-6-(5-chloro-2-pyridinyl)-7-oxo-6,7-dihydro-5H-pyrrolo[3,4-b]pyrazin-5-yl-4-methyl-1-piperazinecarboxylate. Impurities A, B and C are known impurities; Eszopiclone tablets are commercially available in three strengths (1, 2 and 3 mg), with brand name Fulnite tablets (Sun Pharmaceuticals). The objective of this research work was to develop a stability-indicating isocratic LC method for the determination of Eszopiclone and its degradation impurities in tablet dosage form.

4.2. LITERATURE SURVEY:
In this section summarized some of the important analytical methods for the determination of Voriconazole in tablet dosage form or in biological samples.

Gennethel J. P., et. al. (2003)

Gennethel J. P., et. al. reported an analytical method for the determination of voriconazole in plasma. The method utilizes solid-phase extraction technology and high-performance liquid chromatography. The mobile phase consisted of 0.01 M TEMED (N,N,N′,N′-tetramethylethylenediamine) phosphate buffer (adjusted to pH 7.4 with phosphoric acid) added to acetonitrile (55:45, v/v). injection volume was kept 20µL. HPLC conditions: reverse-phase Luna 5-µm C_{18} column (250 by 4.6 mm), preceded by a universal SecurityGuard cartridge (Phomenex, Torrance, Calif.), a detector wavelength of 254 nm, and a mobile-phase flow rate of 1 ml/min. Retention times was approximately 9.0 minutes for voriconazole.
Stability indicating liquid chromatographic methods for the determination of related impurities of drug products.

Keevil B.G., et. al. (2004)\textsuperscript{10}

Keevil B.G., et. al. reported an assay method for voriconazole in serum samples using liquid chromatography. After vigorous mixing and centrifugation, 3 µL of the supernatant was injected into the HPLC-MS/MS system. An HPLC system was used to elute a C18 cartridge (2 mm x 4 mm) at 0.6 ml/min with a step gradient of 50\% to 100\% methanol containing 2 mM ammonium acetate and 0.1\% (v/v) formic acid. The column was maintained at 55 °C, and the retention times were voriconazole 1.50 minutes and ketoconazole 1.47 minutes. Cycle time was 3 minutes, injection to injection. The analytes were monitored using a tandem mass spectrometer operated in multiple reaction monitoring mode using the following transitions: voriconazole m/z 350.0 > 224.1 and ketoconazole m/z 531.1 > 489.1.

Adams A.I.H., et. al. (2005)\textsuperscript{11}

Adams A.I.H., reported a High Performance Liquid Chromatographic Method for the Determination of Voriconazole Content in Tablets. The method employs a Merck LiChrospher\textsuperscript{®} 100 RP-8 (125 × 4.6 mm I.D., 5 µm particle size) column, with a mobile phase of methanol : triethylamine solutions 0.6 \%, pH 6.0 (50:50, v/v) and UV detection at 255 nm. A linear response (r > 0.9999) was observed in the range of 20.0–100.0 µg ml\textsuperscript{−1}. The method showed good recoveries (average 100.4\%) and the relative standard deviation intra and inter-day were ≤ 1.0 \%. Validation parameters as specificity and robustness were also determined.

Srinubabu G., et. al. (2007)\textsuperscript{12}

Srinubabu G., et. al. reported HPLC method for the determination of voriconazole in pharmaceutical formulation using an experimental design. Chromatography was performed with mobile phase containing a mixture of acetonitrile and water (50:50, v/v) with flow rate was of 1.0 ml/min and UV detection at 260 nm. The procedure was validated for linearity (correlation coefficient=0.9999), accuracy, robustness and intermediate precision. Experimental design was used for validation of robustness and intermediate precision. To test robustness, three factors were considered. Percentage of acetonitrile in mobile phase, flow rate and p(H); an increase in the flow rate results in a decrease of the drug found concentration, while the percentage of
organic modifier and pH have no important effect on the response. For intermediate precision measure the variables considered were: analyst, equipment and number of days. The R.S.D. value (0.45%, n=24) indicated a good precision of the analytical method.

**Simmel F., et. al. (2008)**

Simmel F., et al. reported HPLC method for the quantification of voriconazole in plasma. Voriconazole is a very potent antifungal agent used to treat serious fungal infections (candidiasis); it is also the therapy of choice for aspergillosis. Microdialysis is considered to be an outstanding minimally invasive method. For determination of voriconazole in microdialysate and human plasma a new, efficient, reliable, and robust HPLC assay using UV detection at 254 nm has been developed and validated. After simple sample preparation using acetonitrile for plasma and for microdialysate, 20 µL were injected and separated on an RP-18 column. The chromatographic run time was less than 4 min.

**Kheter A.B., et. al. (2009)**

Kheter A.B., et al. reported Stability Indicating R.P-HPIC method for voriconzole. Voriconazole was subjected to stress degradation under different conditions recommended by International Conference on Harmonization. The sample so generated was used to develop a stability-indicating high performance liquid chromatographic method for voriconazole. The peak for voriconazole was well resolved from peaks of degradation products, using a Hypersil C18 (250x4.6 mm) column and a mobile phase comprising of acetonitrile: water (40:60, v/v), at flow rate of 1 ml/min. Detection was carried out using photodiode array detector.

**Gu P., et. al. (2009)**

Gu P., et al. reported stability-indicating HPLC method for determination of voriconazole and its related substances. using an Agilent Zorbax SB-C18 (250mm x 4.6 mm, 5 µ) column maintained at 25°C with a mobile phase of a mixture of ammonium phosphate dibasic buffer (pH adjusted to 6.0 using diluted orthophosphoric acid; 50 mM)-acetonitrile (52:48, v/v). The mobile phase flow rate was 1.0 ml/min, and the detection wavelength was 250 nm.
Bharathi J., et. al. (2010)\textsuperscript{16}

Bharathi J., et al. reported RP-HPLC Method for the Estimation of Voriconazole in Bulk and Tablet Dosage Form. Isocratic elution at a flow rate of 1 ml/min was employed on a Inertsil C8 column (250 x 4.6 mm; 5µ) at ambient temperature. The mobile phase consisted of Buffer (0.01M sodium dihydrogen orthophosphate, pH was adjusted to 5.0): Acetonitrile (50:50 v/v). The UV detection wavelength was 254 nm and 20µl of sample was injected. The retention time for Voriconazole was 6.905 min.

Eldin A.B., et. al. (2010)\textsuperscript{17}

Eldin A.B., el. Al. reported HPLC method for the determination of voriconazole and its degradation products in pharmaceutical formulation. Chromatography was performed with mobile phase containing a mixture of acetonitrile and 0.05M disodium hydrogen phosphate buffer, pH5.5 (1:1, v/v) with flow rate of 1.0 ml/min., C18 column and UV detection at 255 nm. developed method satisfies the system suitability criteria, peak integrity, and resolution for the parent drug and its degradants.

Cheng Y., et. al. (2011)\textsuperscript{18}

Cheng Y., et. al. reported liquid chromatography-electrospray ionization mass spectrometry method for Quantification of voriconazole in human plasma. Identification and Quantification of voriconazole in human plasma has been perform by liquid-liquid extraction, voriconazole and Loratadine were separated using a mobile phase comprised of methanol: water (0.1% formic acid) = 75:25 v/v on a Shimadzu Shim-pack VP-ODS C18 (150 x 2.0 mm ID, 5 microm) column and analyzed by electrospray ionization mass spectrometry. The chromatographic separation was achieved in less than 6 min.

Pauwels S., et. al. (2012)\textsuperscript{19}

Pauwels S., et. al. reported LC-MS/MS method for quantifying plasma voriconazole. Analysis was performed on a Quattro Micro tandem mass spectrometer equipped with an Alliance HPLC 2795 separations module. MRM transitions were m/z 350.0→281.4 for
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voriconazole and m/z 353.0→284.4 for d(3)-voriconazole. Quantification was done by both linear calibration curves and multiplication of the response ratio by a predefined factor.

Lin D., et. al. (2013)²⁰

Lin D., et. al. reported HPLC-ESI-MS method for the determination of voriconazole in human plasma. Chromatographic separation was conducted on an Ultimate C18 column with a mobile phase consisting of acetonitrile-water (containing 0.1% formic acid; 40:60, v/v) at a flow rate of 0.3 ml/min. The detection of voriconazole was performed on a triple-quadrupole tandem mass spectrometer by multiple reaction monitoring with an electrospray ionization source in the positive mode.

Other than above mentioned methods few more methods are also reported for the quantification of voriconazole in human plasma²¹,²²,²³ and HPLC method for the determination of voriconazole in bulk drug ²⁴.

Min M., et. al. (2007)²⁵

Min M., et. al. reported LC/Ms/Ms method for quantitation of Eszopiclone in human Plasma. Chromatographic conditions involves Column: AGP chiral analytical column (50 mm x 2.0 mm,) 5 μm particle size Mobile Phase: 85/15 10 mM ammonium acetate in water (pH unadjusted) / MeOH. LC program: isocratic, flow rate: 0.5 ml/min.

Meng M., et. al. (2010)²⁶

Meng M., et. al. reported the application of computer software ACD Lab to facilitate the development of chiral separation for the quantitation of eszopiclone using LC-MS/MS technology. Assisted by ACD/Chrom Manager and LC Simulator software, the optimal chiral chromatographic development was completed within hours. The baseline chiral separation was achieved with a total cycle time of 3 min. For sample extraction method development, a waters oasis sorbent selection plate containing four different sorbents was utilized. Optimal conditions were determined using a single plate under various load, wash and elution conditions. This was followed by a GLP validation which demonstrated excellent intra- and inter-day accuracy and
precision for the quantitation of eszopiclone in human plasma at 1.00-100 ng/ml range using LC/MS/MS technology.

**Sunil R. D., et.al. (2011)**

Sunil R. D., et. al reported HPLC method for the determination of Eszopiclone, chromatographic conditions consists of A Thermo Hypersil BDS–C18 (250 mm × 4.6 mm, 5.0 µ) column. mobile phase containing methanol : water pH adjusted to 2.5 with ortho phosphoric acid (40 : 60 v/v at flow rate of 1 ml/min using UV detection at 304 nm. The method is validated according to ICH guidelines. The developed method is found to be precise, accurate, specific and selective.

**Yang L.L., et. al. (2011)**

Yang L.L., et. al reported UV spectrophotometry method for the determination of Eszopiclone. method: Taking 0.1mol o L-1 hydrochloric acid as solvent, this paper uses UV spectrophotometric method to determine the contents of Eszopiclone in Eszopiclone tablets. The calibration curve is linear(r = 0.9999) within the range of 2.5~25.0 µg/ml for Eszopiclone. The specificity, precision, recovery rate and stability are good.

**Hotha K.K., et. al. (2012)**

Hotha K.K., et. al reported A rapid LC-MS/MS method for quantitation of Eszopiclone in human plasma. . A simple liquid-liquid extraction process was used to extract ESZ and IS from human plasma. The total run time was 1.5 min and the elution of ESZ and IS occurred at 0.90 min; this was achieved with a mobile phase consisting of 0.1% formic acid-methanol (15:85, v/v) at a flow rate of 0.50 ml/min on a Discover C(18) (50 × 4.6 mm, 5 µm) column. The developed method was validated in human plasma with a lower limit of quantitation of 0.1 ng/ml for ESZ. A linear response function was established for the range of concentrations 0.10-120 ng/ml (r > 0.998) for ESZ. The intra- and inter-day precision values for ESZ were acceptable as per FDA guidelines.
Lanka A.R.P., et.al. (2012)\textsuperscript{30}

Lanka A. R.P., reported UPLC method for the Determination of Eszopiclone and its related impurities. The chromatographic separation was achieved on a C18 stationary phase. The method employed a linear gradient elution and the detection wave-length was set at 303 nm and 240 nm for impurity C. The mobile phases consists of buffer and acetonitrile delivered at a flow rate of 0.5 ml/min. Buffer consists mixture of monosodium and di sodium ortho phosphate. The stress samples were assayed against a qualified reference standard and the mass balance was found to be close to 98.5%. But in the reported method, Impurities B and C are not included, the major degradation impurity, i.e., Impurity D is not identified and the relative response factors (RRFs) of impurities is not established.

Pandya J., et.al. (2013)\textsuperscript{31}

Pandya J., et.al. reported UV Spectrophotometric and RP-HPLC method for the estimation of eszopiclone bulk and tablets The RP-HPLC method for Eszopiclone was developed using Shimadzu HPLC, LC-10, Temperature maintained 25°C, Phenorex Gemini 18 (250 mm x 4.6mm x 5 µm), as stationary particles, isocratic mode. Methanol: Water (80:20 v/v) as mobile phase. Mobile phase was maintained at a flow rate of 1.0 ml/min and detection was carried out at 305nm.

4.3. A VALIDATED STABILITY INDICATING LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF DEGRADATION IMPURITIES AND DIASTEREOMERS IN VORICONAZOLE TABLET.

To the best of our knowledge all reported analytical methods are used only for the quantification of Voriconazole, not for quantification of known related compounds and degradation impurities of Voriconazole in tablet dosage form. Voriconazole degrades significantly in base hydrolysis stress condition as compare with acid hydrolysis. Major degradation impurities have been observed as deschloro and DFH impurity. One HPLC method is available for the determination of voriconazole and its related substances \textsuperscript{15}, but this method is
not mentioning the chromatographic separation of diastereomer of voriconazole along with degradation impurities.

Literature survey reveals that Voriconazole tablet is not official in any pharmacopeia. None of the currently available analytical method is capable of separating all the degradation impurities and process related impurities in voriconazole tablet dosage form. The present work describes analytical parameters aimed to achieve an alternative for the quantification of Voriconazole and its degradation products along with diastereomers in tablets dosage forms, in accordance with ICH recommendations. A reversed-phase gradient liquid chromatographic method has been developed for the quantitative determination of Voriconazole, along with its degradation and diastereomeric impurities in tablet dosage form.

Chromatographic separation has been achieved on an Inertsil ODS 3V, 150 x 4.6 mm, 5 μm column. The mobile phase consisting of solvent A 0.05 molar (M) potassium dihydrogen phosphate (pH 2.5 buffer) and solvent B (mixture of acetonitrile and methanol in the ratio 90:10 (v/v)), was delivered at a flow rate of 1.2 mL min⁻¹ with the detection wavelength at 256 nm. Resolution of Voriconazole and all five potential impurities was achieved at greater than 2.0 for all pairs of compounds. The drug was subjected to stress conditions such as oxidative, acid and base hydrolysis, thermal and photolytic degradation. Voriconazole was found to degrade significantly under base hydrolysis stress conditions compared to acid hydrolysis stress conditions. The degradation products was well-resolved from the main peak and its impurities, thus proving the stability-indicating power of the method. The stressed sample was assayed against a reference standard and the mass balance was found to be close to 99.0%. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision, and robustness.

4.3.1. DRUG PROFILE:
4.3.1.1. Voriconazole:
1. Chemical Name:
   (2R,3S)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)-2-butanol)
2. Chemical Structure:

3. Molecular Formula: C₁₆H₁₄F₃N₅O

4. Molecular Weight: 349.3

5. Description: White to light-colored powder

6. Solubility: Slightly soluble in water and ethanol and soluble in phosphate buffer (pH3.2).

7. Melting Point: 202-204 °C

8. Category: antifungal medicine

4.3.1.2. Voriconazole Related impurities:

Deschloro Impurity: 4-Ethyl-5-trifluoro pyrimidine

DFH Impurity: 1-(2,4-difluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethanone

Impurity A: (2R,3S/2S,3R)-3-(4-chloro-5-fluoropyrimidin-6-yl)-2-(2,4-difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-2-butanol,
4.3.2. EXPERIMENTAL:

4.3.2.1. Working standards:

The working standard was procured from India market having following batch number and potency.

<table>
<thead>
<tr>
<th>Working Standard</th>
<th>Batch Number</th>
<th>Potency (on as is basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole</td>
<td>VE001243</td>
<td>99.6 % w/w</td>
</tr>
</tbody>
</table>

4.3.2.2. Sample:

The sample was procured from Indian market. The test sample bears following details,

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Natco Pharmaceuticals Ltd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Name</td>
<td>Vorizol</td>
</tr>
<tr>
<td>Label Claim</td>
<td>50 mg and 200 mg</td>
</tr>
</tbody>
</table>

4.3.2.3. Instrument / Apparatus Used:

i) All the glassware used for the experiment were certified ‘A’ grade manufactured by SCHOTT Glass India Pvt. Ltd. Mumbai, India

ii) A calibrated high performance liquid chromatography (HPLC), make Agilent-1100 series was used for all the experiments.

iii) A calibrated digital pH meter, manufactured by Mettler-Toledo Inc, Columbus, OH. Private Limited Mumbai, India.

iv) A calibrated analytical balance, manufactured by Sartorius, Germany.

v) A sonicator, manufactured by Amrut Enterprises, Pune, India.
vi) A vacuum oven manufactured by Quality instruments and equipments, Mumbai, India.

4.3.2.4. Reagents and chemicals:
All reagents and chemicals were used from Merck chemicals. Potassium dihydrogen phosphates were used as GR grade. Acetonitrile, Methanol and Water were used as HPLC grade.

4.3.2.5. Preparation of standard Solutions:
A stock solution of voriconazole (300 μg/ml) was prepared by dissolving an appropriate amount in solvent mixture. Standard solutions containing 3 μg/ml were prepared from this stock solution.

4.3.2.6. Preparation of sample solution:
Tablet powder equivalent to 50 mg drug was dissolved in solvent with rotary shaking for 10 min and sonication for 10 min to give a solution containing 1000 μg/ml. This solution was filtered through a 0.45 μm pore size Nylon 66 membrane filter.

4.3.2.7. Chromatographic conditions:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>mobile phase A</td>
<td>0.05 molar (M) potassium dihydrogen phosphate (pH 2.5) buffer</td>
</tr>
<tr>
<td>mobile phase B</td>
<td>mixture of acetonitrile and methanol in the ratio 90: 10 (v/v); respectively.</td>
</tr>
<tr>
<td>Column</td>
<td>Inertsil ODS 3V 150x4.6mm, 5μm</td>
</tr>
<tr>
<td>Column oven temp.</td>
<td>35°C</td>
</tr>
<tr>
<td>Flow</td>
<td>1.2 ml/min</td>
</tr>
<tr>
<td>Wavelength</td>
<td>256 nm</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 μL</td>
</tr>
<tr>
<td>Runtime</td>
<td>45 minutes.</td>
</tr>
</tbody>
</table>

Mobile phase gradient program:
Stability indicating liquid chromatographic methods for the determination of related impurities of drug products.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (ml/min)</th>
<th>Mobile phase A (%)</th>
<th>Mobile phase B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>1.2</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>38</td>
<td>1.2</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>40</td>
<td>1.2</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>45</td>
<td>1.2</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

4.3.2.8. Procedure:

HPLC system was set up as described under chromatographic conditions. Standard and sample solution was prepared according to 4.3.5 and made single injection of each of solvent mixture as a blank, standard solution (six injections), placebo solution and sample solution in to the chromatographic system.

- Recorded the chromatograms at 256 nm and measure the peak area counts for all eluting peaks.
- Examined the blank and placebo chromatogram for any extraneous peaks and disregard corresponding peaks observed in the chromatogram of the sample solution.

4.3.2.9. Calculations:

Known Impurity % (w/w) = \( \frac{AT \times WS \times DT \times P \times AW}{AS \times DS \times WT \times 100 \times LC} \)

Unknown Impurity % (w/w) = \( \frac{AT_1 \times WS \times DT \times P \times AW}{AS \times DS \times WT \times 100 \times LC} \)

Total Impurities = Sum of all known and unknown impurities

Where,

\( AT \) : Area of Known impurity peak in sample solution.

\( AT_1 \) : Area of Unknown impurity peak in sample solution.
Stability indicating liquid chromatographic methods for the determination of related impurities of drug products.

AS : Average area of Voriconazole peak in standard solution.
WS : Weight of Voriconazole standard in mg.
DS : Dilution of Voriconazole standard in ml.
DT : Dilution of Voriconazole sample solution.
WT : Weight of Voriconazole sample in mg.
P : Potency of Voriconazole working standard on as is basis.
LC : Label claim of Voriconazole in mg per tablet.
AW : Average weight of tablet in mg.

4.3.3. RESULTS AND DISCUSSION:

The important criteria for development of successful RP-HPLC method for determination of Voriconazole related substances in tablet dosage form was the method should be able to determine all impurities of the drug in single run with the good amount of resolution. Method should be accurate, reproducible, robust, stability indicating, free from interference (blank/placebo/ other unknown degradation product) and straight forward enough for routine use in quality control laboratory. The main objective of the chromatographic method development was to separate voriconazole impurities and main peak with good amount of resolution.

Initial method development was started with Isocratic mobile phase. Different combination of buffer : acetonitrile in the range of 90:10 to 10:90 v/v has been tried, it has been observed that Deschloro impurity and DFH impurity are most polar in nature where as Impurity A is nonpolar in nature. Increase in buffer concentration more than 50% in the mobile phase leads to more retention of impurity A on the column, which leads to increase in run time more than 60 minutes also peak shape of impurity A is not proper. By decrease in buffer concentration less than 50% in mobile phase retention of impurity A is reduced but resolution between Deschloro impurity and DFH impurities decreases also both the peak elutes near to void volume. So Switch to gradient mobile phase where potassium dihydrogen phosphate (pH 2.5) buffer used as mobile phase A and Mobile phase B used as acetonitrile and methanol in the ratio 90:10 v/v. Different gradient programs has been tried to improves the run time less than 60
minutes with good retention of deschloro impurity and DFH impurity on the column. Peroxide stressed sample and impurity spiked sample injected on column to check good amount of resolution between known, unknown impurities and voriconazole.

During the optimization of the method the gradient program has been finalized as time (min) / % solution B: 0/20, 20/40, 38/60, 40/20 and 45/20. The column temperature was maintained at 35 °C and the detection was monitored at a wavelength 256 nm. The injection volume was 20 µL. The relative response factor for all the five impurities has been determined with respect to voriconazole (Table 4.1). An HPLC chromatogram obtained from a sample of Voriconazole spiked with all five impurities at the 0.30% level is shown in Figure 4.1.

Table 4.1: Chromatographic performance data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (Min)</th>
<th>RRT (n = 3)</th>
<th>Resolution (n = 3)</th>
<th>Tailing factor (n = 3)</th>
<th>RRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deschloro impurity</td>
<td>7.189</td>
<td>0.33</td>
<td>---</td>
<td>1.01</td>
<td>1.3</td>
</tr>
<tr>
<td>DFH/TAP</td>
<td>9.063</td>
<td>0.42</td>
<td>8.7</td>
<td>1.08</td>
<td>1.7</td>
</tr>
<tr>
<td>Voriconazole Diasterio.</td>
<td>16.051</td>
<td>0.74</td>
<td>22.3</td>
<td>1.09</td>
<td>0.8</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>21.784</td>
<td>1.00</td>
<td>16.9</td>
<td>1.02</td>
<td>1.0</td>
</tr>
<tr>
<td>Impurity A Diasterio.</td>
<td>25.559</td>
<td>1.17</td>
<td>10.5</td>
<td>1.14</td>
<td>0.9</td>
</tr>
<tr>
<td>Impurity A</td>
<td>30.642</td>
<td>1.41</td>
<td>13.9</td>
<td>1.16</td>
<td>1.1</td>
</tr>
</tbody>
</table>

![Fig. 4.1 Chromatogram of Placebo, Blank and Sample solution spiked with impurities.](image)
4.3.3.1. Validation of method:

The optimized chromatographic conditions were validated by evaluating specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and system suitability, in accordance with ICH guidelines Q2 (R1)\textsuperscript{46-48}.

4.3.3.1.1. Specificity:

The specificity of a method is its suitability for analysis of a substance in the presence of potential impurities. Stress testing of a drug substance can help to identify likely degradation products, which can help to establish degradation pathways and the intrinsic stability of the molecule. It can also be used to validate the stability-indicating power of the analytical procedures used. The specificity of the LC method for voriconazole has been determined in the presence of five impurities.

4.3.3.1.2. Forced degradation study of drug product:

The stress degradation study has been performed on the drug product includes acid hydrolysis (5ml of 0.1 M HCl at 60°C for 2hr), base hydrolysis (5ml of 0.1 M NaOH at 60°C for 30min), oxidation (2ml of 1% H₂O₂ at bench top for 30min), thermal (60°C for 24hrs), humidity (40°C, 75% RH for 7days) and photolytic degradation (drug product exposed UV and Visible light so has to complete 1.2 million lux hours and 200 watt h/m\(^2\)). The stress study was performed as per international conference on harmonization (ICH) recommendation\textsuperscript{49-60}. Peak purity has been checked for the Voriconazole peak by using PDA detector in stress samples. Assay of stressed samples has been performed by comparison with reference standard and the mass balance (% assay + % impurities + % degradation products) were calculated.

There was no peak found at the retention time of voriconazole and its all five impurities in blank and placebo blend chromatograms proves no interference from blank and placebo. Slight degradation was observed when drug product was subjected to acid hydrolysis and peroxide stress conditions and stable in photolytic and humidity stress condition. Voriconazole was sensitive in Basic and Heat conditions and significantly degraded into Deschloro, DFH impurity (Figure 4.2,4.3,4.4). Peak-purity test results from the PDA detector confirmed that
Voriconazole peak obtained from all the stress samples analyzed was homogeneous and pure. Peak purity results from the PDA detector for the peaks produced by degradation of Voriconazole confirmed that all these peaks were homogeneous and pure for all the stress samples analyzed (Table 4.2). The mass balance for the stressed samples was close to 99% (Table 4.2). Assay of Voriconazole was unaffected by the presence of the impurities/degradation products, confirming the stability-indicating power of the method.

Table 4.2: Stress testing (forced degradation) data of Voriconazole.

<table>
<thead>
<tr>
<th>Stress Condition</th>
<th>% Net Degradation</th>
<th>Purity Angle</th>
<th>Purity threshold</th>
<th>Purity flag</th>
<th>Mass Balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Hydrolysis</td>
<td>1.6</td>
<td>0.341</td>
<td>0.873</td>
<td>No</td>
<td>99.8</td>
</tr>
<tr>
<td>Base Hydrolysis</td>
<td>7.2</td>
<td>0.567</td>
<td>0.912</td>
<td>No</td>
<td>99.5</td>
</tr>
<tr>
<td>Peroxide Oxidation</td>
<td>1.4</td>
<td>0.456</td>
<td>0.767</td>
<td>No</td>
<td>99.1</td>
</tr>
<tr>
<td>Photolytic-sunlight</td>
<td>Stable</td>
<td>0.256</td>
<td>0.834</td>
<td>No</td>
<td>99.5</td>
</tr>
<tr>
<td>Heat Stress</td>
<td>5.0</td>
<td>0.342</td>
<td>0.781</td>
<td>No</td>
<td>99.2</td>
</tr>
<tr>
<td>Humidity Stress</td>
<td>Stable</td>
<td>0.456</td>
<td>0.621</td>
<td>No</td>
<td>99.8</td>
</tr>
</tbody>
</table>

Mass balance = % assay + % impurities + % degradation products.

Fig. 4.2: A Typical HPLC Chromatogram of Acid Stressed Blank, Placebo and Sample.
Stability indicating liquid chromatographic methods for the determination of related impurities of drug products.

4.3.3.1.3. Limits of Detection and Quantification:

LOD and LOQ for the five impurities and voriconazole were estimated as the amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively, by injecting a series of dilute solutions of known concentration. Precision was also determined at the LOQ level by analysis of six individual preparations of the five impurities and calculating the RSD (%) of the peak area for each impurity.

4.3.3.1.4. Linearity:

Solutions for testing linearity for the related substances were prepared by diluting the impurity stock solution to five different concentrations from the LOQ to 200% of the permitted
maximum level of the impurity (i.e. the LOQ to 0.6% for Voriconazole and all impurities for an
analyte concentration of 1000 μg/ml). The correlation coefficients, slopes, and y-intercepts of the
calibration plots are reported. Calibration plots for the Five related substances were linear over
the ranges tested. The correlation coefficients were >0.999 for all the components (Table 3).
These results show there was an excellent correlation between the peak area and concentration
for the five impurities.

4.3.3.1.5. Precision:

The precision of the method verified by repeatability and by intermediate precision.
Repeatability was checked by injecting five individual preparations of Voriconazole real sample
spiked with 0.30% of its five impurities . The intermediate precision of the method was also
evaluated using different analyst and different instrument, and performing the analysis on
different day. %RSD of area for each impurity was calculated for both precision as well as
intermediate precision and was found within 2%. These results confirmed the precision and
ruggedness of the method (Table 4.3).

Table. 4.3: Regression and precision data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOQ μg/ml</th>
<th>LOD μg/ml</th>
<th>Regression equation (y)</th>
<th>Correlation coefficient</th>
<th>Precision (Intermediate Precision) (% RSD)</th>
<th>Precision LOQ (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole</td>
<td>0.25</td>
<td>0.09</td>
<td>21285.2 196.3</td>
<td>0.9998</td>
<td>1.5 (1.6)</td>
<td>1.2</td>
</tr>
<tr>
<td>Deschloro</td>
<td>0.22</td>
<td>0.06</td>
<td>28308.9 271.0</td>
<td>1.0000</td>
<td>1.2 (1.5)</td>
<td>1.3</td>
</tr>
<tr>
<td>DFH</td>
<td>0.15</td>
<td>0.05</td>
<td>36980.4 373.4</td>
<td>1.0000</td>
<td>1.6 (1.3)</td>
<td>1.7</td>
</tr>
<tr>
<td>Voriconazole Diastereo.</td>
<td>0.30</td>
<td>0.08</td>
<td>18197.2 421.4</td>
<td>0.9996</td>
<td>1.8 (1.6)</td>
<td>1.2</td>
</tr>
<tr>
<td>Imp. A Diastereo.</td>
<td>0.28</td>
<td>0.07</td>
<td>19278.9 77.3</td>
<td>0.9999</td>
<td>1.4 (1.5)</td>
<td>1.3</td>
</tr>
<tr>
<td>Imp. A</td>
<td>0.26</td>
<td>0.07</td>
<td>24032.3 270.7</td>
<td>0.9997</td>
<td>1.7 (1.1)</td>
<td>1.2</td>
</tr>
</tbody>
</table>
4.3.3.1.6. **Accuracy:**

For the impurities, recovery was determined in triplicate for LOQ, 0.15, 0.30 and 0.45% of the analyte concentration (1000 μg/ml) for Voriconazole and recovery of the impurities was calculated. (Table 4.4).

**Table 4.4: Evaluation of accuracy.**

<table>
<thead>
<tr>
<th>Ammount Spiked</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Voriconazole</td>
</tr>
<tr>
<td>LOQ</td>
<td>99.7 ± 0.11</td>
</tr>
<tr>
<td>50%</td>
<td>98.9 ± 0.31</td>
</tr>
<tr>
<td>100%</td>
<td>99.9 ± 0.10</td>
</tr>
<tr>
<td>150%</td>
<td>99.2 ± 0.21</td>
</tr>
</tbody>
</table>

4.3.3.1.7. **Robustness:**

To determine the robustness of the method the experimental conditions were deliberately changed and the resolution of Voriconazole and the five impurities was evaluated. To study the effect of flow rate on resolution it was changed to 1.0 and 1.4 ml/ min. The effect of pH was studied at pH 2.3 and 2.7. The effect of column temperature was studied at 30 and 40 °C. In all these experiments the mobile phase components were not changed. The effect of the percent organic strength on resolution was studied by varying acetonitrile by −10 to +10% while other mobile phase components were held constant. In all the deliberate varied chromatographic conditions the selectivity as well as the performance of the method were unchanged proves the robustness of the method.

4.3.3.1.8. **Stability in Solution and in the Mobile Phase:**

No significant changes in the amounts of the five impurities were observed during solution stability and mobile phase stability experiments when performed using the related substances method. The results from solution stability and mobile phase stability experiments
confirmed that standard solutions and sample were stable for up to 24 h during determination of related substances. The mobile phase was stable up to 48h.

4.3.4. CONCLUSION:

The gradient RP-HPLC method developed for quantitative analysis of Voriconazole and related impurities in tablet dosage form is precise, accurate, linear, robust, rugged and specific. Satisfactory results were obtained from validation of the method. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of Voriconazole tablet dosage form.

4.4. STABILITY-INDICATING LC–UV METHOD FOR THE DETERMINATION OF ESZOPICLONE AND DEGRADATION IMPURITIES IN TABLET DOSAGE FORM.

A literature survey reveals that Eszopiclone tablet for related substances is not official method in any pharmacopeia. A forced degradation study was performed on the drug product to show the stability-indicating capability of the method. The developed method is stability-indicating, simple, robust, precise and accurate for the determination of Eszopiclone and degradation impurities in tablet dosage form. The method is capable of separating the peaks due to the degradation products from the main peak. The method was validated as per ICH requirements and thus is useful for routine analysis in quality control laboratories.

The chromatographic separation was achieved on an Inertsil C18 column (250 3 4.6 mm, 5 mm), using a mobile phase consisting of 0.05M monobasic sodium phosphate buffer containing 0.8% sodium lauryl sulfate (pH 3.5) and acetonitrile in the ratio of 60:40 (v/v), at a flow rate of 1.5 mL/min and temperature of 40°C. Quantification was achieved with photodiode array detection at 303 nm. The described method showed excellent linearity over a range of limits of quantification to 4.8 mg/mL (150% of specification limit; i.e., 3.2 mg/mL). The drug product was subjected to the stress conditions of oxidative, acid, base, thermal and photolytic degradation. Eszopiclone degradation was observed in acid hydrolysis, base hydrolysis and peroxide stress conditions. Eszopiclone was stable in thermal and photolytic degradation.
Stability indicating liquid chromatographic methods for the determination of related impurities of drug products.

conditions. The developed method is simple, selective and accurate for the quantification of impurities and degradation products of eszopiclone in tablet dosage form.

4.4.1. DRUG PROFILE:

4.4.1.1. Eszopiclone:

1. Chemical Name: (S)-6-(5-Chloro-2-pyridinyl)-7-oxo-6,7-dihydro-5H-pyrrolo[3,4-b]pyrazin-5-yl-4-methyl-1-piperazincarboxylate,

2. Chemical Structure:

![Chemical Structure Image]

3. Molecular Formula: \( C_{17} H_{17} ClN_{6}O_{3} \)

4. Molecular Weight: 388.808

5. Description: White to light yellow crystalline solid

6. Solubility: Slightly soluble in water and ethanol and soluble in phosphate buffer (pH3.2).

7. Melting Point: 202-204 °C

8. Category: Nonbenzodiazepine hypnotic drug

4.4.1.2. Eszopiclone related impurities:

![Impurity A Image]

Impurity A: (5RS)-6-(5-chloropyridin-2-yl)-7-oxo-6,7-dihydro-5H-pyrrolo[3,4-b]pyrazin-5-yl 4 methylpiperazine-1-carboxylate 4 oxide.
Stability indicating liquid chromatographic methods for the determination of related impurities of drug products.

Impurity B: (7RS)-6-(5-chloropyridin-2-yl)-7-hydroxy-6,7-dihydro-5H-pyrrolo[3,4-b]pyrazin-5-one.

Impurity C: 6-(5-chloropyridin-2-yl)-6,7-dihydro-5H-pyrrolo[3,4-b]pyrazin-5-one.

Impurity D: 2 amino-5-chloropyridine.

4.4.2. EXPERIMENTAL:

4.4.2.1. Working standards:

The working standard was procured from India market having following batch number and potency.

<table>
<thead>
<tr>
<th>Working Standard</th>
<th>Batch Number</th>
<th>Potency (on as is basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eszopiclone</td>
<td>EZ00258</td>
<td>99.2% w/w</td>
</tr>
</tbody>
</table>

4.4.2.2. Sample:

The sample was procured from India market. The test sample bears following details,

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Sun pharmaceutical LTD.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Name</td>
<td>Fulinte tablets</td>
</tr>
<tr>
<td>Label Claim</td>
<td>1 mg, 2 mg and 3 mg</td>
</tr>
</tbody>
</table>
4.4.2.3. **Instrument / Apparatus Used:**

i) All the glassware used for the experiment were certified ‘A’ grade manufactured by SCHOTT Glass India Pvt. Ltd. Mumbai, India

ii) A calibrated high performance liquid chromatography (HPLC), make Agilent-1100 series was used for all the experiments.

iii) A calibrated LC/MS, Agilent 1100 Sereies LC/MSD Trap SL system

iv) A calibrated digital pH meter, manufactured by Mettler-Toledo Inc, Columbus, OH.

v) A calibrated analytical balance, manufactured by Sartorius, Germany.

vi) A sonicator, manufactured by Amrut Enterprises, Pune, India.

vii) A vacuum oven manufactured by Quality instruments and equipments, Mumbai, India.

4.4.2.4. **Reagents and chemicals:**

All reagents and chemicals were used from Merck chemicals. Heptafluorobutyric acid Sodium lauryl sulfate and Sodium dihydrogen phosphate were used as GR grade. Acetonitrile, and water were used as HPLC grade.

4.4.2.5. **Standard preparation:**

A standard stock solution of Eszopiclone was prepared by dissolving 40 mg of the drug in 10 ml of mobile phase. Working standard solution (3.2 mg/ml) was prepared from the stock solution by dilution with the mobile phase.

4.4.2.6. **Sample preparation:**

The average weight of 20 tablets was determined. Accurately weighed sample powder was transferred in the amount of 16 mg of Eszopiclone into a 10 ml volumetric flask, to which was added 5 ml of the mobile phase. This was sonicated for approximately 20 min to dissolve and the mobile phase was added to obtain a volume of 10 ml.
4.4.2.7. Chromatographic conditions:

mobile phase: A mobile phase consisting of 0.05 M monobasic sodium phosphate buffer containing 0.8 % Sodium lauryl sulphate (pH 3.5) and acetonitrile in the ratio of (60:40, v/v)

Column: Inertsil C18, 250 x 4.6 mm, 5µm

Column oven temperature: 40°C

Flow: 1.5 ml/min

Wavelength: 303 nm

Injection volume: 20 µL

Runtime: 55 minutes.
4.4.2.8. **HPLC method:**

The chromatographic system used was an Agilent-1100 series comprised of degasser, quaternary pump, auto injector, column compartment, PDA detector. Chromatographic separation was achieved in isocratic mode. Inertsil C18, 250 x 4.6 mm, 5µm column was used for isocratic separation. A mobile phase consisting of 0.05 M monobasic sodium phosphate buffer containing 0.8% Sodium lauryl sulphate (pH 3.5) and acetonitrile in the ratio of (60:40, v/v), at a flow rate of 1.5 ml/min and 40°C temperature. Quantification is achieved with photodiode array detection at 303nm.

4.4.2.9. **LC-MS method:**

Agilent 1100 Series LC/MSD Trap SL system and Inertsil C18, 250 x 4.6 mm, 5µm column was used. A mobile phase consisting of Purified water (pH 3.5 adjusted with heptafluorobutyric acid) and acetonitrile in the ratio of (60:40, v/v), at a flow rate of 1.5 ml/min. Quantification is achieved at 303nm, by keeping following mass parameters. Ion spray type: Electron spray ionization, Ion polarity: positive mode, Dry temperature: 325°C, Nebulizer: 20 psi and dry gas: 10 ml/min.

4.4.2.10. **Procedure:**

HPLC system was set up as described under chromatographic conditions. Standard and sample solution was prepared according to 4.2.3.5 and made single injection of each of solvent mixture as a blank, standard solution (six injections), placebo solution and sample solution in to the chromatographic system.

- Recorded the chromatograms at 256 nm and measure the peak area counts for all eluting peaks.
- Examined the blank and placebo chromatogram for any extraneous peaks and disregard corresponding peaks observed in the chromatogram of the sample solution.

4.4.2.11. **Calculations:**

\[
\text{Known Impurity} \% \ (w/w) = \frac{\text{AT} \times \text{WS} \times \text{DT} \times \text{P} \times \text{AW}}{\text{AS} \times \text{DS} \times \text{WT} \times 100 \times \text{LC}}
\]
Stability indicating liquid chromatographic methods for the determination of related impurities of drug products.

Unknown Impurity % (w/w) = \[
\frac{\text{AT}_1 \times \text{WS} \times \text{DT} \times \text{P} \times \text{AW}}{\text{AS} \times \text{DS} \times \text{WT} \times 100 \times \text{LC}}
\]

Total Impurities = Sum of all known impurities and unknown impurities

Where,

- \( \text{AT} \): Area of Known impurity peak in sample solution.
- \( \text{AT}_1 \): Area of Unknown impurity peak in sample solution.
- \( \text{AS} \): Average area of Eszopiclone peak in standard solution.
- \( \text{WS} \): Weight of Eszopiclone standard in mg.
- \( \text{DS} \): Dilution of Eszopiclone standard in ml.
- \( \text{DT} \): Dilution of Eszopiclone sample solution.
- \( \text{WT} \): Weight of Eszopiclone sample in mg.
- \( \text{P} \): Potency of Eszopiclone working standard on as is basis.
- \( \text{LC} \): Label claim of Eszopiclone in mg per tablet.
- \( \text{AW} \): Average weight of tablet in mg.

4.4.3. RESULTS AND DISCUSSION:

4.4.3.1. Identification of unknown impurity:

Stability indicating capability of method can be established by achieving the 20–80% of degradation. During 6 month stability study of drug product at 40°C/75% RH it has been observed that one unknown impurity at 0.33 RRT is increasing above the identification threshold. Acid Hydrolysis stress condition (5 ml, 2 N HCl, 60 °C for 2 Hrs) about 50% of impurity at RRT 0.33 is formed (Figure 4.9). LC/ MS analysis of same impurity has been carried out which gives the \( m/z \) 129.2 (Figure 4.7). During further investigation it has been observed that pure form of 2 amino 5 chloropyridine is \( m/z \) 129.2, which is key starting material for the synthesis of Eszopiclone as well as degradation impurity formed during acid hydrolysis. Its presence in the sample has been confirmed by injecting pure form of 2 amino 5 chloropyridine in HPLC method. Which is eluting at the same RRT of 0.33. Fragmentation pattern of molecular ion peak in positive mode of LC-MS analysis is as shown in Table 4.5.
Stability indicating liquid chromatographic methods for the determination of related impurities of drug products.

4.4.3.2. **Optimization of chromatographic conditions:**

The important criteria for the development of the successful RP-HPLC method is that method should be accurate, reproducible, robust, stability-indicating, free from interference (blank/placebo/other unknown degradation product), and straightforward enough for routine use in quality control laboratories. Reversed-phase columns are silica based bonded phases, and C18-type bonded phase is most frequently used so Inertsil C18, 250 x 4.6 mm, 5µm column is used for analytical method development. Keeping the pH of the mobile phase on the acidic side i.e. pH 3.5, the silanol interaction with basic analyte was minimized and peak symmetry and sensitivity was improved but basic analytes were protoned in acidic mobile phase and eluted more quickly. As ion-pairing reagents are potentially capable of ion-pairing with the positively charged basic

---

**Table 4.5: Fragmentation Pattern.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Observed molecular ion peak in positive mode</th>
<th>Fragmentation Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eszopiclone Tablets 40°C/5% R, 6 M.</td>
<td>129.2</td>
<td>129.2→ 93.4</td>
</tr>
<tr>
<td>2 amino 5 chloropyridine</td>
<td>129.1</td>
<td>129.2→ 93.1</td>
</tr>
</tbody>
</table>

**Fig. 4.7: Mass spectra of Impurity D (2-amino-5-chloropyridine).**
analytes, thus reduces hydrophilicity and increases their retention time so sodium lauryl sulphate is used as ion pairing reagent. Impurity B is having hydroxyl group as functional group so most polar in nature and elutes first. Impurity C is having the ketone group as functional group so less polar compare to Impurity B and elutes second. Impurity D is having the secondary amine group as functional group so more nonpolar compare to Impurity C and elutes next. Impurity A and eszopilcone are very close structures except negatively charged oxygen i.e. N-Oxide group, due to negatively charged oxygen impurity A is more polar in nature compare to Eszopiclone and elutes fourth and Eszopiclone elutes lastly. The flow rate of the mobile phase was so adjusted to obtain the expected retention time of the analytes. Finally buffer and acetonitrile was adjusted in the ratio of 60:40 v/v in order to obtain better resolution between impurities and Eszopiclone. Chromatographic performance of finalized method is as shown in Table 4.6.

Table 4.6: Chromatographic performance data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (Min)</th>
<th>RRT (n = 3)</th>
<th>Resolution (n = 3)</th>
<th>Tailing factor (n = 3)</th>
<th>RRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imp. B</td>
<td>3.660</td>
<td>0.85</td>
<td>-</td>
<td>1.01</td>
<td>1.3</td>
</tr>
<tr>
<td>Imp. C</td>
<td>6.620</td>
<td>0.10</td>
<td>14.25</td>
<td>1.08</td>
<td>1.7</td>
</tr>
<tr>
<td>Imp. D</td>
<td>11.960</td>
<td>0.18</td>
<td>32.36</td>
<td>1.09</td>
<td>0.8</td>
</tr>
<tr>
<td>Imp. A</td>
<td>32.087</td>
<td>0.32</td>
<td>62.14</td>
<td>1.02</td>
<td>1.1</td>
</tr>
<tr>
<td>Eszopiclone</td>
<td>37.570</td>
<td>1.0</td>
<td>4.50</td>
<td>1.16</td>
<td>1.0</td>
</tr>
</tbody>
</table>

4.4.3.3. Validation of method:

The optimized chromatographic conditions were validated by evaluating specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and system suitability, in accordance with ICH guidelines Q2 (R1) 46-48.

4.4.3.3.1. Specificity:

The specificity of a method is its suitability for analysis of a substance in the presence of potential impurities. Stress testing of a drug substance can help to identify likely degradation products, which can helps to establish degradation pathways and the intrinsic stability of the molecule. The peak purity of the Eszopiclone was found satisfactory under different stress
conditions. The specificity of the LC method for Eszopiclone has been determined in the presence of four impurities.

4.4.3.3.2. **Forced degradation study of drug product**: Forced degradation studies were performed to demonstrate the selectivity and stability indicating capability of the proposed method. The stress conditions engaged for degradation studies as per ICH recommendation. The stress degradation study was performed on the drug product includes acid hydrolysis (5ml of 2 M HCl at 60°C for 2hr), base hydrolysis (5ml of 2 M NaOH at 60°C for 30min), oxidation (2ml of 5% H2O2 at bench top for 2 hr), thermal (60°C for 24hrs), humidity (40°C, 75% RH for 7days ) and photolytic degradation (drug product exposed UV and Visible light so has to complete 1.2 million lux hours and 200 watt h/m²). The stress study was performed as per International Conference on Harmonization (ICH) recommendation. Peak purity has been checked for the Eszopiclone peak by using PDA detector in stress samples. There was no peak found at the retention time of Eszopiclone and its all four impurities in blank and placebo blend chromatograms proves no interference from blank and placebo. Slight degradation was observed when drug product was subjected to Peroxide stress conditions and stable in photolytic and humidity stress condition. Eszopiclone was sensitive for Acid stress conditions and Significantly degraded into unknown impurity which further identified as impurity D. Peak-purity test results from the PDA detector confirmed that Eszopiclone peak obtained from all the stress samples analyzed was homogeneous and pure. Peak purity results from the PDA detector for the peaks produced by degradation of Eszopiclone confirmed that all these peaks were homogeneous and pure for all the stress samples analyzed (Table-4.7).
**Table 4.7: Stress testing (forced degradation) data.**

<table>
<thead>
<tr>
<th>Stress Condition</th>
<th>% Net Degradation</th>
<th>Purity Angle</th>
<th>Purity threshold</th>
<th>Purity flag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Hydrolysis</td>
<td>50.5</td>
<td>0.456</td>
<td>0.679</td>
<td>No</td>
</tr>
<tr>
<td>Base Hydrolysis</td>
<td>17.2</td>
<td>0.553</td>
<td>0.765</td>
<td>No</td>
</tr>
<tr>
<td>Peroxide</td>
<td>5.1</td>
<td>0.345</td>
<td>0.644</td>
<td>No</td>
</tr>
<tr>
<td>Photolytic-sunlight</td>
<td>1.5</td>
<td>0.213</td>
<td>0.567</td>
<td>No</td>
</tr>
<tr>
<td>Heat Stress</td>
<td>Stable</td>
<td>0.344</td>
<td>0.741</td>
<td>No</td>
</tr>
<tr>
<td>Humidity Stress</td>
<td>Stable</td>
<td>0.179</td>
<td>0.491</td>
<td>No</td>
</tr>
</tbody>
</table>

**Fig. 4.8:** Typical chromatogram of unstressed sample solution.

**Fig. 4.9:** Typical chromatogram of Acid stressed sample solution.
4.4.3.3. Limits of detection and quantification:

LOD and LOQ for the four impurities and Eszopiclone were estimated as the amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively, by injecting a series of dilute solutions of known concentration. Precision was also determined at the LOQ level by analysis of six individual preparations of the four impurities and calculating the RSD (%) of the peak area for each impurity.

4.4.3.4. Linearity:

Linearity was established by analyzing six concentrations of Eszopiclone and all impurities ranging between LOQ to 150% of respectively of the permitted maximum level of the impurity, by plotting the peak area ratio against the corresponding concentration. The correlation coefficients, slopes, and y-intercepts of the calibration plots are reported. Calibration plots for the Four related substances were linear over the ranges tested. The correlation coefficients were >0.999 for all the components (Table 4.8). These results show there was an excellent correlation between the peak area and concentration for the four impurities.

4.4.3.5. Accuracy:

For the impurities, recovery was determined in triplicate analysis of spiked sample for LOQ, 0.10, 0.20 and 0.30% of the analyte concentration (1600 μg/ml) for Eszopiclone and recovery of the impurities was calculated. (Table 4.8).

Table 4.8. Evaluation of accuracy.

<table>
<thead>
<tr>
<th>Amount spiked</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOQ</td>
<td>99.0 ± 0.23</td>
</tr>
<tr>
<td>50%</td>
<td>98.3 ± 0.31</td>
</tr>
<tr>
<td>100%</td>
<td>99.1 ± 0.67</td>
</tr>
<tr>
<td>150%</td>
<td>99.5 ± 0.87</td>
</tr>
</tbody>
</table>
Stability indicating liquid chromatographic methods for the determination of related impurities of drug products.

4.4.3.3.6. Precision:

Precision of the method was determined in relation to repeatability (intraday) and intermediate precision (interday). In order to evaluate the repeatability of the methods, six samples were determined during the same day. The intermediate precision of the method was also evaluated using different analyst and different instrument, and performing the analysis on different day. % RSD of area for each impurity was calculated for both precision as well as intermediate precision and was found within 2%. These results confirmed the precision and ruggedness of the method (Table 4.9).

Table 4.9: Regression and precision data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOQ µg/ml</th>
<th>LOD µg/ml</th>
<th>Regression equation (y)</th>
<th>Correlation coefficient (r)</th>
<th>Precision (% RSD)</th>
<th>Precision LOQ (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slope (b)</td>
<td>Intercept (a)</td>
<td></td>
<td>Intra day</td>
</tr>
<tr>
<td>Eszopiclone</td>
<td>0.64</td>
<td>0.17</td>
<td>153168</td>
<td>+234.0</td>
<td>0.9997</td>
<td>0.81</td>
</tr>
<tr>
<td>Imp. A</td>
<td>0.59</td>
<td>0.14</td>
<td>168481</td>
<td>+628.13</td>
<td>0.9996</td>
<td>0.67</td>
</tr>
<tr>
<td>Imp. B</td>
<td>0.30</td>
<td>0.08</td>
<td>207297</td>
<td>+871.2</td>
<td>0.9999</td>
<td>0.85</td>
</tr>
<tr>
<td>Imp. C</td>
<td>0.31</td>
<td>0.09</td>
<td>261891</td>
<td>+451.3</td>
<td>0.9995</td>
<td>0.91</td>
</tr>
<tr>
<td>Imp. D</td>
<td>0.68</td>
<td>0.09</td>
<td>125132</td>
<td>+514.7</td>
<td>0.9998</td>
<td>0.96</td>
</tr>
</tbody>
</table>

4.4.3.3.7. Robustness:

To determine the robustness of the method the experimental conditions were deliberately changed and the resolution of Eszopiclone and the four impurities was evaluated. To study the effect of flow rate on resolution it was changed to 1.2 and 1.7 ml/min. The effect of pH was studied at pH 3.3 and 3.7. The effect of column temperature was studied at 35 and 45 °C. In all these experiments the mobile phase components were not changed. The effect of the percent organic strength on resolution was studied by varying acetonitrile by −10 to +10% while other mobile phase components were held constant. In all the deliberate varied chromatographic conditions the selectivity as well as the performance of the method were unchanged proves the robustness of the method.
4.4.3.3.8. Stability in solution and in the mobile phase:

No significant changes in the amounts of the four impurities were observed during solution stability and mobile phase stability experiments when performed using the related substances method. The results from solution stability and mobile phase stability experiments confirmed that standard solutions and sample were stable for up to 24 h during determination of related substances. The mobile phase was stable up to 48h.

4.4.4. CONCLUSION:

The Isocratic RP-HPLC method developed for quantitative analysis of Eszopiclone and degradation impurities in tablet dosage form is precise, accurate, linear, robust, rugged and specific. Satisfactory results were obtained from validation of the method. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of Eszopiclone tablet dosage form.

4.5. REFERENCES:


[8] European pharmacopoeia 5.0, 2735-2736.


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