5. MATERIALS AND METHODS

1. Reagents and Chemicals used

Acetonitrile, methanol, ortho phosphoric acid, potassium di hydrogen ortho phosphate, trifluoro acetic acid, perchloric acid and triethylamine were supplied by Qualigens Fine Chemicals and S.D. Fine chemicals. Water (HPLC grade) was obtained from Milli-QR system. All the reagents and chemicals used were of HPLC or Analytical grade.

Working standards of Zolmitriptan was procured from Orchid pharmaceuticals (Chennai, India) and Rizatriptan was a gift sample from Dr.Reddy’s laboratory (Hyderabad, India). HPMC (Methocel – K100–CR, apparent viscosity, 2% in water at 20°C is 80,000-12000 cP) was a gift sample from Colorcon Asia Pvt Ltd (Goa, India). Polyvinyl pyrrolidine (PVP-K-30) was purchased from Sd fine chemicals (Mumbai, India).

1.1. Instruments used

i. Sartorius single pan digital balance

ii. Systronics pH meter, μ pH system 361

iii. Shimadzu LC 2010A HT HPLC system with the following configurations;

- Low pressure gradient quaternary pump
- Auto injector
- Multi wavelength UV array detector
- Column oven and Degasser
- Class-VP 6.01 data station

iv. Electrolab dissolution testing apparatus

- USP Type II apparatus TDL-08L

v. Shimadzu UV 1700 spectrophotometer

vi. Shimadzu FT IR 8400S spectrophotometer

vii. Ultra Sonicator
viii. Analytical column such as,

- VYDAC Monomeric C\textsubscript{18} (250× 4.6mm, 5µ)
- Princeton SPHER HPLC C\textsubscript{18} (250× 4.6mm, 5µ)
- Phenomenex Luna C\textsubscript{18} (250× 4.6mm, 5µ)
- Kromasil C\textsubscript{18} (250× 4.6mm, 5µ)
- Zorbax C\textsubscript{8} (250× 4.6mm, 5µ)
- Hypersil C\textsubscript{4} (250× 4.6mm, 5µ)

2. Experimental

This chapter describes the experimental details of the preformulation study, tablet manufacture, bio availability study design & data handling, optimization and validation of the bio analytical methods for the estimation of zolmitriptan and rizatriptan in rabbit plasma samples, preparation of standard and sample solutions, development of \textit{in vitro} dissolution methods, \textit{in vitro} data analysis, \textit{in vivo} data analysis, statistical analysis of pharmacokinetic data and development & validation of level A \textit{In Vitro - In Vivo} Correlation (IVIVC).

2.1. Preformulation Study

Preformulation in the broadest sense encompasses all the activities and studies that are required to convert an active pharmacological substance into a suitable dosage form. It can be defined as an investigation of the physical and chemical properties of a drug substance alone and also when combined with the excipients. In the present study, therefore, evaluation of granulations, development of \textit{in vitro} dissolution method and the compatibility between the drug and the selected polymer were determined.

2.1.1. Evaluation of Granulations

The following parameters were used for the characterization of prepared granules

(1) Flow properties
(2) Granular densities
(3) Percentage of fines
2.1.1. Flow Properties

The flow properties are critical for an efficient tableting operation. A good flow of the powder or granulation is necessary to assure efficient mixing and acceptable weight uniformity for the compressed tablets. In some cases, dry powder has to be pre-granulated to improve their flow properties. During the pre-formulation, the flow ability of the drug and granulation should be studied especially when the anticipated dose of the drug is large.

When a heap of powder is allowed to stand with only the gravitational force acting on it, the angle between the free surface of the static heap and the horizontal plane can achieve a certain maximum value for a given powder. This angle is defined as the static angle of repose and is a common way of explaining flow characteristics of powder granulation. In most pharmaceutical powders and granules, the angle of repose values range from 25-40°, with lower values indicating better flow characteristics.

The angle of repose is defined as the maximum angle possible between the surface of a pile of powder or granules and the horizontal plane.

\[ \tan \theta = \frac{h}{r} \]

where,

- \( h \) and \( r \) are the height and radius of the powder cone

\[ \theta = \tan^{-1} \frac{h}{r} \]

2.1.1.2. Bulk Density

The weighed amount of the powder was introduced into a graduated measuring cylinder. The cylinder was fixed on the bulk density apparatus and the timer knob was set for 100 tapping. The volume occupied by the powder was noted. Further, another 50 tapping may be continued and final volume was noted. This final volume is called as bulk volume. Bulk density is defined mathematically as given below:
Bulk density = mass of powder / Bulk volume

Bulkiness = 1 / Bulk density

2.1.1.3. Percentage of fines

Percentage of fines was determined by passing the granules through sieves 16 and 22. The particles which pass through # 22 are considered fines.

2.2. Differential Scanning Calorimetry (DSC)

The possibility of drug-excipient interaction was investigated by differential scanning calorimetry. The DSC thermograms of pure drugs zolmitriptan, rizatriptan respectively, individual excipients and drug-excipient mixtures were recorded.

2.3. Compatibility studies

Infrared spectral matching approach was employed to detect any possible chemical interaction between zolmitriptan, rizatriptan and the polymer. Physical mixtures of the drug and the polymer (1:1) were mixed with 400 mg of potassium bromide (IR grade). About 100 mg of the mixture was taken and compressed to form a transparent pellet in a hydraulic press at 15 tonnes pressure. The samples were scanned from 4000 to 400 cm\(^{-1}\) in a Shimadzu FT IR spectrophotometer. Similarly, the IR spectra of zolmitriptan, rizatriptan and the polymer were also recorded. Physical appearance of the samples and appearance/disappearance of peaks in the spectra were observed to assess any possible physical and chemical interactions.

3. Tablet Manufacture

3.1. Development of Zolmitriptan Sustained Release (SR) tablets

Zolmitriptan SR tablets were prepared by the wet granulation method (Figure 1). All the composition, with the exception of magnesium stearate and talc were thoroughly mixed in a tumbling mixer for 5 min and wetted in a mortar with isopropyl alcohol. The wet mass was sieved (16 mesh) and granules were dried at 60°C for 2 h. The dried granules were sieved (22 mesh)
and these granules were lubricated with a mixture of magnesium stearate and talc (2:1). The zolmitriptan tablets were prepared using an electrically operated punching machine. Compression was performed after granulation process with a single punch press applying a compression force of a 9 KN (preliminary work) or 12 KN (experimental design), equipped with a 6 mm concave punch. For the preliminary work, batches of 100 tablets were prepared. Each batch of experimental design consisted of 100 tablets (drug content in the tablet was 0.5 mg). Three batches were prepared for each formulation and the compositions of different batches of zolmitriptan SR tablets are given in Table 1. The compressed tablets were evaluated for average weight and weight variation, thickness, diameter, drug content & content uniformity, hardness, friability, disintegration and in vitro drug release.

3.2. Development of Zolmitriptan Immediate Release (IR) tablets

Zolmitriptan immediate release (IR) tablets were prepared by wet granulation method. All the ingredients, with the exception of magnesium stearate and talc were mixed in a tumbling mixer for 5 min and wetted in a mortar separately using PVP-K-30 in isopropyl alcohol as granulating fluid. The wet mass was passed manually through BSS with a 1.7 mm (sieve no 10) opening and granules were dried at 60° for 3-4 h until the loss on drying was not more than 2% w/w. The dried granules were passed through BSS 1.0 mm (sieve no.16). The granules were blended with magnesium stearate and talc (2:1). The blends were compressed using 6 mm concave punches on a 10 station rotary tablet press (Rimek, Ahmedabad) and the compression force of a 9 KN (preliminary work) or 12 KN (experimental design), was applied for all the formulations. For the preliminary work, a batch of 50 tablets were prepared (drug content in the tablet was 0.175 mg). The composition of Zolmitriptan immediate release tablets are given in Table 2.
3.3. Development of Rizatriptan Sustained Release (SR) tablets

Rizatriptan SR tablets were prepared by the wet granulation method (Figure 1). All the composition, with the exception of magnesium stearate and talc were thoroughly mixed in a tumbling mixer for 5 min and wetted in a mortar with isopropyl alcohol. The wet mass was sieved (16 mesh) and granules were dried at 60°C for 2 h. The dried granules were sieved (22 mesh) and these granules were lubricated with a mixture of magnesium stearate and talc (2:1). The rizatriptan tablets were prepared using an electrically operated punching machine. Compression was performed after granulation process with a single punch press applying a compression force of a 9 KN (preliminary work) or 12 KN (experimental design), equipped with a 6 mm concave punch. For the preliminary work, batches of 100 tablets were prepared. Each batch of experimental design consisted of 100 tablets (drug content in the tablet was 1.5 mg). Three batches were prepared for each formulation and the compositions of different batches of rizatriptan SR tablets are given in Table 3. The compressed tablets were evaluated for average weight & weight variation, thickness, diameter, drug content and content uniformity, hardness, friability, disintegration and in vitro drug release.

3.4. Development of Rizatriptan Immediate Release (IR) tablets

Rizatriptan immediate release (IR) tablets were prepared by wet granulation method. All the ingredients, with the exception of magnesium stearate and talc were mixed in a tumbling mixer for 5 min and wetted in a mortar separately using PVP-K-30 in isopropyl alcohol as granulating fluid. The wet mass was passed manually through BSS with a 1.7 mm (sieve no 10) opening and granules were dried at 60° for 3-4 h until the loss on drying was not more than 2% w/w. The dried granules were passed through BSS 1.0 mm (sieve no.16). The granules were blended with magnesium stearate and talc (2:1). The blends were compressed using 5 mm concave punches on a 10 station rotary tablet press (Rimek, Ahmedabad) and the compression force of a 9 KN (preliminary work) or 12 KN (experimental design), was applied for all the formulations. For the preliminary work, a batch of 50 tablets were prepared.
Materials and methods

(drug content in the tablet was 0.5 mg). The composition of Rizatriptan immediate release tablets are given in Table 4.

3.5. Evaluation of Tablets

The prepared tablets were evaluated for the following properties:

1. Thickness
2. Hardness
3. Friability

3.5.1. Hardness

The hardness of a tablet is indication of its strength. It is tested by measuring the force required to break the tablet across the diameter. The force is measured in kg and the hardness of about 4 kg is considered to be satisfactory for uncoated tablets. Monsanto hardness tester is used for this purpose. The hardness of 10 tablets was measured and the average hardness was calculated.

3.5.2. Friability Test

Friability is the loss of weight of tablets in the container, due to removal of fine particles from their surfaces. Friability test is carried out to assess the ability of the tablet to withstand abrasion in packing, handling and transport. Roche friability tester was used for finding out the friability of the tablet. A number of tablets (10) were weighed accurately and placed in the chamber of the apparatus. After 100 rotations, the tablets were taken out from the apparatus, re-dusted and weighed. The loss in weight indicates the friability of the tablets. A maximum friability of 1% is acceptable for tablets as per Indian Pharmacopoeia (IP). Percentage friability was determined by using the formula given below:

\[
\% \text{ friability} = \left( \frac{W_1 - W_2}{W_1} \right) \times 100
\]

where  
\[W_1 = \text{weight of tablets before test}\]
\[W_2 = \text{weight of tablets after test}\]
3.5.3. In vitro drug release

Dissolution was performed using an Electro lab – Tablet dissolution Tester, USP XXIII Model. The media used was 0.1N HCl at pH 1.2 and a volume of 700 ml for the first 2 h after which 200 ml of 0.2 M sodium phosphate tribasic, was added to give a final pH of 6.8 and maintained at 37°C. Dissolution tests were performed on six tablets and the amount of drug released was analyzed by UV spectrophotometer.

3.5.4. Stability studies as per the ICH guidelines

Developed SR tablets were packed in High Density Poly Ethylene (HDPE) containers and were subjected to stability studies at the following different temperature and humidity conditions as prescribed by the International Conference on Harmonization (ICH)\textsuperscript{53}.

- 25°C with 60 % RH
- 40°C with 75 % RH

Samples were withdrawn at 1, 2, 3 and 6 months intervals and evaluated for their physical properties and in vitro drug release.

4.0. Bioavailability studies

A randomized single dose bioavailability study was conducted in rabbits for the developed modified release formulations containing zolmitriptan & rizatriptan and in house developed immediate release formulations containing zolmitriptan & rizatriptan (Table 5).

The protocol of the study was submitted to the Institutional Animal Ethical Committee and the approval for conducting the same was obtained (Proposal no. JSSCP/IAEC/PH.ANALYSIS/Ph.D/01/2012-13). A reproducible HPLC analytical technique was developed for the estimation of the drugs in the plasma samples. Various pharmacokinetic parameters such as $C_{max}$, $T_{max}$, $t_{1/2}$, $k_{el}$, $AUC_{0-t}$ and $AUC_{0-\infty}$ were estimated.
Albino rabbits were used for bioavailability studies. All the rabbits were fasted for overnight before the experiments but had free access to water and the rabbits were divided into four groups of 2 animals each with 1 animal as control.

Group 1 In house developed immediate release formulation treated group
Group 2 Developed slow release formulation treated group
Group 3 Developed medium release formulation treated group
Group 4 Developed fast release formulation treated group

Drug formulations were administered orally at the dose which is calculated based on human dose. Blood (0.5ml) was collected using disposable syringes in preheparinised centrifugal tubes at 0 (before drug administration), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 18.0 and 24.0 h post dosing. The samples were centrifuged at 3500 rpm for 10 min to separate the plasma. They were transferred into air tight containers and stored at deep freeze condition until starting of analysis. A similar procedure adopting cross over design in drug treatment was repeated after 7 days of wash out period. The plasma samples of drugs were extracted protein precipitation technique and their drug levels were quantified using HPLC techniques.

Pharmacokinetic parameters namely, \( C_{\text{max}} \), \( T_{\text{max}} \) and \( k_{\text{el}} \) were determined for individual drug treatments from the observed plasma concentration-time data. AUC were calculated by trapezoidal rule from time zero to the last observed concentration.

Pharmacokinetic parameters such as peak plasma concentration (\( C_{\text{max}} \)), Time to peak concentration (\( T_{\text{max}} \)), Area under the plasma concentration - time curve (\( \text{AUC}_{0-t} \) & \( \text{AUC}_{0-\infty} \)), elimination rate constant (\( k_{\text{el}} \)) and Elimination half-life (\( t_{1/2} \)) were calculated separately and the blood level data of the reference product and the test products were compared.

5.0. Estimation of drugs

5.1. Optimization of chromatographic conditions for the estimation
Proper selection of the chromatographic method depends upon the nature of the sample (ionic or neutral molecule), its molecular weight and solubility. The drugs selected for the present study are polar in nature and hence either reverse phase or ion pair or ion exchange chromatography can be used. For the present study reverse phase HPLC methods are considered to be more suitable because they are extremely specific, linear, precise, accurate, sensitive and rapid methods.

5.1.1. Selection of detection wavelength for zolmitriptan and rizatriptan

10 µg/ml of zolmitriptan and rizatriptan were prepared, individually in solvent mixtures of methanol and water (1:1). These solutions were scanned in the UV region of 200 - 400 nm and the UV spectrums were recorded (Figures 2 and 3). From the spectra, detection wavelengths of 221 and 224 nm were selected for zolmitriptan and rizatriptan, respectively.

5.1.2. Initial separation conditions

A isocratic run was performed for the initial separation. From this the approximate ratio of the organic phase in the buffer solution required to elute the drugs from the column was determined. An aliquot of the standard solution was prepared and chromatogrammed using the following chromatographic conditions;

Stationary phase : Hiber C18 Column, (5 µ, 25 cm X 4.6 mm i.d and 5 µ)

Mobile phase : Solvent A: Di-pottasium hydrogen ortho phosphate

Solvent B : Methanol

Solvent ratio : 50 : 50

Flow rate : 1.0 ml/min

Sample size : 50 µl
Temperature : Room temperature of 20\(^0\) ± 1\(^0\)C

From the above isocratic run, the approximate percentage of methanol in the di-pottassium hydrogen ortho phosphate buffer required to elute the drugs from the column was determined (Table 6). This ratio was used for subsequent separation and the chromatograms were recorded.

5.1.3. Effect of chromatographic variables

To optimize the chromatographic conditions, the effect of chromatographic variables such as mobile phase pH, solvent strength, flow rate, solvent ratio and the nature of stationary phase on the peak separation were studied. The resulting chromatograms were recorded and the chromatographic parameters such as capacity factor, asymmetric factor, resolution and column efficiency were calculated. The conditions that gave the best symmetry and capacity factor were selected for the estimation.

The standard solution of zolmitriptan and rizatriptan were chromatogrammed for 20 min using methanol in buffer solutions of different pH ranging from 2.0 to 7.0 as the mobile phase at a flow rate of 1.0 ml/min. It was observed that an increase in pH decreases the retention time of rizatriptan and increases retention time of zolmitriptan.

5.1.4. Nature of the stationary phase

Different reverse phase stationary phases (C\(_4\), C\(_8\) and C\(_{18}\)) were used and the chromatograms were recorded. When C\(_4\) and C\(_8\) columns were used, the retention times of the drugs were reduced.

Based on the retention and peak shape, Hiber C\(_{18}\) column was selected for zolmitriptan and rizatriptan.

Different mobile phases, namely, acetonitrile and methanol were used at a flow rate of 1.0 or 0.5 ml/min. For the initial separation conditions, methanol was used. When methanol was substituted by other solvents, the solvents to
buffer ratios were calculated using solvent strength. The resulting ratios of the mobile phase were prepared and the drugs were chromatogrammed. These mobile phases gave well retained and symmetrical peaks. Methanol was used as the mobile phase for further studies because it gave reproducible peaks.

5.1.5. Selection of internal standard

Internal standards may be used along with the standard drugs to minimize the error in the assay due to loss of drugs that occur during extraction procedure. Internal standards were selected on the basis of purity, polarity, solubility and absorption characteristics. The internal standards selected for the present study was Rizatriptan for Zolmitriptan and Zolmitriptan for Rizatriptan respectively. These internal standards provide well resolved and symmetrical peaks.

5.2. Optimized chromatographic conditions

Based on the above studies, the following chromatographic conditions were selected for the estimation of selected drugs in plasma samples and dissolution samples.

5.2.1. Chromatographic Conditions for Zolmitriptan

Stationary phase : Hiber C\textsubscript{18} (250 x 4.6 mm i.d., 5 μ)
Mobile Phase : Methanol : 10 mM di-pottasium hydrogen ortho phosphate pH 3.2
Mobile phase ratio : 23:77 % v/v
Flow rate : 1.0 ml/min
Sample volume : 50 μl using Rheodyne 7725i injector
Detection : 231 nm
Data station : Class VP data station
Materials and methods

5.2.2. Chromatographic Conditions for Rizatriptan

Stationary phase: Hiber C$_{18}$ (250 x 4.6 mm i.d., 5 µ)
Mobile Phase: Methanol : 10 mM di-pottasium hydrogen ortho phosphate pH 3.2
Mobile phase ratio: 23:77 % v/v
Flow rate: 1.0 ml/min
Sample volume: 50 µl using Rheodyne 7725i injector
Detection: 231 nm
Data station: Class VP data station
Internal Standard: Zolmitriptan

6.0. Preparation of standard and sample solutions

6.1. Preparation of standard and sample Zolmitriptan solutions

a. Standard stock solution of Zolmitriptan

10 mg of Zolmitriptan working standard was accurately weighed and transferred into a 10 ml volumetric flask and dissolved in methanol - water mixture (1:1) and made up to the volume with the same solvent to produce a 1 mg/ml of zolmitriptan. The stock solution was stored in refrigerator at –20 ± 2°C until analysis.

The stock solution was diluted to suitable concentrations for spiking plasma to obtain calibration curve (CC) standards and quality control (QC) samples.

b. Calibration Curve Standards and Quality Control Samples

Working solutions for calibration and controls were prepared from the stock solution by an adequate dilution using water. Calibration standards for control plasma were prepared by spiking this stock solution to obtain the concentration levels of 18.73, 18.95, 37.46, 74.92, 112.38, 168.57, 337.14 and 374.6 ng/ml in rabbit plasma. Quality control samples were prepared as bulk, at a
concentration of 18.73 ng/ml (LLOQ QC), 56.19 ng/ml (LQC), 187.3 ng/ml (MQC) and 337.14 ng/ml (HQC).

These samples were stored below -50°C until use.

c. Standard stock solution of Rizatriptan (Internal Standard)

10 mg of rizatriptan internal standard was accurately weighed and transferred into a 10 ml volumetric flask, dissolved in methanol - water mixture (1:1) and made up to the volume with the same solvent to produce a 1 mg/ml of rizatriptan. The stock solution was stored in refrigerator at 20 ± 2°C until analysis.

The stock solution was diluted to suitable concentration (100 μg/ml) with HPLC grade water, prior to use as internal standard.

d. Plasma samples

Calibration standards, validation QC samples and rabbit plasma samples were prepared by adding 0.5 ml plasma to Eppendorf tube followed by adding 10.0 μl internal standard solution (1.0 μg/ml). All samples were mixed by vortexed for 30 s. After these procedures, 0.5 ml of 10% perchloric acid solution was added and vortexed for 30 s and centrifuged at 3500 rpm. The resulting supernatant solution was used for the analysis.

6.2. Preparation of standard and sample Rizatriptan solutions

a. Standard stock solution of Rizatriptan

10 mg of rizatriptan working standard was accurately weighed and transferred into a 10 ml volumetric flask and dissolved in methanol and water mixture (1:1) and made up to the volume with the same solvent to produce a 1mg/ml of rizatriptan. The stock solution was stored in refrigerator at −20 ± 2°C until analysis.

The stock solution was diluted to suitable concentrations for spiking plasma to obtain calibration curve (CC) standards and quality control (QC) samples.
b. Calibration Curve Standards and Quality Control Samples

Working solutions for calibration and controls were prepared from the stock solution by an adequate dilution using water. Calibration standards for control plasma were prepared by spiking this stock solution to obtain the concentration levels of 12.55, 12.69, 25.10, 50.19, 75.29, 112.94, 225.88, 250.98 ng/ml in rabbit plasma. Quality control samples were prepared as bulk, at a concentration of 12.55 ng/ml (LLOQ QC), 35 ng/ml (LQC), 115 ng/ml (MQC) and 225 ng/ml (HQC). These samples were stored below -50°C until use.

c. Standard stock solution of Zolmitriptan (Internal Standard)

10 mg of zolmitriptan internal standard was accurately weighed and transferred into a 10 ml volumetric flask, dissolved in methanol and water mixture (1:1) and made up to the volume with the same solvent to produce a 1 mg/ml of zolmitriptan. The stock solution was stored in refrigerator at –20 ± 2°C until analysis.

The stock solution was diluted to suitable concentration (100 μg/ml) with HPLC grade water, prior to use as internal standard.

d. Plasma samples

Calibration standards, validation QC samples and rabbit plasma samples were prepared by adding 0.5 ml plasma to Eppendorf tube followed by adding 10.0 μl internal standard solution (1.0 μg/ml). All samples were mixed by vortexed for 30 s. After these procedures, 0.5 ml of 10% perchloric acid solution was added and vortexed for 30 s and centrifuged at 3500 rpm. The resulting supernatant solution was used for the analysis.

7.0. Validation of HPLC methods

Validation is a process which involves confirmation or establishment by laboratory studies that a method / procedure / system / analyst can give the required accuracy, precision, sensitivity, ruggedness, etc. In the most basic form, validation of an analytical procedure demonstrates that the procedure
Validation parameters tested were,

1. Selectivity/ Specificity
2. Sensitivity
3. Linearity
4. Precision and Accuracy
   a. Within-batch precision and accuracy
   b. Intra-day precision and accuracy
   c. Between batch / Inter-day precision and accuracy
5. Stabilities
   a. Short Term Stock Dilution Stability
   b. Long Term Stock Solution Stability
   c. Freeze Thaw Stability
   d. Bench Top (BT) Stability
   e. Long-Term (LT) Stability
   f. Autosampler Stability
6. Recovery
7. Ruggedness
8. Robustness

7.1. Selectivity/ Specificity

A method is said to be specific when it produces a response only for a single analyte. Method selectivity is the ability of the method to produce a response for the analyte in the presence of other interferences. In order to prove that the method chosen was specific and selective the following two sets of samples were processed and injected into the HPLC using the extraction procedure.

1. Blank samples from six different lots of biological matrix (plasma containing K₂EDTA as anticoagulant).
2. Samples from the same six lots of biological matrix mentioned in step 1 spiked with the analyte at the lower limit of quantification (LLOQ) of the calibration curve and with the internal standard at the concentration level in the study.

To calculate % interference, the response obtained for each sample in step 1 was compared with the response obtained for each corresponding sample in step 2.

\[
\% \text{ Interference} = \left( \frac{\text{Peak area response of blank}}{\text{Peak area response of LLOQ}} \right) \times 100
\]

7.2. Sensitivity

It is expressed as limit of quantitation. It is the lowest amount of analyte in a sample matrix that can be determined.

The lower limit of quantification for zolmitriptan was 18.73 ng/ml and rizatriptan was 12.55 ng/ml.

7.3. Linearity

Linearity and range of the methods were analyzed by preparing calibration curves using different concentrations of the standard solution containing the internal standard. The calibration curve was plotted using response factor and concentration of the standard solutions.

Linearity was established using two calibration curves over the range of (18.73 to 374.6 ng/ml for zolmitriptan, 12.55 to 250.98 ng/ml for rizatriptan) using the weighted least square regression analysis.

A calibration curve consisted of

- Aqueous standard at middle concentration level to check retention time of analyte and internal standard.
- Blank sample (matrix sample processed without internal standard and analyte)
- Zero sample (matrix sample processed with internal standard but without analyte)
• Eight non-zero standards covering the expected range. The lowest and highest standards were prepared in duplicates.

7.4. **Precision and Accuracy**

The precision and accuracy of the method was determined by analyzing two batches each consisting of one set of calibration curve with six replicates of quality control samples at four concentration levels [Quality Control samples at the lower limit of quantification (QCLLQ), Low (QCL), Middle (QCM) and High(QCH)].

**Precision**

Precision is expressed as the percentage coefficient of variation (%CV) which is calculated as per the following expression:

\[
\text{% CV} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100
\]

*Intra-run Precision*

Intra-run precision was determined by calculating the percentage coefficient of variation (% CV) of the results obtained in the same run.

*Intra-day Precision*

Intra-day precision was determined by calculating the percentage coefficient of variation (% CV) of the results obtained in the same day.

*Inter-day Precision*

Inter-day precision was determined by calculating the percentage coefficient of variation (% CV) of the results obtained over at least two days.

**Accuracy**

Accuracy is reported as % nominal of the analyzed concentration which is calculated as:

\[
\text{% Nominal} = \frac{\text{Measured Concentration}}{\text{Actual Concentration}} \times 100
\]

*Intra-run Accuracy*

Intra-run accuracy was determined by calculating the percentage nominal of the calculated concentration from the actual values for quality control samples at
each concentration level analyzed in a single run and the mean of percentage nominal at each level was reported.

*Intra-day Accuracy*

Intra-day accuracy was determined by calculating the percentage nominal of the calculated concentration from the actual values for quality control samples at each concentration level analyzed in a single day and the mean of percentage nominal at each level was reported.

*Inter-day Accuracy*

Inter-day accuracy was determined by calculating the percentage nominal of the calculated concentration from the actual value for quality control samples at each concentration level analyzed over at least two days and the mean of percentage nominal at each level was reported.

### 7.5. Stock Solution Stability

#### 7.5.1. Short Term Stock Dilution Stability

The stability of stock dilutions of analyte and the internal standard was evaluated at room temperature. Aqueous stock dilution of the analyte and the internal standard were prepared. One portion of the stock dilution was placed in the refrigerator between 2-8°C, while the other portion was placed at room temperature for 24 h. Stock dilution stored at room temperature (stability samples) was compared with refrigerated stock dilutions considered as ‘comparison samples’. Six replicate injections of the above solutions were made.

Acceptance Criteria: % stability should be within 90 to 110% or the % change should be ± 10%.

% Stability = (Mean response of stability samples / Mean response of comparison samples) × 100

% Change = 100 – (Mean response of stability samples / Mean response of comparison samples × 100)
7.5.2. **Long Term Stock Solution Stability**

The stability of the stock solution when stored in the refrigerator for a given period of time was determined. Stock solutions of the analyte and internal standard were prepared and stored in the refrigerator between 2 - 8°C for 7 days (stability stock). The stock solution stabilities of the analyte and the internal standard were determined with a comparison stock solution, which was prepared freshly. Five replicate injections of the above solutions were made. The response of comparison samples were corrected by multiplying with correction factor to nullify the difference between the measured weights or the dilutions made.

Correlation factor (CF) = Concentration of comparison stock / Concentration of stability stock

\[
\text{% Stability} = \left( \frac{\text{Mean response of stability samples}}{\text{Mean response of comparison samples}} \right) \times \text{CF} \times 100
\]

\[
\text{% Change} = 100 - \left( \frac{\text{Mean response of stability samples}}{\text{Mean response of comparison samples}} \right) \times \text{CF} \times 100
\]

7.5.3. **Freeze Thaw Stability**

This test was done to ensure that the analyte was stable in the biological matrix even after multiple freeze-thaw cycles.

1. Six quality control samples each at low and high concentrations stored below -50°C for at least 24 h were removed from the deep freezer and were allowed to thaw unassisted at room temperature (FT4 samples). These samples were frozen again below -50°C for at least 12 h.

2. Another set of six quality control samples at low and high concentration levels (FT3 samples) were removed from the deep freezer along with the FT4 samples and thawed unassisted. Both sets of samples were replaced back into the deep freezer.
3. At least after 12 h of freezing, fT4, fT3 and another set of six samples each at low and high concentration levels (fT2 samples) were removed from the deep freezer and thawed unassisted. All the samples were replaced back into the deep freezer.

4. At least after 12 h of freezing, fT4 samples were taken out from deep freezer, thawed unassisted to room temperature and analyzed with freshly prepared calibration curve (CC) solutions.

7.5.4. Bench Top (BT) Stability

Six quality control samples each at Quality Control sample at Low concentration (QCL) and Quality Control sample at High concentration (QCH) levels were stored at room temperature for 3 and 6 h. The above samples were analyzed along with freshly prepared calibration curve standards by using the method being validated.

7.5.5. Long-Term (LT) Stability

To assess the stability of the analyte in the biological matrix under the same conditions of storage as that of the study samples for the time period between the date of first sample collection and the date of last sample analysis, the following test was performed.

Six samples of each quality control samples at low and high concentrations were stored below -50°C in the deep freezer. The stability of the analyte was evaluated by comparing each of the back calculated concentrations of stability Quality Control sample (QCs) with the mean concentrations of the respective QCs analyzed in the first accepted precision and accuracy batch.

7.5.6. Auto sampler Stability

To evaluate the stability of the samples in the autosampler after processing for the anticipated run time, six sets of quality control samples each at low and high concentrations were placed in the auto sampler for 24 h and 48 h. The quality control samples were retained in the autosampler to prove auto
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sampler stability. After the lapse of the test time, the samples placed in the auto sampler were injected into the system along with freshly prepared calibration curve standards. The stability of the analyte was evaluated by comparing the back calculated concentration of stability samples from the freshly prepared calibration curve with their respective nominal concentrations.

To determine the auto sampler stability of the internal standard, the mean peak area obtained for the internal standard of the stability samples was compared with that of the mean of internal standard area of accepted non-zero calibration curve solution (CC’s) and the percent change was calculated using the following expression:

\[
\% \text{ Change} = 100 - \frac{(\text{Mean of internal standard peak area in the stability samples})}{(\text{Mean of internal standard peak area of the accepted non-zero CCs})} \times 100
\]

7.6. Recovery

Absolute recovery of a bio analytical method is the measured response obtained from a certain amount of analyte added to and extracted from the biological matrix, expressed as a percentage of the response obtained for the true concentration of the pure authentic standard which has not been subjected to the extraction procedure.

To determine the recovery of this method, six replicates of aqueous quality control samples (un extracted) with concentrations close to spiked Quality Control sample at Low concentration (QCL), Quality Control sample at Middle concentration (QCM) and Quality Control sample at High concentration (QCH) concentration (extracted) were prepared. These un extracted samples were injected along with precision and accuracy batch.

% Recovery of analyte at each level was calculated using the following expression:

\[
\left\{ \frac{\text{Individual analyte peak area of extracted QCs} \times \text{Concentration of analyte added (un extracted sample)}}{\text{Mean analyte peak area of aqueous QCs} \times \text{Concentration of analyte added (extracted sample)}} \right\} \times 100
\]
The mean and standard deviation for the percent recovery obtained and thereby the percent variation (%CV) was calculated at each concentration level. The overall percent recovery was calculated as the mean of recoveries obtained at the three quality control levels (QCL, QCM and QCH). The overall percent variation (% CV) was also calculated.

The percentage recovery for the internal standard was also calculated. The peak area response of the internal standard obtained for the extracted QCM sample (analyzed in the precision and accuracy batch) was compared with the mean area response of the internal standard obtained for the unextracted QCM samples.

7.7. Ruggedness

Ruggedness of the method was studied by changing the experimental conditions such as operators, instruments, source of reagents, solvents and column of similar type. Chromatographic parameters such as retention time, asymmetric factor, capacity factor and selectivity factor were evaluated.

7.8. Robustness

Robustness of the method was studied by injecting the standard solutions with slight variations in the optimized conditions namely, ± 1% in the ratio of methanol in the mobile phase, ± 0.5 units in the pH of the buffer and ± 0.1 ml of the flow rate.

8.0. Method of analysis

The processed standards and samples were analysed using optimised chromatographic conditions mentioned earlier and the chromatograms were recorded. The quantification of the chromatogram was performed using peak area ratios (response factor) of the drug to internal standard. The calibration curves were constructed routinely during the process of pre-study validation and in-study validation.

Analytical batch organization:

Samples were injected in the following order,
i) Aqueous standard
ii) Plasma blank
iii) Zero sample
iv) Calibration curve samples
v) Quality control samples
vi) Subject samples

9.0. Development of in vitro dissolution methods

The release characteristics of test and reference formulations of zolmitriptan and rizatriptan were determined using USP XXIII dissolution apparatus (type II, paddle), at 50 and 75 rpm. The dissolution medium used were pH 1.2 and 6.8 buffers maintained at 37±0.5°C. Dissolution tests were performed on six tablets. Five ml of the samples were withdrawn at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h time intervals. Equal quantity of the dissolution medium was replaced to the dissolution jar after each sampling. The amount of the drug released was estimated by optimized and validated HPLC methods described in section 5.2. Percentage drug release and cumulative release at various time intervals were calculated and compared.

10.0. In vivo and in vitro data analysis

10.1. In vivo data analysis

The pharmacokinetic parameters C_{max}, t_{max}, AUC_{0-t}, AUC_{0-\infty}, t_{1/2} and k_{el} were determined using WinNonlin Standard edition version 5.1 for individual drug treatments from the observed plasma concentration-time data.

The measured plasma concentrations were used to calculate the area under the plasma concentration-time profile from time zero to the last concentration time point (AUC_{(0-t)}). The AUC_{(0-t)} was determined by the trapezoidal method. AUC_{(0-\infty)} was determined by the following equation:

\[
AUC_{(0-\infty)} = AUC_{(0-t)} + \frac{C(t)}{k_{el}}
\]
\( k_{el} \) was estimated by fitting the logarithm of the concentrations versus time to a straight line over the observed exponential decline. The Wagner-Nelson method\textsuperscript{54} was used to calculate the percentage of the dose absorbed,  

\[
F(t) = C(t) + k_{el} \text{AUC}(0-t) \quad \text{where} \quad F(t) \text{is the amount absorbed.}
\]

The percent absorbed is determined by dividing the amount absorbed at any time by the plateau value, \( k_{e} \text{AUC}(0-\infty) \) and multiplying this ratio by 100  

\[
\% \text{ dose absorbed} = \frac{C(t) + (k_{el} \text{AUC}(0-t) / k_{el} \text{AUC}(0-\infty))}{100}
\]

10.2. \textit{In vitro} dissolution data analysis

Percentage drug released or dissolved at various time intervals were calculated using the following formula,

Percentage release = \( \frac{[\text{Concentration (mg/ml) x bath volume (ml)}]}{\text{Drug content (mg)}} \times 100 \)

The dissolution profiles were determined by plotting the cumulative percentage drug dissolved at various time points. The \textit{in vitro} drug release profiles of the slow and fast modified release formulations (test formulations) were compared using the similarity factor, \( f_{2} \), presented in the following equation,

\[
f_{2} = 50 \log \left( 1 + \frac{1}{n} \sum_{t=1}^{n} (R_{t} - T_{t})^{2} \right)^{-0.5} \times 100
\]

where \( R_{t} \) and \( T_{t} \) are the cumulative percentage dissolved at each time point for the reference product and the test product, respectively.

The evaluation of the similarity is based on the following conditions;

- a minimum of three points (zero excluded),
- 12 individual values for every time points for each formulation,
- not more than one mean value of more than 85\% dissolved for each formulation,
- the standard deviation of the mean of any formulation is less than
10% from the second to last time points and

- in cases where more than 85% of the drugs are dissolved within 15 min, dissolution profiles may be accepted without mathematical evaluation.

11.0. **In Vitro-In Vivo Correlation (IVIVC)**

11.1. **IVIVC Model development**

Linear regression analysis was used to examine the relationship between percentage of the drug dissolved and the percentage of drug absorbed. The percentage of the drug unabsorbed was calculated from the percentage absorbed. The slope of the best-fit line for the semi-log treatment of this data was taken as the first order rate constant for absorption. The dissolution rate constants were determined from % released versus the square root of time. Linear regression analysis was applied to the *in vitro-in vivo* correlation plots and the coefficient of correlation ($r^2$), slope and intercept values were calculated.

Level A correlation was estimated by a two-stage procedure, deconvolution followed by comparison of the percentage drug absorbed to the percentage drug dissolved.