CHAPTER 3

METHODS
SYNTHESIS OF TAZQ & DB-TAZQ:

Anthranilic acid was treated with thionyl chloride to give corresponding sulfonamide anhydride which was further condensed with caprolactam to give the 7, 8, 9, 10-tetrahydroazepino [2, 1-b] quinazolin-12(6H)-one. Figure-15 represents the mechanism showing the synthesis of TAZQ. Modification in ring A (di-bromo substitution) was done as shown in figure-16.

![Figure 15: Mechanism showing the formation of TAZQ](image)

![Figure 16: a) NaBH4, C2H5OH, stirring, r.t b) CCl4, Br2, Stirring](image)

PROCEDURE FOR THE SYNTHESIS OF TAZQ:

13.7g (0.1 M) of anthranilic acid was dissolved in dry benzene (200 ml) in a 500 ml round bottom flask and 29.5 ml of thionyl chloride (0.25 M) was added to it. The reaction mixture was refluxed for 3 hours on a water bath under anhydrous conditions. After completion of the reaction excess of thionyl chloride and benzene were distilled off. Benzene (100 ml) was again added to remove residual thionyl chloride. 11.3g (0.1 M) of caprolactam was dissolved in benzene in a 100 ml round bottom flask. The solution was added slowly to the sulfanylanthraniloyl chloride under cold conditions and kept overnight. Progress of the reaction was monitored on TLC. The reaction mixture was basified with aqueous ammonia and extracted with chloroform. The chloroform extract was dried and distilled under reduced pressure, which yielded crude
product. The crude product was purified by column chromatography on neutral alumina with increasing percentage of ethyl acetate in hexane (hexane: ethyl acetate: 90:10) to give 7, 8, 9, 10-tetrahydroazepino [2, 1-b] quinazolin-12(6H)-one.

PROCEDURE FOR THE SYNTHESIS OF DB-TAZQ:

2 g (0.009 M) of TAZQ was dissolved in ethanol in a 250 ml round bottom flask. The reaction mixture was kept on stirring and a pinch of sodium borohydride was added to it. The reaction was monitored by TLC and sodium borohydride was added until the completion of reaction. The reaction mixture after completion was extracted with water and ethyl acetate. The ethyl acetate layer was concentrated under reduced pressure. The residue obtained was dissolved in 20 ml of carbon tetrachloride and the solution was kept in a 100 ml round bottom flask containing a magnetic stirring piece. Bromine (2.99 g, 0.018 M) was dissolved in 10 ml of carbon tetrachloride and the solution was added to the reaction mixture drop wise accompanied by constant stirring. Completion of the reaction was monitored on TLC. The precipitate thus obtained were filtered and washed with water. The residue obtained was crystallized from ethanol to give 2, 4-Dibromo-7, 8, 9, 10-tetrahydroazepino [2, 1-b] quinazolin-12(6H)-one.

2. CHARACTERISATION OF TAZQ AND DB-TAZQ:

NMR SPECTROSCOPY:

5 mg of sample compound was dissolved in 0.6-0.8 ml of CDCl3 in a clean and dry NMR tube. The tube was cleaned and the spectrum was recorded with D1 of 03 seconds. The experiment was carried out using Bruker® 400 MHz (AVANCE-400) NMR spectrometer. For 1H-NMR, maximum 8 scans were recorded whereas 4000 scans were recorded for 13C-NMR. The spectra were processed with Bruker Topspin 2.1 software.

MELTING POINT BY DSC ANALYSIS:

The DSC curve of TAZQ was recorded on Mettler Toledo®–DSC-822e (USA) model of differential scanning calorimeter. The thermal behavior was studied by heating all samples in sealed aluminum pans, and for reference empty sealed pan was used, over a temperature range of 30-200 ºC at heating rate of 10 ºC/min. The DSC thermo gram of the compounds were analysed for a melt endothermic peak that represents the melting point. The melting point was recorded in 0 ºC.
INFRA RED SPECTROSCOPY:

The IR study was performed for identification of the compounds using Thermo® Nicolet FTIR Spectrometer. A total of 2 % (w/w) of sample was mixed in dry KBr. The mixture was grounded into a fine powder using an agate mortar/pestle before compressing into KBr disc under a hydraulic press at 10,000 psi. Each KBr disc was scanned 32 times at 4 mm s\(^{-1}\) at a resolution of 2 cm\(^{-1}\) over a wave number region of 500–4000 cm\(^{-1}\). The characteristic bands were recorded.

MS analysis:

The electro-spray ionization (ESI) studies were performed on triple quadrupole mass spectrometer (Varian® 1200L series). The samples were dissolved in methanol and injected into the direct inlet probe. The positive ES/MS data was recorded by varying the cone voltage. The needle voltage was kept at 4.8KV with nitrogen at 197.1°C as drying gas. The spectra were processed using Varian MS Workstation 6.8 software.

3. EVALUATION OF ANTITUSSIVE ACTIVITY

All the compounds were evaluated for their antitussive effect using citric acid induced cough model in guinea pig (Laude et al., 1994). The animals were treated with the compounds on individual as well as combination basis in order to study the effect of various combinations.

ANIMALS:

Albino guinea pigs weighing 500-700 g of either sex were employed for the study. Animals were maintained in animal house and exposed to normal cycles of day and night under standard conditions, temperature of 25 ± 2°C and relative humidity 55-65%. The protocol of the present study was approved by the Institutional Animal Ethics Committee (IAEC) (CPCSEA/IAEC/ISF/2010/0041 & 0045)

SCREENING OF THE ANIMAL SUBJECTS:

Animals underwent a screening procedure before pre-treatment with drugs. On the first day after a 3 min acclimatisation period animals were first exposed to normal saline and subsequently 5 min later to aerosolized 7.5% citric acid for a period of 10 min. Animal
selection criterion was made either on the basis of number of coughs (< 7 or > 15) on exposure to aerosolized 7.5% citric acid or their tendency to cough on exposure to normal saline. Animal producing cough outside the stated limit of citric acid or on exposure to aerosolised saline was excluded as this was taken as an indication of infection or hyper-reactivity.

STUDY DESIGN AND TREATMENTS:

The animals passing the selection criterion were randomly divided into 10 groups. Each group was comprised of 6 animals (n=06). All the drugs were dissolved in 4% HCl and then diluted in saline. Their pH values were adjusted to 6.5-7.0 with 1% Na₂CO₃. Codeine 10mg/kg was administered as standard. The samples were intraperitoneally administered 30 min. before the second challenge of aerosolized citric acid (7.5% w/v) solution. Table-5 represents the study design and groups assignment.

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>GPS</th>
<th>TREATMENT (i.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NC</td>
<td>Normal saline treated</td>
</tr>
<tr>
<td>2</td>
<td>TAZQ-10</td>
<td>TAZQ-10mg/kg</td>
</tr>
<tr>
<td>3</td>
<td>TAZQ-20</td>
<td>TAZQ-20mg/kg</td>
</tr>
<tr>
<td>4</td>
<td>DBTAZQ-10</td>
<td>DBTAZQ-10mg/kg</td>
</tr>
<tr>
<td>5</td>
<td>DBTAZQ20</td>
<td>DBTAZQ-20mg/kg</td>
</tr>
<tr>
<td>6</td>
<td>AH-50</td>
<td>AH-50mg/kg</td>
</tr>
<tr>
<td>7</td>
<td>AH-100</td>
<td>AH-100mg/kg</td>
</tr>
<tr>
<td>8</td>
<td>TAZQ-10+DBTAZQ-10</td>
<td>TAZQ-10mg/kg+DBTAZQ-10mg/kg</td>
</tr>
<tr>
<td>9</td>
<td>TAZQ-10+AH-50</td>
<td>TAZQ-10mg/kg+AH-50mg/kg</td>
</tr>
<tr>
<td>10</td>
<td>CODEIN-10 (ST)</td>
<td>CODEIN-10mg/kg</td>
</tr>
</tbody>
</table>

Figure: 6: Experimental study design and treatments (Antitussive studies)

EVALUATION OF COUGH RESPONSE:

After 30 minutes of intraperitoneally administration of all the drugs the cough challenge was given at the same time of day for each animal and minimum of 24 h interval was allowed between challenges to eliminate any short term prophylaxis. Animals were allowed free access to food and water up to the time of testing. Each animal was placed in a Perspex chamber, dimensions 30 cm X 20 cm X 20 cm and
exposed to an aerosolized aqueous solution of 7.5% w/v citric acid for a period of 10 min. The output of the aerosolizer (INCO Laboratories, Ambala, India) was 0.25 ± 0.02 mL per minute and same aerosolizer was used throughout the experiment. The animals were watched continuously by the trained observer, and number of coughs and latency time to initial cough response were noted.

STATISTICAL ANALYSIS:

All the values were expressed as Mean ± SEM and were statistically analyzed using one way ANOVA followed by Tukey's multiple comparison test. The p<0.05 was considered to be statistically significant.

4. EVALUATION OF ANTIASTHMATIC ACTIVITY:

All the compounds were evaluated for their antiasthmatic activity in systemically antigen induced asthmatic subjects. The animals were sensitised using ovalbumine for a period of 14 days. The antiasthmatic potential of TAZQ, DBTAZQ and ambroxol hydrochloride was evaluated individually as well as in different combinations by the means of thorough in-vivo, in- vitro experimentation.

ANIMALS:

Male Hartley guinea pigs weighing 500 to 700 g were purchased from IIIM, Jammu, India. The animals were maintained in ordinary animal cages in a constant 12-h light/dark cycle. Food and water were available ad libitum. All protocols were in accordance with guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA) in India.

ANTIGEN INDUCED SENSITIZATION:

The animals were actively sensitized by the method of Andersson et al., 1981. Briefly the intraperitoneal injection of ovalbumine 20 µg and Al (OH)₃ 100 mg in normal saline (0.2 ml) was administered twice in a gap of seven days. Two weeks later (on 14th day) from the first day of sensitization, the animals were placed in histamine chamber equipped with an ultrasonic nebulizer (Inco Ltd., Ambala, India) and challenged with 0.5% ovalbumine aerosol to verify that the sensitization has occurred. The animals were withdrawn from antigen exposure at the first sign of respiratory abnormality. The
animals showing airway hyper-responsiveness to the inhaled antigen were referred to as sensitized animals. The success rate of sensitization method was 85 % as 67 of 80 animals showed airway abnormalities to inhaled antigen.

STUDY DESIGN AND TREATMENTS

The sensitized animals were randomly divided into 10 groups. Each group was comprised of 6 animals (n=06). All the drugs were dissolved in 4% HCl and then diluted in saline. Their pH values were adjusted to 6.5-7.0 with 1% Na₂CO₃. Aminophylline 50mg/kg was administered as standard treatment. Sensitized control group animals were treated with normal saline and an unsensitized normal control group was also studied. All the animals got daily treatment of drugs for 7 days. The drugs were injected intraperitoneally once a day for seven days. Table-6 represents the study design and groups assignment.

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>GPS</th>
<th>TREATMENT (i.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NC</td>
<td>Non sensitized/Normal saline treated</td>
</tr>
<tr>
<td>2</td>
<td>SC</td>
<td>Sensitized Control/Normal saline treated</td>
</tr>
<tr>
<td>3</td>
<td>TAZQ-10</td>
<td>TAZQ-10mg/kg</td>
</tr>
<tr>
<td>4</td>
<td>TAZQ-20</td>
<td>TAZQ-20mg/kg</td>
</tr>
<tr>
<td>5</td>
<td>DBTAZQ-10</td>
<td>DBTAZQ-10mg/kg</td>
</tr>
<tr>
<td>6</td>
<td>DBTAZQ20</td>
<td>DBTAZQ-20mg/kg</td>
</tr>
<tr>
<td>7</td>
<td>AH-50</td>
<td>AH-50mg/kg</td>
</tr>
<tr>
<td>8</td>
<td>AH-100</td>
<td>AH-100mg/kg</td>
</tr>
<tr>
<td>9</td>
<td>TAZQ-10+DBTAZQ-10</td>
<td>TAZQ-10mg/kg+DBTAZQ-10mg/kg</td>
</tr>
<tr>
<td>10</td>
<td>TAZQ-10+AH-50</td>
<td>TAZQ-10mg/kg+AH-50mg/kg</td>
</tr>
<tr>
<td>11</td>
<td>AMN-50</td>
<td>AMINOPHYLLINE-50mg/kg</td>
</tr>
</tbody>
</table>

Figure: 7: Experimental study design and treatments (Anti asthmatic studies)

EVALUATION OF RESPIRATORY HYPERREACTIVITY:

Final challenge with ovalbumin aerosol (0.5% in normal saline) was performed on seventh day of treatment, 30 minutes after the last dose. The guinea pigs were placed in the histamine chamber and challenged with an aerosol of ovalbumine (5mg/ml in water) for 30 min. Airway abnormalities of the animals were visibly monitored by two trained observers who were blinded to the group assignment of the animals for the changes in the respiratory activity of the animals subjected to different treatments.
Evaluation of latency time (min) for the appearance of first respiratory abnormalities assessed as the time between onset of aerosolization and the first stroke of cough and the frequency of cough till 10 min after first cough stroke was measured according to the method of Gupta et al., 1968.

BAL FLUID AND LUNG TISSUE HOMOGENATE PREPARATION

Twenty four hours after aerosolised ovalbumine challenged, animals were sacrificed with sodium pentobarbitone (200 mg/kg i.p.). The trachea was immediately cannulated and the lungs were lavaged with 5 x 4 mL aliquots of Ca $^{2+}$ and Mg $^{2+}$ free 0.1M phosphate buffered saline solution of pH-7.0. To standardize the lavage technique, 50% of the instilled medium (the maximum volume that could be consistently recovered) was withdrawn from each animal. The collected BAL fluid was centrifuged and used for further biochemical estimations.

The lung tissue was dissected out, washed in cold saline, blotted dry and homogenated (10%) in 10 ml phosphate buffer saline pH 7.4. The homogenate was centrifuged using refrigerated centrifuge and the supernatant was used for further biochemical estimations.

ASSESSMENT OF AIRWAY INFLAMMATION:

The BAL fluid was centrifuged at 150Xg for 10 min at 4$^\circ$C. using refrigerated centrifuge (Sigma 3K30 laborzentrifugen). The supernatant was discarded and the cells were resuspended in 1 mL 0.1M PBS containing 0.1 M EDTA. Total and differential leukocyte count was performed manually with a haemocytometer under DMWB Series Motic digital microscope.

ESTIMATION OF LIPID PEROXIDATION: (TBARS)

The TBARs levels lung tissue homogenates and BAL fluid (Oliveira et al, 2006) were estimated spectrophotometrically as described by Ohkawa et al 1979. Briefly, to each test tube, 0.5 ml supernatant of 1g lung tissue homogenate in 5 ml PBS, 0.5 ml of normal saline (0.5 ml of BAL fluid in case of measurement of TBARS level in BAL fluid), 1 ml of 20% trichloroacetic acid (TCA) and 0.25 ml of TBA reagent (200 mg of thiobacbituric acid dissolved in a mixture of 30 ml distilled water and 30 ml of acetic
acid) were added. The test tubes were kept for boiling at 95ºC for one hour. To each of the test tubes, 3 ml of n-butanol was added, vortexed for 5 min and centrifuged at 200Xg for 10 minutes. The separated butanol layer was read spectrophotometrically against reagent blank at 535 nm. TBARS concentration is expressed in terms of nmol of malondialdehyde per mL of BAL fluid and per grams of wet lung tissue.

ESTIMATION OF NOX LEVEL IN BAL AND LUNG TISSUE:

The amount of stable nitrite (nitrite and nitrate), the end product of NO generation by RPE cells was determined in BAL fluid and lung tissue homogenates by a spectrophotometric assay according to the method of Green et al., 1982. Briefly, 50 µl supernatant of 1g tissue homogenated in 5 ml PBS (supernatant of BAL fluid in case of determination in BAL fluid) was mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride, 5% H3PO4), and incubated at room temperature for 10 min. The absorbance was read at 540 nm. The amount of nitrite was calculated from a NaNNO2 standard curve and expressed as µmol per ml of BAL fluid and lung tissue homogenates.

DETERMINATION OF GLUTATHIONE CONTENT

The determination of glutathione content in BAL fluid and lung tissue homogenates was measured spectrophotometrically according to the method of Ellmann et al., 1959. Briefly, 1ml supernatant of 1g tissue homogenated in 5 ml PBS (supernatant of BAL fluid in case of determination in BAL fluid) was mixed with 1 ml of protein precipitant (20% trichloro acetic acid + 0.1 mM EDTA) and centrifuged at 150Xg for 10 min. 200 µL of the supernatants obtained were mixed with 1.8 ml of Ellmann’s reagent (10mM solution of 5, 5’–Dithiobis- 2-nitrobenzoic acid of pH 7.0). The resulting solutions were read spectrophotometrically at 412 nm after 5 min against blank (1.8 ml Ellmann’s reagent mixed with 200µL protein precipitant).

LUNG TISSUE HISTOLOGY:

After BALF was obtained, the lung tissue was fixed in 10% neutral buffered formalin for 24 h. The lung tissue was embedded in paraffin, and then cut into 4 mm thickness sections, stained with H&E solution (haematoxylin; Sigma MHS-16 and eosin, sigma HT110-1-32). The tissue was subsequently mounted and cover-slipped with Dako-
mounting medium (Dakocytomation; Denmark Carpinteria CA). The lung tissue sections were studied for Peribronchial cellular infiltration by acute and chronic inflammatory cell infiltrate comprising of neutrophils, eosinophils and lymph mononuclear cell infiltrate, widening of interalveolar septa because of vasodilatation, edema & increase in no. of macrophages, goblet cell hyperplasia and bronchodilatation.

**OVALBUMIN INDUCED CONSTRICTION OF ISOLATED BRONCHIAL SMOOTH MUSCLE**

The method of isolating tracheal smooth muscle was essentially the same as that described previously by Baba et al., 1986. OVA sensitized guinea pigs were killed by a sharp blow on the head and exsanguinations followed by removal of the trachea. The tracheas were removed and cut spirally into two strips 15 mm long and 3 mm wide. The tracheal strips were mounted vertically in a 30-mL water-jacketed organ bath filled with Krebs-bicarbonate buffer aerated with a mixture of 95% O₂ and 5% CO₂ at 37°C. The composition of the Krebs-bicarbonate buffer was as follows (in mM): NaCl-124, KCl-5, MgSO₄-1.3, CaCl₂-2.5, NaHCO₃-25, NaH₂PO₄-0.6, and glucose-10. Before each experiment, each strip was subjected to a load of 2 g for at least 2 h, with frequent changes of Krebs-bicarbonate buffer, until a stable baseline tension was obtained. Thereafter, each strip was exposed to 10⁻⁴ M OVA to test the responsiveness of the muscles. Any strip that developed, 1 g of tension after exposure to OVA was excluded from the study. Immediately after the peak tension developed, the strips were washed thoroughly with Krebs-bicarbonate buffer and were kept unstimulated until a stable baseline tension was obtained again. In all experiments, each drug was added into the organ bath in a quantity of not more than 300 µL. All drug concentrations indicated are expressed as the final concentration in the organ bath. After the initial equilibration phase, the tracheal strips (n=6) were studied to examine the effect of TAZQ and DBTAZQ on tracheal contraction induced by OVA. Concentration-response curves for OVA (10⁻⁹ to 10⁻⁴ M) was obtained in the absence or presence of all the drugs. Three concentrations of TAZQ (10⁻⁵, 10⁻⁴ and 10⁻³ M) and two concentrations of DBTAZQ (0.1 & 0.2 M) were studied against ovalbumine induced contraction. The effect on concentration response curve of TAZQ (10⁻³ M) in combination with ambroxol 10⁻¹ M was studied. The concentration response curve for OVA was fitted by nonlinear
regression, and the concentration giving 50% of the maximal response (EC50) was determined according to the method of Hashiba et al., 1999.

STATISTICAL ANALYSIS:

All the values were expressed as Mean ± SEM and were statistically analyzed using one way ANOVA followed by Tukey's multiple comparison tests. The p<0.05 was considered to be statistically significant.

5. PRE-FORMULATION STUDIES OF TAZQ AND AH:

CHARACTERISATION OF THE COMPOUNDS

TAZQ was synthesized and characterised as given in result section. AH was kind gift from Zoetic pharmaceuticals Ltd. and was characterised as given in result section.

SOLUBILITY STUDIES:

Quantitative solubility of TAZQ was determined using saturation solubility method. Excess amount of drug was dissolved in measured amount of different solvents in eppendorff tubes at room temperature and shaken well for 24hr on a mechanical shaker to achieve the equilibrium. Appropriate aliquots were then withdrawn, filtered and suitably diluted for spectrophotometric analysis at the 305 nm. Concentrations were calculated from the standard curves plotted in the respective solvent. It was further confirmed by visual inspection. Solubility was determined in different solvents such as acetonitrile, methanol, water, hexane and buffers within the range of 1-7.4 and put in a particular solubility class as per USP.

PARTITION COEFFICIENT

UV calibration curve of TAZQ and AH were plotted at 305 nm. Three different ratios of n-octanol and water (1:1, 1:2 and 2:1) were equilibrated on mechanical shaker for 24 hours. After 24 hours weighed amount of the compound was spiked (in each ratio) and the flasks were shaken for 15-20 min. Mixture was then centrifuged & the concentration of drug in both phases was estimated.
<table>
<thead>
<tr>
<th>S. No.</th>
<th>n-octanol:water</th>
<th>TAZQ (mg)</th>
<th>AH (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5:5 (1:1 ratio)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>II</td>
<td>3.3:6.6 (1:2 ratio)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>III</td>
<td>6.6:3.3 (2:1 ratio)</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Table: 8: Composition of Partition mixtures.

DISSOCIATION CONSTANT (PKa) DETERMINATION

For TAZQ:

TAZQ was dissolved in methanol to give 0.01 M solution and titrated with standard solution of HCl (0.01 N). Added volume of titrant and pH was noted down until pH showed 10-11, further, pH vs. added volume of titrant was plotted and the derivative of the same was obtained i.e. ΔpH/ΔV vs. volume of HCL. Volume of HCl consumed at inflection point was noted down from the derivative curve and pH was noted down at half of the volume of HCl at inflection point, which is equivalent to pKa of drug.

For AH:

AH was dissolved in water to give 0.01 M solution and titrated against standard solution of HCl (0.01 N). Added volume of titrant and pH was noted down until pH showed 10-11, further, pH vs. added volume of titrant was plotted and the derivative of the same was obtained i.e. ΔpH/ΔV vs. volume of HCL. Volume of HCl consumed at inflection point was noted down from the derivative curve and pH at half of the volume of HCl at inflection point was noted, which is equivalent to pKa of drug.

HPLC-UV METHOD DEVELOPMENT AND VALIDATION

Normal phase chromatography can be used for the separation of non-ionic and/or non-polar, while reversed phase chromatography C8 and C18 columns can be used for the separation of non-ionic as well as ion forming non-polar to medium polar substances. Thus, TAZQ and AH (weakly polar bases) can be satisfactorily separated by reversed phase chromatography. However, C8 columns are less retentive as compared to C18. Majority of the ionisable pharmaceutical compounds can be very well separated on octadecylsilane reversed phase columns. Hence, C18 was selected. A rapid and sensitive HPLC method for simultaneous determination of TAZQ and AH was developed and
validated. The experiment was performed on WATERS®515 binary HPLC system equipped with 2998 Photo Diode Array detector. The sample was injected through a Rheodyne® manual 20µl loop injector and the elution was carried out using WATERS C₁₈ stationary phase (250 mm X 4.6 mm ID; 5µm).

Preparation of stock solutions

Stock solution (1 mg/ml) of TAZQ and AH were freshly prepared in CAN and water respectively. Test solutions were prepared by diluting required quantity of stock solution with mobile phase. Stock solution of 1.0 mg/ml of pure Pantoprazole as Internal Standard was freshly prepared in ACN. Test solution of Internal Standard (10.0 µg/ml) was prepared by diluting required quantity of stock solution with water.

Optimization of chromatographic conditions:

The optimization of experimental conditions was guided by the requirement of obtaining chromatograms with better resolution of adjacent peaks, especially when numerous similar components were to be analysed. Optimized chromatographic conditions were achieved after a series of screening experiments with elution systems of ACN-Water, ACN-PBS pH 5.5, in various proportions such as 50:50, 30:70, 20:80; it was found that ACN and phosphate buffer of pH-5.5 in the ratio of 40:60 gave better peaks than other mobile phase composition. The effects of different levels of all these factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, separation factor and asymmetry etc. The appropriate internal standard selected that eluted in same chromatographic conditions was Pantoprazole that gave a well defined sharp peak with good resolution. Prior to injecting solutions, the column was equilibrated for at least 30-45 parts column volume with the mobile phase flowing through the system. Each solution was injected in triplicate, and the relative standard deviation (R.S.D.) was measured. The optimum mobile phase, consisting of ACN-phosphate buffer pH-5.5 in the ratio 40:60 at a flow rate of 0.6ml/min was subsequently employed which leads to good resolution with well defined sharp peaks for TAZQ, AH and Pantoprazole.
METHOD VALIDATION

After the optimization of the chromatographic conditions, the analytical method was validated according to ICH (Q2) guidelines for parameters such as linearity, precision, LOD, LOQ, specificity and sensitivity.

Linearity

The linearity was determined by using eight different concentration of TAZQ and AH with a constant concentration of Internal Standard in triplicate and calibration curve was plotted in the specified concentration range. The regression equation was calculated in the form of \( y = mx + c \), where \( y \) and \( x \) are the peak area ratio of drug to internal standard and concentration of each standard drug, respectively. The calibration curve was plotted by 06 replicate analysis at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® software.

Detection and Quantification limits (LOD and LOQ):

The limits of detection (LOD) were determined according to following equation:

\[
\text{LOD} = 3.3 \frac{\text{S.D}}{\text{S}}
\]

\[
\text{LOQ} = 10 \frac{\text{S.D}}{\text{S}}
\]

Where S.D. is standard deviation of the analytical signal and S is slope of the concentration/response curve.

Precision:

The reproducibility (relative standard deviation, R.S.D.) of the developed method in terms of peak area in five replicate samples was detected in intra-day and inter-day precision for the drugs. Solutions containing lowest, intermediate and highest concentration of the calibration curve were prepared. The concentration values for both intra-day precision and inter-day precision were calculated three times separately and percent relative standard deviation were calculated.
Specificity:
The specificity criterion tries to demonstrate that the result of the method is not affected by the presence of interferences, i.e. whether the compound elutes without any interfering compounds or not. The specificity of the method was determined by comparing the chromatograms obtained from samples containing TAZQ, AH and IS with those obtained from blank. Five blank samples were injected with and without IS to evaluate the presence of interfering peaks.

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION

Analytical method for simultaneous estimation of TAZQ & AH in rat plasma and lung tissue homogenates was developed. The method was validated as per the ICH (Q2) guidelines. The validated HPLC method was utilised for the plasma pharmacokinetics and lung bioavailability of TAZQ and AH on liposomal dry powder administration to the rats.

Chromatographic Conditions

Analysis was done in isocratic mode at flow rate of 0.6 ml/min with ACN: Phosphate Buffer adjusted to pH 5.5 (40:60 %v/v) as mobile phase. The mobile phase was prepared freshly, premixed and filtered through a 0.2 µm membrane filter to remove any particulate matter and degassed by sonication before use. The drugs were quantified using the peak area ratio of analytes to IS. Prior to injecting solutions, the column was equilibrated for at least 30-45 column volumes with the mobile phase flowing through the system. Each solution was injected in triplicate, and the relative standard deviation (R.S.D.) was measured.

Plasma Sample Pre-Treatment

The plasma samples were pre-treated by the method of protein precipitation. Briefly, to 200 µl plasma 800 µl of protein precipitant (ACN) was spiked. The solution was vortexed and centrifuged at 10000 rpm. The measured quantity of supernatant was transferred to sampling vials containing IS to produce 10 µg/ml concentration of IS.
Lung tissue sample pre-treatment

The lung tissue homogenates were pre-treated by the method of protein precipitation followed by liquid-liquid extraction. Briefly, measured volume of lung tissue homogenate (1ml) was added to 1 ml solution of protein precipitant (20% TCA & 1mM EDTA) the mixture was vortexed and centrifuged. Maximum possible supernatant layer was separated and extracted thrice with equal volume of chloroform (HPLC grade). The chloroform layer was separated and evaporated to dryness under flow of nitrogen. The residue was spiked with IS and reconstituted with measured volume of mobile phase. After sonication the sample was injected into chromatograph for simultaneous determination of TAZQ and AH.

VALIDATION PARAMETERS

The developed chromatographic method was validated according to CDER guidelines for accuracy, linearity, precision, sensitivity, stability and recovery. Consequently, the following were performed:

Linearity

The linearity was determined for five concentrations of TAZQ and AH ranging from 0.1-4 μg/ml & 0.2-6.4 μg/ml respectively. The ratio AUC_{drug} to AUC_{IS} was plotted against concentration of spiked standards. The calibration plot was generated by replicate analysis (n=6) at all concentration levels and a linear relationship was evaluated using the least square method within Microsoft Excel® program.

Detection and Quantification limits (LOD and LOQ):

The limits of detection (LOD) were determined according to following equation:

\[ \text{LOD} = 3.3 \times \text{S.D./S} \]

\[ \text{LOQ} = 10 \times \text{S.D/S} \]

Where S.D. is standard deviation of the analytical signal and S is slope of the concentration/response curve.
Precision:

Both repeatability (intra-day precision) and reproducibility (inter-day precision) were determined as follows:

Solutions containing lowest, intermediate and highest concentrations (LQC MQC and HQC) of the calibration curve, i.e. 0.1, 1 and 4 μg/ml and 0.2, 3.2 and 6.4 μg/ml were prepared for TAZQ and AH respectively. Six injections at each of the specified concentration levels were injected within the same day for repeatability (intra-day), and over a period of 3 days (6 injections/day) for reproducibility (inter-day). Mean and relative standard deviations were calculated and used to judge precision of the method. Both intra-day and inter-day samples were calibrated with standard curves concurrently prepared on the day of analysis. Intra-day precision and inter-day precision for the developed methods were measured in terms of % R.S.D. which was taken for conclusion.

Extraction efficiency (Recovery)

Spiked plasma and tissue homogenates were prepared in triplicate at three different standard concentrations of TAZQ & AH and assayed as described above. The extraction efficiency was determined by comparing the peak areas measured after analysis of spiked plasma samples with those found after direct injection of non-biological samples into the chromatographic system at the same concentration levels.

Specificity:

The specificity criterion tries to demonstrate that the result of the method is not affected by the presence of interferences, i.e. whether the compound elutes without any interfering compounds or not (Stulzer et al, 2008). The specificity of the method was determined by comparing the chromatograms obtained from samples containing TAZQ, AH and IS with those obtained from blank plasma. Five blank samples were injected with and without IS to evaluate the presence of interfering peaks.

Stability:

Blank plasma and tissue homogenates were spiked with the known amount of TAZQ & AH to achieve the concentration of LQC, MQC and HQC (n = 3) and stored at −4 °C. The stability of these samples was checked up to 24 hr by comparing the fresh stock prepared on the day of analysis to find out short term stability. Further, the freeze–thaw
(−20 °C/room temperature) stability was determined for three cycles. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision.

ESTABLISHMENT OF BCS CLASS OF TAZQ:

Calculating the Therapeutic Dose of TAZQ in Human-Beings:

It is reported that TAZQ at a dose of 5-30 mg/kg showed marked antagonism to histamine induced broncho-constriction in-vivo as well as in-vitro in Guinea-pigs.

The effective dose responsible for therapeutic action in guinea-pigs can be converted into human dose (USFDA Guidance, 2005) for which BCS characteristics as Solubility, Permeability can be determined.

To calculate HED for a particular dose in animals, one can calculate the animal dose in mg/m² by multiplying the dose in mg/kg by the km factor for that species. The dose can then be converted back to mg/kg in humans by dividing the dose in mg/m² by the km factor for humans indicated in formula below:

\[(\text{Animal mg/kg dose} \times \text{Animal km}) \div \text{Human km} = \text{Human mg/kg dose}\]

Solubility Determination:

A drug is considered as highly soluble if the highest therapeutic dose is soluble in less than 250ml of aqueous media within the pH range 1-7.5. Equilibrium Solubility of this dose was determined in Simulated Gastric Fluid (0.1N HCl pH 1.2), Acetate Buffer (pH 4.5), Simulated Intestinal Fluid (pH 6.8), Phosphate Buffer Saline (pH 7.4).Composition of these buffers is as mentioned in USP 28.

Permeability Determination:

A drug is considered as highly permeable if extent of absorption of the drug is more than 90% or the value of Permeability Coefficient at pH 6.5 is more than 2 x 10⁻⁴ cm/sec in human jejunum i.e. at pH 6.5 or more than 1x10⁻⁵ cm/sec in Caco-2 cells. Ex-vivo intestinal permeation techniques were employed for permeability determination as:
Franz-Diffusion Cell Technique:

In this method, the intestinal tissue was mounted between donor and receiver compartment and equilibrated with Phosphate Buffer Saline pH 7.4. Drug solution in PBS was added into donor compartment which was then covered to prevent evaporation. The samples were collected at definite time intervals till 24 hrs and replaced with the fresh PBS each time the sample is withdrawn to maintain sink conditions in the donor compartment. The samples withdrawn were quantified by validated HPLC-UV method and the percentage of drug permeated with time was calculated out.

Non-everted gut sac technique:

Wistar rats weighing 160-200 g were starved overnight in cages while water was provided ad libitum. Animals were sacrificed by spinal dislocation after overnight fasting. The small intestine was removed by cutting across the upper end of the duodenum and the lower end of the ileum and manually stripping the mesentery. The small intestine was washed out carefully with cold normal oxygenated saline solution (0.9%, w/v, NaCl) using a syringe equipped with blunt end. The clean intestinal tract was prepared into 8 ± 0.2 cm long sacs having a diameter of 3.0 ± 0.5 mm. Each sac was filled with TAZQ-HCl solution via a blunt needle, and the two sides of the intestine were tied tightly with thread. Each non-everted intestinal sac was placed in a glass cylindrical tube containing 60 ml of Krebs Ringer Solution. The entire system was maintained at 37 ºC in an organ bath and aerated with oxygen (10–15 bubble/min) using laboratory aerator. From outside of the sac 2 ml samples were withdrawn every 30 min for 4 h and replaced with fresh 2 ml of medium. The samples were analyzed using validated HPLC-UV method for the determination of TAZQ at 267 nm. The percentage drug permeation in the receptor compartment was calculated.

The electrolyte composition (mM) of Ringer Locke were as follows: NaCl 153.8, KCl 6.10, NaHCO₃ 4.64, CaCl₂ 3.33.
PREPARATION OF EMPTY AND DRUG LOADED LIPOSOMAL DISPERSION

The molar ratios of phosphotidyl choline to cholesterol and drugs to lipid concentration was established in order to produce best fit liposomal structure desired for pulmonary administration. Various parameters like percent drug entrapment, drug loading capacity, particle size and Polydispersity index were studied with liposomal formulations prepared using different ratios. Various process parameters like pH, volume of hydrating medium, time, temperature of hydration and instrumental parameters like RPM and vacuum were studied to reach the optimum formulation. The ratio which provided a vesicular size between 0.5 to 5 µm was selected for preparation of liposomal dispersion. Various parameters were studied and optimised that are given in table-8.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC (mg)</td>
<td>52.5 (7M)</td>
</tr>
<tr>
<td>CHOLESTEROL (mg)</td>
<td>11.4 (3M)</td>
</tr>
<tr>
<td>TAZQ : lipid ratio (M)</td>
<td>1:5</td>
</tr>
<tr>
<td>TAZQ (mg)</td>
<td>25 (0.12mM)</td>
</tr>
<tr>
<td>Chloroform (ml)</td>
<td>10</td>
</tr>
<tr>
<td>AH (mg)</td>
<td>150 (0.36mM)</td>
</tr>
<tr>
<td>AH: lipid ratio (M)</td>
<td>2:5</td>
</tr>
<tr>
<td>Hydrating medium (phosphate buffer, pH 7) (mL)</td>
<td>10</td>
</tr>
<tr>
<td>HYDRATION TIME</td>
<td>1 Hr</td>
</tr>
<tr>
<td>TEMP (°C)</td>
<td>40</td>
</tr>
<tr>
<td>RPM (Film Preparation /Hydration)</td>
<td>100/25</td>
</tr>
<tr>
<td>VACCUMIE (in Hg)</td>
<td>40</td>
</tr>
<tr>
<td>CRYOPROTECTANT</td>
<td>Trehalose</td>
</tr>
<tr>
<td>LIPID : CRYOPROTECTANT MASS RATIO</td>
<td>01:12</td>
</tr>
</tbody>
</table>

Table: 9: Formulation variables.
Purification of liposomal dispersions:

The purification method was done by diffusing the unentrapped drug with the help of dialysis tubing. Various methods of purification were studied such as use of Sephadex G-50 and centrifugation.

Determination of encapsulation efficiency:

The liposomal dispersions were suitably diluted with buffer and centrifuged at 15000 rpm for 30 min in cooling centrifuge (3K30 Sigma Laborzentrifugen, Germany). The supernatant was diluted further with mobile phase and analyzed using HPLC-UV method for simultaneous estimation of free (unentrapped) TAZQ and ambroxol. The amount of the entrapped drug was then calculated by substituting the resulted concentration from total drug content in the preparation. Encapsulation efficiency (EE) was expressed as mg of TAZQ and ambroxol entrapped per mM of lipid (mg/mM).

In another technique to determine the percentage of entrapped drugs, the liposomal dispersion was centrifuged at 15000 rpm for 30 min in cooling centrifuge (3K30 Sigma Laborzentrifugen, Germany) and was washed several times to remove the unentrapped drug and finally the vesicles pellet was lysed with the addition of 0.1 ml of 0.1 % X-100 solution in water. The solution was soniacted until clear and centrifuged at 15000 rpm for 10 min. the supernatants were diluted with the mobile phase and injected into chromatograph for determination of entrapped TAZQ and AH. Empty liposomal dispersions were used for the preparation of blank.

STABILITY OF THE LIPOSOMAL DISPERSIONS

Purified liposomal dispersions (empty and drug loaded, quantitatively analysed for drug content per ml of dispersion) were stored at 4, 25 and 45 °C for 1 month and were followed by visual and microscopic observation. In order to study the stability with respect to drug leakage, the measured volume of liposomal dispersions were stored at 4, 25 and 45 °C for 1 month and sampling (1 ml) was done at the end of 30 days from the date of preparation. The samples were suitably diluted with 10 ml PBS and centrifuged at 15000 rpm for 30 min. The supernatant was diluted appropriately with mobile phase and the final solution was injected onto the HPLC column in triplicate. The content of TAZQ and AH was then computed from the respective standard curve for any drug
leakage from the vesicles. This experiment was carried out in triplicate for each sampling point. Particle size and PDI of liposomal dispersion stored at 48°C was studied after 2 months.

LYOPHILIZATION OF LIPOSOMES

The different formulation depicting their percent (%) entrapment efficiency, particle size (µm), zeta potential (mV) and polydispersity index were lyophilized with different cryoprotectants. The selection of cryoprotectants was done by studying different sugars such as sucrose, lactose and trehalose at various mass ratios to lipid. Mass ratio of cryoprotectant to the lipid was optimized on the basis of percent drug retention (PDR) after lyophilization of liposomes. Trehalose was found to protect the vesicles and provide maximum vesicular retention of the drugs as compared to other sugars studied. Increased sugar concentration leads to hygroscopic nature of the DPI whereas reduced amount of sugar in the formulation caused liposomal rupture at the time of lyophilization.

In these studies, the liposomal pellet obtained after centrifuging liposomal dispersion was suspended in 0.1 M phosphate buffer pH-7 containing lactose, maltose, trehalose, sucrose, or dextrose in mass ratio of lipid: sugar (1:4). PDR of liposomes following dehydration-rehydration cycle was determined and the influence of sequence of cryoprotectant addition and mass ratio of lipid: sucrose on PDR also was studied.

Finally, liposomal dispersions were prepared by hydrating a dry lipid film with aqueous phase containing trehalose. Non encapsulated drugs were removed by dialysis for 30 min and the trehalose solution in phosphate buffer pH-7 was added to the purified dispersion to achieve the lipid: sugar mass ratio of 1:12. The dispersions were frozen to –68°C and freeze drying was performed in freeze drier (Alfa 1-2 LO Plus Lyophilizer, Christ Germany) till a dry powder was obtained. The whole drying process took 24 h. The formed porous cake was sized through ≠ 120 and ≠ 240 sieves. The lyophilized liposomal powder was mixed with lactose carrier in mass ratio of 1:5 (Mishra et al, 2004) and filled in capsules (size 2). The capsules were packed in umber colored bottles containing silica bags as desiccant, purged with nitrogen and covered with PVC coated aluminium foil. Bottles were placed in a desiccators kept in refrigerator till further use.
CHARACTERIZATION OF LDPI FORMULATION

Angle of repose:

It was determined by the fixed funnel method. The powder material was allowed to flow through a funnel tip from height of 2 cm. and the pile of powder was carefully built. The angle of repose was calculated by inverting tangentially the ratio of height and radius of the formed pile.

Compressibility Index: (Carr, 1965)

The compressibility index was obtained by tapping the powder for 500 tap (taps sufficient to obtain the plateau condition).

Moisture content: The moisture content of the DPI formulation (100 mg) was determined in triplicate on two consecutive days by Karl Fischer volumetric titration method using (reagent composition) Autotitrater, Metler Toledo.

Percentage drug encapsulation (PDE):

After lyophilization the liposomal powder was re-suspended in buffer solution with an addition of 0.1 ml of 0.1 % Triton X-100 solution in water. The solutions were made up to 1 ml with buffer and were analyzed using RP-HPLC method for simultaneous estimation of entrapped TAZQ and AH. The empty liposomal dispersion was treated in the same manner and used as blank whereas the standards for calibration curve were prepared by spiking standard drug solution into blank liposomal dispersion containing 0.1 ml of 0.1 % Triton X-100 solution in water.

The PDE was calculated as fraction of drug in a pellet expressed as percentage of total drug content. The percentage of entrapped drug was determined before (%Encaps(0)) and after freeze-drying (%Encaps) so that the leakage of the drug was expressed as the percentage of release as follows,

\[
\%\text{Release} = \frac{[\%\text{Encaps} (0) - \%\text{Encaps}] / \%\text{Encaps} (0))} \times 100
\]
Determination of Particle size and zeta potential:

The mean particle size of the prepared liposomes was obtained by photon correlation spectroscopy (PCS) using Delsa Nano particle analyzer (Beckman Coulter, Japan) before and after lyophilization. The average particle size was measured after performing the experiment in triplicates and polydispersity index (PDI) of liposomal dispersions were studied. The potential difference between dispersion medium and stationary layer of fluid attached to the dispersed particle was measured by electrophoretic light scattering (ELS) using Delsa Nano particle analyzer (Beckman Coulter, Japan) equipped with a special cuvette for zeta potential measurement.

Photomicrography

All the batches of prepared liposomes were viewed under B1 series Motic Microscope (BX40F4, Japan) with polarizing attachment to study their shape and lamellarity.

ASSAY:

The LDPI formulations were quantitatively analysed for % drug content. Accurately weighed amount of LDPI was diluted in phosphate buffer with the addition of 0.1 ml of 0.1 % Triton X-100 solution in water and sonicated until the solution becomes clear. The solution was centrifuged at 15000 rpm for 10 min. The supernatants were diluted with the mobile phase and injected into chromatograph for determination of TAZQ and AH content. Empty LDPI was treated in the same manner in order to prepare the blank.

IN VITRO RELEASE STUDIES:

In vitro drug release from the liposomal dispersions was determined by using dialysis bags 12 KD having flat width 35 mm and inflated diameter 21 mm (Sigma, U.S.A.). The assayed LDPI formulation was re-suspended in PBS and centrifuged at 15000 rpm for 30 min in (3K30 Sigma Laborzentrifugen, Germany). The pellet formed after centrifugation was re-suspended in PBS and 2 ml dispersion was transferred to the dialysis bag, which was tied to the paddle of the dissolution apparatus, Programmable Tablet Dissolution Tester (USP XXI/ XXII) TDT-06P (Electrolab, India), which was lowered into a 100 ml beaker containing 50 ml PBS (pH 7.4) as dissolution medium. The contents of the beaker were stirred at 50 rpm at the temperature of 37±1°C
throughout the experiment. 5 ml samples were withdrawn periodically at the end of 10, 20, 30, 45, 60, 90, 120, 180 min, every hour thereafter for 6 h and finally at 24 h. Every withdrawal was followed by replacement with fresh medium. The samples were analyzed using RP-HPLC method for simultaneous estimation of TAZQ and ambroxol against the samples withdrawn at respective time interval from empty liposomal dispersion treated in a similar manner. In vitro release of plain drug solutions of same concentration as in liposomal dispersion was also studied.

Evaluation of in-vitro release kinetics:

Preparation of dialysis sac: A 10cm long portion of the dialysis tubing was made into dialysis sac by folding and tying up one end of the tubing with thread, taking care to ensure that there would be no leakage of the contents from the sac. The sac was soaked overnight in PBS.

Dialysis set up:

The wet sac was gently opened and washed with PBS. The sac (donor compartment) was filled with 2 mL of RIF loaded liposomes and was suspended in a container containing 200 mL of methanolic PBS (receptor compartment). The temperature was maintained at about 37°C± 2°C in the incubator shaker, with 150 rpm, the container was closed with the aluminium foil to prevent any evaporative losses during experimental run.

Various models (zero- order, first- order, higuchi model and Korsemeyer-Peppas) were applied according to the parameters tabulated in table in order to evaluate the release data. Regression coefficient (R²), elimination rate constant and diffusion exponent (n) were calculated and proximity to 1 for (R²) and 0.45 for (n) was the reference.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameter</th>
<th>Kinetic order</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cumulative% drug release v/s time</td>
<td>Zero- order</td>
</tr>
<tr>
<td>2</td>
<td>log cumulative % drug release v/s time</td>
<td>First- order</td>
</tr>
<tr>
<td>3</td>
<td>% drug release v/s √time</td>
<td>Higuchi model</td>
</tr>
<tr>
<td>4</td>
<td>log cumulative % drug release v/s log time</td>
<td>Peppas model</td>
</tr>
</tbody>
</table>

Table: 10: Different parameters with kinetic orders.
MORPHOLOGY

The lyophilized powder was coated with gold and then kept in the sampling unit as a thin film and the photographs were taken at different magnifications using scanning electron microscopy (SEM).

DETERMINATION OF AERODYNAMIC PROPERTIES:

Aerodynamic particle sizing of the liposomal dry powder was measured using an Andersen I ACFM nonviable ambient particle sizing sampler equipped with eight stage size compartments and an artificial throat as a simulator of the human respiratory system (Andersen Instruments Inc, Atlanta, GA). After determination of the drug concentrations for each stage by HPLC, the MMAD and geometric standard deviation (GSD) of the liposomal dry powder was computer calculated by a log probability plot. The MMAD and GSD were determined by the liposomal drug content distributed within the array of particles comprising the airflow.

STABILITY OF LDPI:

Liposomal dry powders (empty and drug loaded, quantitatively analysed for drug content per ml of dispersion) were stored at 4, 25 and 45 °C for 1 month and were followed by visual and microscopic observation. In order to study the stability with respect to drug leakage, the weighed amount of LDPI was stored at 4, 25 and 45 °C for 1 month and sampling was done at the end of 30 days. The samples was suitably diluted with 10 ml PBS and centrifuged at 15000 rpm for 30 min. The supernatant was diluted appropriately with mobile phase and the final solution was injected onto the HPLC column in triplicate. The content of TAZQ and AH was then computed from the respective standard curve for any drug leakage from the vesicles. This experiment was carried out in triplicate for each sampling point. Particle size and PDI of liposomal dispersion stored at 48°C was studied after 2 months.
IN-VIVO BIO-AVAILABILITY:

Lyophilised LDPI powders were studied for lung bioavailability and plasma pharmacokinetics.

ANIMALS:

Adult albino rats (Wistar origin) of either sex weighing 200 ± 20 g were housed in animal house of I.S.F. College of pharmacy with free access to pelletized chow and tap water. The temperature was maintained at approximately 26°C to 28°C.

Healthy rats were selected randomly and divided into 6 groups (n=18) which were exposed to the formulations to study the bioavailability of the drugs. In vivo bioavailability studies were performed by administering the formulations to the lungs with the help of a suitable delivery device (cannula) that was inserted up to the tracheal bifurcation. The animals were anaesthetized using ketamine solution (1.2 gm/kg) by intra-peritoneal administration. The experimental groups were designed as given in table-10. Plasma pharmacokinetic was studied by comparing the systemic concentration of the drugs at different time points with that of intravenous administration of the drug solution.

<table>
<thead>
<tr>
<th>Group</th>
<th>no of animals (n)</th>
<th>formulation administered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>TAZQ (IV)</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>AH (IV)</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>TAZQ (INH)</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>AH (INH)</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>TAZQ LDPI</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>AH LDPI</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>COMB LDPI</td>
</tr>
</tbody>
</table>

Table: 11: Bio-availability study design.

The sampling protocol was developed in such a way so that only three animals were scarified at each sampling point. At each time point only three animals were studied for bio-availability. After the collection of blood sample, the animals were scarified and tissue sample was collected.
Blood sampling:

After administration of various formulations, 0.4 ml of blood sample was collected from retro orbital plexus at 1, 2, 4, 8, 12 and 24 hr in heparinised tubes. In case of free drugs administered to the lungs, 0.4 ml of blood samples were collected from retro orbital plexus of at 0.25, 0.5, 1, 2, 4, 8, and 12 hr. Blood samples were immediately centrifuged (5000-8000 rpm) for 10 min. at an ambient temperature. The supernatant plasma was separated and stored at –68°C until analyzed. The Plasma samples were treated with protein precipitation and analysed using validated bio-analytical HPLC-UV method and drug plasma concentration values were determined from calibration curve.

Lung Tissue Sampling:

Animals (three animals at each time point) were sacrificed after 1, 2, 4, 8, 12 and 24 h. The animals were dissected and right lobe of the lungs of all the animals were taken out. The lung samples were washed to remove any adhered debris and dried using a tissue paper. The isolated organs were weighed separately, and homogenized in phosphate buffer using tissue homogenizer. The homogenized tissues (20%) were stored in a deep freezer for analysis. The samples were treated with protein precipitant followed by liquid-liquid extraction. The prepared samples were analysed using validated bio-analytical HPLC-UV method. The concentrations of drugs were calculated at different time intervals.

Plasma pharmacokinetic Analysis:

The plasma concentration v/s time data, obtained after administration of free RIF and different formulations were analyzed. The various pharmacokinetic parameters estimated were: maximum plasma concentration (Cmax), time to reach maximum concentration (Tmax), area under the curve from time zero to last time point of the detection (AUC0−t). The calculation of pharmacokinetic parameters was as follows: Maximal plasma concentration and time to maximal plasma concentration were taken directly from the plasma concentration v/s. time curve. The area under the curve (AUC) was calculated by the linear trapezoidal rule from measured data points from the time of administration until the time of the last quantifiable concentration, half- life (t1/2) and the elimination rate constant (Ke) was also determined.