### 4.1. MATERIALS

**Table 4.1. List of chemicals**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Chemicals/ Reagents</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Terbutaline Sulfate (TBS; Purity &gt;99.54%)</td>
<td>Netco Ltd., Hyderabad, India</td>
</tr>
<tr>
<td>2</td>
<td>Propranolol hydrochloride (99.9% purity)</td>
<td>Jubilant Clinsys, Noida, India</td>
</tr>
<tr>
<td>3</td>
<td>Pluronics (F68, F108, F127)</td>
<td>Sigma Aldrich Ltd., Bangalore, India</td>
</tr>
<tr>
<td>4</td>
<td>Polyethylene glycol (PEG 400, PEG 6000)</td>
<td>SD Fine Chem, India</td>
</tr>
<tr>
<td>5</td>
<td>Polyvinyl alcohol (molecular weight 125,000)</td>
<td>SD Fine Chem, India</td>
</tr>
<tr>
<td>6</td>
<td>Tween 80</td>
<td>SD Fine Chem, India</td>
</tr>
<tr>
<td>7</td>
<td>Lactose (Pharmatose)</td>
<td>DMV International, Netherlands</td>
</tr>
<tr>
<td>8</td>
<td>D-mannitol</td>
<td>Sigma-Aldrich, India</td>
</tr>
<tr>
<td>9</td>
<td>D-trehalose dihydrate</td>
<td>Sigma-Aldrich, India</td>
</tr>
<tr>
<td>10</td>
<td>L-leucine</td>
<td>Fluka, Switzerland</td>
</tr>
<tr>
<td>11</td>
<td>Precoated silica gel 60F254 plates</td>
<td>E. Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>12</td>
<td>Acetonitrile (HPLC grade)</td>
<td>Merck India Ltd</td>
</tr>
<tr>
<td>13</td>
<td>Acetone (HPLC grade)</td>
<td>Merck India Ltd</td>
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<td>14</td>
<td>Isopropyl alcohol (HPLC grade)</td>
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<tr>
<td>15</td>
<td>Methanol (LC grade)</td>
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<td>16</td>
<td>Ethanol (LC grade)</td>
<td>Merck India Ltd</td>
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<td>17</td>
<td>Ethyl acetate (LC grade)</td>
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<td>18</td>
<td>Dichloromethane (HPLC grade)</td>
<td>Merck India Ltd</td>
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<td>19</td>
<td>Ammonium acetate (MS grade)</td>
<td>Fluka analytical, Netherland</td>
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<td>20</td>
<td>Ammonium formate (MS grade)</td>
<td>Fluka analytical, Netherland</td>
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<tr>
<td>21</td>
<td>Formic acid (&gt;98.0% purity)</td>
<td>Fluka analytical, Netherland</td>
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<tr>
<td>22</td>
<td>Distilled water</td>
<td>Millipore, Millipore Corp., USA</td>
</tr>
</tbody>
</table>
### 4.2. EQUIPMENTS AND INSTRUMENTS

**Table 4.2. List of equipments/ Instruments**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Equipment/ Instrument</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UV-Vis Spectrophotometer</td>
<td>Shimadzu UV-1601</td>
</tr>
<tr>
<td>2</td>
<td>High Performance Thin layer chromatography</td>
<td>CAMAG (Muttenz, Switzerland)</td>
</tr>
<tr>
<td>3</td>
<td>Ultra High Performance Liquid Chromatography (UHPLC)</td>
<td>Waters ACQUITY UPLC™ system (Waters Corp., MA, USA)</td>
</tr>
<tr>
<td>4</td>
<td>Mass Spectrophotometer (Synapt ESI-qTOF)</td>
<td>Waters Q-TOF Premier™ (Micromass MS Technologies, (Manchester, UK))</td>
</tr>
<tr>
<td>5</td>
<td>Fluorescence Spectrophotometer</td>
<td>Hitachi 650-10 S</td>
</tr>
<tr>
<td>6</td>
<td>Infra-red spectroscopy</td>
<td>FT-IR JASCO (MD spectrometer Easton, Maryland)</td>
</tr>
<tr>
<td>7</td>
<td>Nuclear magnetic spectroscopy</td>
<td>Bruker BioSpin GmbH (Rheinstetten, Germany)</td>
</tr>
<tr>
<td>8</td>
<td>Powder X-Ray diffraction</td>
<td>Shimadzu XRD-6000, Mumbai, India</td>
</tr>
<tr>
<td>9</td>
<td>Particle size analyzer</td>
<td>Zetasizer-1000 HAS, Malvern Instruments, UK</td>
</tr>
<tr>
<td>10</td>
<td>Particle size analyzer for nebulizer</td>
<td>Lasair II, USA</td>
</tr>
<tr>
<td>11</td>
<td>Transmission Electron Microscopy</td>
<td>TOPCON 002B, Netherland</td>
</tr>
<tr>
<td>12</td>
<td>Scanning electron microscopy</td>
<td>Leo 435 VP, Japan</td>
</tr>
<tr>
<td>13</td>
<td>Andersen Cascade Impactor</td>
<td>Copley Instruments (Nottingham, UK)</td>
</tr>
<tr>
<td>14</td>
<td>Gamma Counter</td>
<td>Capintec, USA</td>
</tr>
<tr>
<td>15</td>
<td>Differential Scanning Calorimetry</td>
<td>Perkin Eimer (Pyris 6DSC)</td>
</tr>
<tr>
<td>16</td>
<td>Bath Sonicator</td>
<td>Altrasonics India US-250W</td>
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<tr>
<td>17</td>
<td>Probe sonicator</td>
<td>Electrosonic India Ltd</td>
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<tr>
<td>18</td>
<td>Spray drier</td>
<td>SM Scientech, India</td>
</tr>
<tr>
<td>19</td>
<td>Freeze drier</td>
<td>Labconco™, Japan</td>
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<tr>
<td>20</td>
<td>Oven</td>
<td>Metrex Scientific Instrument Ltd</td>
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<tr>
<td>21</td>
<td>Stability chamber</td>
<td>Thermolab, India</td>
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<tr>
<td>22</td>
<td>Rotary evaporator</td>
<td>Hahnshin Scientific Co. Japan</td>
</tr>
<tr>
<td>23</td>
<td>Magnetic stirrer</td>
<td>Remi Equipment Pvt Ltd.</td>
</tr>
<tr>
<td>24</td>
<td>Freeze</td>
<td>Samsung, India</td>
</tr>
<tr>
<td>25</td>
<td>Vacuum dryer</td>
<td>Hahnshin Scientific Co. Japan</td>
</tr>
</tbody>
</table>
4.3. PREFORMULATION STUDY

4.3.1. Physical characterization

1. Description
The physical property of TBS was determined on preliminary identification based on general appearance, color and odor.

2. Loss on drying (LOD)
Approximately weighed drug sample (2.0 gm) was put in a clean and dried pan (Alumina pan) and load to moisture analyzer (HB-43 Halogen Moisture Analyzer) at 105 °C for 5 min. After the defined time the moisture content was estimated.

4.3.2. Identification of drug candidate

1. Fourier Transform Infrared Spectrometry (FTIR)
Spectra were recorded in the range of 400–4000 cm⁻¹ using a resolution of 4 cm⁻¹ and 10 scans. Samples were mixed with potassium bromide (KBr) and were pressed to obtain supporting disks. It was compared with reference spectrum and the peaks were found according to functional groups present in the TBS.

2. Nuclear Magnetic Resonance (NMR)
For NMR study, the drug samples were dissolved in 200 μL D₂O containing 0.1% ethylene glycol (99%, Cambridge Isotope Laboratories, Woburn, Massachusetts, USA) and pH was adjusted to values between 6.6 and 7.2. The samples were transferred into 5 mm NMR microtubes (Shigemi Inc., PA, USA). Power gated proton decoupled ¹³C spectra were obtained on an advance DRX500, 11.7T spectrometer and a WALTZ-16 decoupling sequence were used (30° pulse angle, acquisition time: 1.3 s, number of scans: 30 000, sweep width: 25 000 Hz, 32 K data points and a relaxation delay: 0.5 s). Relevant carbon signals in the ¹³C spectra were identified and integrated using XWIN-NMR software. The amounts of ¹³C were quantified from the integrals of the peak areas, using ethylene glycol as internal standard and corrected for nuclear Overhauser enhancement effects and relaxation by applying correction factors that were obtained from samples run with and without nuclear Overhauser enhancement plus 20 sec relaxation time.
3. Melting point
Melting point of the drug was determined using scientific melting point apparatus (Scientific apparatus, India) and further confirmed by DSC.

4.3.3. Solubility study
The solubility of TBS was determined in various solvents including distilled water and ethanol. In this determination, an excess amount of the drug was added to the solvent present in different flasks till saturation. The drug was exposed to various organic solvents and the solubility phenomena were monitored. Then, it was placed in a mechanical shaker 37°C in mechanical shaker for 72 h to attain the equilibrium. The samples were then centrifuged at 4000 rpm for 10 min, and the supernatants were assayed by UV spectroscopy after appropriate dilution.

4.4. ANALYTICAL METHODOLOGY

4.4.1. Ultra-Violet Spectrophotometer (UV)
All UV spectrophotometric measurements were made using Shimadzu UV-Vis spectrophotometer. The wavelength was selected at 281.5 nm. Calibration curves in different solvents/ physiological buffers were prepared by using UV Spectrophotometer. A definite amount of the TBS (10 mg) was transferred to 100 mL volumetric flask, dissolved in 100 mL distilled water, filtered and volume was completed with distilled water and solution was prepared with final concentration of 100 μg mL⁻¹ (stock solution). Then different sets of working standards at different concentrations were prepared by appropriate dilution of the stock solution.

4.4.2. High Performance thin layer chromatography (HPTLC)
HPTLC has advantages over other available analytical methods because of the rapidity, selectivity, economy and overall versatility in the quality control aspects of drugs (Ahmad et al., 2009). An ideal stability indicating method is one that quantifies the drug and also resolves its degradation products. Hence we attempted to develop a rapid, specific, reproducible and stability-indicating HPTLC method for the determination of TBS in the presence of degradation products and related impurities.
from a pharmaceutical formulation in the form of submicronized dry powder inhalers and to establish validation as per ICH guidelines.

### 4.4.2.1. Apparatus and chromatographic condition

Chromatography was performed on precoated silica gel aluminum plates 60F254 (20 cm × 10 cm, 200 μm thickness; E. Merck, Darmstad, Germany) were used as stationary phase. Samples were spotted in the form of distinct bands (4 mm in width; 10 mm apart) with a CAMAG 100 μL syringe using a Linomat V (CAMAG, Muttenz, Switzerland) sample applicator. Equipment parameters were optimized for smooth working (constant application rate: 160 nL s⁻¹; slit dimension: 3 mm × 0.45 mm; and scanning speed: 20 mm s⁻¹). Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland), previously saturated with optimized mobile phase for 15 min at room temperature (25 ± 2°C) and relative humidity (RH) of 60±5%. The development includes band space of 10 mm, chromatogram run of 8 cm, 20 mL of mobile phase and time duration of 10 min. The mobile phase was chloroform-methanol (9.0:1.0 v/v) with densitometric analysis at 366 nm in fluorescent mode with CAMAG TLC scanner III; a tungsten lamp was used as a radiation source and the procedure were operated by winCATS software (Ver. 1.2.0).

### 4.4.2.2. Stock solution and QC samples

A stock solution of TBS (100 ng μL⁻¹) was prepared in methanol. Different volumes of stock solution ranging from 1–10 μL⁻¹ were spotted in triplicate on TLC plates to obtain a final concentration range of 100–1000 ng spot⁻¹. The data of peak areas plotted against corresponding concentration were treated by linear-square regression. QC samples were prepared at concentrations of 400 and 800 ng spot⁻¹. The data of peak area versus drug concentration were treated by linear least-square regression.

### 4.4.2.3. Method validation

The proposed analytical method was validated as per the latest ICH guidelines Q2A and Q2B.

#### a. Precision and Accuracy

The intra-day precision and accuracy of the assays was evaluated by performing replicate analyses (n=6) of QC samples (200, 400 and 800 ng spot⁻¹). The inter-day precision and accuracy of the assay was determined by repeating the intra-day assay.
on three different days. Precision was expressed as the percentage coefficient variation (%CV) of measured concentrations for each calibration level, whereas accuracy was expressed as percent recovery [TBS found/TBS applied] × 100.

b. Robustness

The robustness of the method was determined by introducing a small change in optimized mobile phase system compositions, mobile phase volume and duration of mobile phase saturation; we observed the effects on the results. Mobile phases having different compositions of chloroform-methanol (8.75:1.25 and 9.25:0.75 v/v) were tried for one QC sample (800 ng spot⁻¹) and chromatograms were run. Mobile phase volume and duration of saturation were varied at 20 ± 2 mL (18, 20 and 22) and 30 ± 10 min (20, 30 and 40 min), respectively.

c. Linearity and Sensitivity

In order to estimate detection (LOD) and quantification (LOQ) limits, we spotted blank methanol (n=6) following the same method as explained under the section Apparatus and chromatographic conditions and the standard deviation (σ) of the magnitude of analytical response was determined. The LOD was expressed as (LOD = 3.3σ/slope of TBS calibration curve), whereas LOQ was expressed as (LOQ = 10σ/slope of TBS calibration curve).

d. Recovery

Recovery studies for the method were carried out by applying the method to drug samples to which known amounts of TBS corresponding to 50, 100 and 150% of the TBS label claim had been added. At each level of the amount, six determinations were performed. This was done to check for the recovery of the drug at different levels in the formulations.

4.4.2.4. Forced degradation study

A stock solution containing 50 mg of TBS and submicronized dry powder (SµTBS) equivalent to 50 mg of TBS was separately prepared in 50 mL methanol. The stock solution (1000 µg mL⁻¹) was used for forced degradation to provide an indication of the stability-indicating property and the specificity of the proposed method.

a. Acid and Base induced degradation

For acid and base induced degradation, to the five mL of methanolic stock solutions (TBS and SµTBS), a 5 mL of 2 N HCl (acid) and 2 N NaOH (alkali) was added
separately and the resultant mixtures were refluxed for 3 h at 80 °C in the dark (to exclude the possible degradative effect of light). Two microliters (1000 ng spot⁻¹) of the resultant solutions were carefully applied on the TLC plate and the chromatograms were run as described in the Section Apparatus and chromatographic conditions. The average peak area for TBS after application (1000 ng spot⁻¹) of six replicates was obtained.

b. Hydrogen-peroxide induced degradation

For hydrogen-peroxide induced degradation study, to the 5 mL of methanolic stock solutions of TBS and SµTBS (equivalent to 50 mg of TBS), a 5 mL of hydrogen peroxide (H₂O₂; 30.0%, v/v) were added separately. The solutions were gently heated in a boiling water bath for 20 min and then refluxed for 3 h at 80 °C to remove excess hydrogen peroxide. Two Microliters (1000 ng spot⁻¹) of the resultant solutions were spotted on the TLC plate and the chromatograms were run as described in the Section on Apparatus and chromatographic conditions. The average peak area for TBS after application (1000 ng spot⁻¹) of six replicates was obtained.

c. Photolytic degradation

For photochemical induced degradation study, 5 mL of methanolic stock solutions of (TBS and SµTBS) equivalent to 50 mg of TBS, a 5 mL of methanol was added and the solution was exposed to UV irradiation at 254 nm for 8 h in a UV chamber. Two microliters (1000 ng spot⁻¹) of the resultant solutions were applied on the TLC plate and the chromatograms were run as described in the Section on Apparatus and chromatographic conditions.

d. Dry & Wet heat degradation

For dry heat treatment, the powdered drug (TBS and Sµ-TBS) was stored separately in oven at 100°C for 8 hrs (dry heat conditions) to study the inherent stability of drug to dry heat-induced degradation (Ahmad et al., 2009). The stock solution was prepared containing 10 mg of dry heat exposed drug in 20 mL methanol (500 ng spot⁻¹). 2 µL (1000 ng spot⁻¹) of the resultant solutions were applied on the TLC plate and the chromatograms were run as described earlier and the average peak area for TBS after application (1000 ng spot⁻¹) of six replicates was obtained. However for the wet heat treatment, 5 mL of methanolic stock solution of (TBS and Sµ-TBS) equivalent to 50 mg of TBS, was diluted with 5 mL of methanol and the resulting mixture was then...
refluxed separately for 2 hrs at boiling water bath to study wet heat degradation. 2 µL (1000 ng spot⁻¹) of samples were applied on the TLC plate and the chromatograms were run as described above and the average peak area for TBS after application (1000 ng spot⁻¹) of six replicates was obtained.

d. Related Impurities
To estimate unknown impurities, TBS solution was prepared at concentration of 1000 µg mL⁻¹ in methanol; and this solution was specified as “Sample solution”. One milliliter of the sample solution was diluted to 5 mL of methanol termed as “Standard solution” (200 µg mL⁻¹). Two microlitres of the sample solution (2000 ng spot⁻¹) and 2 µL of the standard solution (400 ng spot⁻¹) were gently spotted on TLC plate in triplicates (n=3) and chromatography was performed as previously discussed earlier.

4.4.3. Ultra High Performance Liquid Chromatography–Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry (UHPLC/ESI-Q-TOF-MS)

4.4.3.1. UHPLC conditions
UHPLC was performed with a Waters ACQUITY UPLC™ system (Serial No# F09 UPB 920M; Model Code# UPB; Waters Corporation, MA, USA) equipped with a binary solvent delivery system, an auto-sampler, column manager and a tunable MS detector (Serial No# JAA 272; Synapt; Waters, Manchester, UK). Chromatographic separation was performed on a Waters ACQUITY UPLC™ BEH C18 (100.0 mm × 2.1 mm; 1.7 µm) column. The mobile phase for UHPLC analysis consisted of Acetonitrile–2 mM Ammonium acetate (1: 9; v/v), which was degassed. For isocratic elution, the flow rate of the mobile phase was kept at 0.25 mL min⁻¹ and 10 µL of sample solution was injected in each run. The total chromatographic run time was 3.0 min. The column and auto-sampler were maintained at 40±2 ºC and 4±2 ºC respectively and the pressure of the system was kept in between 2457-2440 psi.

4.4.3.2. Q-TOF-MS conditions
Mass spectrometry was performed on a Waters Q-TOF Premier Premier (Micromass MS Technologies, Manchester, UK) mass spectrometer. The nebulization gas was set to 500 L h⁻¹, the cone gas set to 50 L h⁻¹ and the source temperature set to 100 ºC. The capillary voltage and sample cone voltage were set to 3.0 KV and 40 V respectively.
The Q-TOF Premier Premier™ was operated in V mode with resolution over 8500 mass with 1.0 min scan time and 0.02 s inter-scan delay. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the MassLynx V 4.1 software incorporated in the instrument. Argon was employed as the collision gas at a pressure of $5.3 \times 10^{-5}$ Torr. Quantitation was performed using Synapt Mass Spectrometery (Synapt MS) of the transitions of $m/z$ 226.19$\rightarrow$152.12 for TBS and $m/z$ 260.34$\rightarrow$183.11 for IS (Propranolol) respectively with a scan time of 1.0 min scan time, and 0.02 s inter-scan per transition. The optimum values for compound-dependent parameters like trap collision energy (Trap CE) and transfer collision energy (Tran CE) were set to 13.2 and 11.9 V, respectively for fragmentation information. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the MassLynx V 4.1 software.

4.4.3.3. Quality control (QC) samples
The standard stock solution of 100 µg mL$^{-1}$ of TBS was prepared by dissolving requisite amount in water (sonicated: 44 kHz, 250W at 25 °C for 20 min). Calibration curve standards consisting of a set of ten non-zero concentrations (A-J) were prepared by 5% aqueous analytes spiking in blank rat plasma (50 µL aqueous aliquots to 950 µL blank rat plasma) yielding concentration range from 1–1000 ng mL$^{-1}$ for TBS. The final concentrations for each analyte were prepared to be 1, 2, 5, 10, 20, 50, 100, 250, 500, 750 and 1000 ng mL$^{-1}$. QC samples were prepared independently at three levels; 800 ng mL$^{-1}$ (HQC, high quality control), 402 ng mL$^{-1}$ (MQC, middle quality control) and 2 ng mL$^{-1}$ (LQC, low quality control). A 100 ng mL$^{-1}$, internal standard working solution was prepared by diluting the stock solution in methanol-water (50: 50 v/v). All the solutions were stored at 2–8 °C until use.

4.4.3.4. Sample preparation protocol
All the solutions (CC standards, QC samples and unknown plasma samples) were freshly prepared before carrying out the experiments. The 200 µL aliquot of each samples were taken into glass tube, 50 µL of IS (100 ng mL$^{-1}$) was added in each sample, and further 500 µL of formic acid (5% w/v) was incorporated to mixture and vortexed at 300 rpm for 5 min). Finally, 10 µL of plasma was extracted with 1 mL of ethyl acetate, and the reaction mixture was evaporated to 900 µL. 50 µL of reconstitution solution (acetonitrile: ammonium acetate buffer; 10: 90, v/v) was then
added and vortexing for 10 s at 300 rpm, the solution was transferred into the clean autosampler vials and 10 µL was injected into UHPLC/MS system for analysis.

4.4.3.5. Bioanalytical method validation

The method validation of TBS in Wistar rat plasma was performed according to USFDA guideline (USFDA, 2011). The linearity of the method was determined by analysis of five standard plots containing ten non-zero concentrations. Peak area ratios of analyte/ IS were utilized for the construction of calibration curves, using weighted ($1/x^2$) linear least squares regression of the plasma concentrations and the measured peak area ratios. The lower limit of quantification (LLOQ) is the lowest concentration of the calibration curve, which could be measured with acceptable accuracy and precision. The LLOQ was determined based on the signal-to noise ratio of 10:1. The extraction efficiency (recovery) of TB was performed at LQC, MQC and HQC levels. It was evaluated by comparing the mean area response of six replicates of extracted samples (spiked before extraction) to that of extracted drug free plasma samples (spiked after extraction) at each QC levels. The recovery of IS was similarly estimated. For determining the intra-day accuracy and precision, replicate analysis of plasma samples of TB was performed on the same day. The run consisted of a calibration curve and six replicates of LQC, MQC and HQC samples. The inter-day accuracy and precision were assessed by analysis of six precision and accuracy batches on three consecutive validation days. However, for evaluating the ruggedness of the method, one batch of precision and accuracy was run using a different column (same type) by a different analyst employing the same or another instrument. The six replicates were run for LQC, MQC and HQC samples.

4.4.3.6. Matrix effect

To study the effect of matrix on analyte quantification 6 samples were prepared from 6 different batches of plasma at LQC and HQC levels and checked for the % accuracy and precision (%CV) in both the QC samples. This was assessed by comparing the back calculated value from the QC’s nominal concentration. After specified storage conditions, samples were processed and analyzed. The matrix effect was investigated by postextraction spike method. Peak area (A) of the analyte in spiked blank plasma with a known concentration (MQC) was compared with the corresponding peak area (B) obtained by direct injection of standard in the mobile phase. The ratio ($A/B \times 100$)
is defined as the matrix effect. The six replicates were run for LQC, MQC and HQC samples.

4.4.3.7. Ex vivo Stability

The stability of TBS in Wistar rat plasma was evaluated by analyzing six replicates of plasma samples at the concentrations of 2 ng mL\(^{-1}\) (LQC) and 800 ng mL\(^{-1}\) (HQC) which were exposed to different conditions (time and temperature). Percentage stability was determined as; % Stability = mean corrected response of stability stock / mean response of comparison stock × 100.

The long term stability (LTS) was assessed after storage of the standard spiked plasma samples at deep freeze (−80 °C) for one month. Six replicates of LQC and HQC were used for analysis. However, the freeze–thaw stability (FTS) in plasma was evaluated for three consecutive freeze–thaw cycles from −20 °C to room temperature (+25 °C). Six replicates of LQC and HQC were analyzed after undergoing three freeze–thaw cycles. The bench top stability (BTS) was determined for 24 h storage in optimized conditions, using six sets each of LQC and HQC. The QC samples were quantified against the freshly spiked calibration curve standards. Short-term stability was determined after the exposure (of processed samples) at 10 °C for 24 h in autosampler using six sets each of LQC and HQC. After specified storage conditions, samples were processed and analyzed. The analytes are considered to be stable when the precisions are below 15% and the accuracies are in the range of 85–115% respectively for both levels [12]. After specified storage conditions, samples were processed and analyzed.

4.4.3.8. Pharmacokinetics

The developed UHPLC/ESI-qTOF-MS method was applied to determine the plasma concentrations of TBS in treated Wistar rats.

Animal: Wistar rats (n=6; 200–250 g, 8–10 weeks old) were provided from Experimental Central Animal House, Hamdard University and kept in an environmentally controlled room (Temperature: 25±2°C, humidity: 60±5%, 12 h dark–light cycle) for at least 1 week before the experiments. Animals were fed on standard pellet diet (Ashirwad Industries, Chandigarh) and water was provided ad libitum. The rats were fasted overnight before the day of the experiment.

Experimental design: The animal protocol used in this study was approved by Hamdard University’s Institutional Animal Ethics Committee. Rats were fasted for 12
h with free access to water prior to the pharmacokinetic investigation. A single oral dose (5mg kg\(^{-1}\) dissolved in distilled water/oral feeding tube) was given to the animal. Using venous catheter, 1 mL of blood samples were withdrawn and procured in pre-heparinized glass tubes at different time intervals (0, 0.083, 0.166, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, and 16 h). Further, blood samples were centrifuged (2500×g; 10 min; 20ºC) to separate plasma fractions.

The collected plasma samples were preserved for investigation at −80 ºC until analysis. Concentration–time curves were established for each analyte and used for the determination of pharmacokinetic parameters such as peak plasma concentration (\(C_{\text{max}}\)), peak time (\(T_{\text{max}}\)), extent of absorption (AUC) and half-life (\(T_{0.5}\)), by a non-compartmental analysis using PK Solutions Version 2.0; Summit Research Services, USA.

### 4.5. FORMULATION DEVELOPMENT

Fig. 4.1 summarizes the processes that have been used to produce different inhaled nanotherapeutics agents. In general, the processes could be grouped into two categories, top-down and bottom-up, depending on the starting materials (Rabinow BE, 2004; Bailey & Berkland, 2009).

![Particle formation scheme](image-url)
4.5.1. Nanosizing techniques

Particle designing is the prime focus to improve pulmonary drug targeting with the splendor of nanomedicines. For the effective lungs deposition, the particle size must be in the range of 0.5–5 µm (Choi et al., 2010; Muller et al., 2004). Because of the cohesive nature and poor flow characteristics of existed DPI’s, they are difficult to redisperse upon aerosolization with breath. These problems are illustrious in aerosol research, much of which is vastly pertinent to pulmonary therapeutics. Advances that have been utilized in production of submicron drug particles which could affect aerosol behavior and regional lung deposition of inhaled particles are experimentally confirmed here (Fig. 4.2).

![Diagram of nanosizing techniques](image)

**Fig. 4.2.** Techniques utilized in formation of submicron particles.

4.5.1.1. Simple stirring

A schematic view of stirring method is presented in Fig. 4.3. Simple stirring is the conventional method of the size reduction, in which the plain drug is stirred at high speed in an organic solvent (Eerdenbrugh et al., 2008). In brief, the weighed amount of TBS (250 mg) was passed through 400-mesh sieve. The sieved drug was slowly added in different antisolvent containing different stabilizers (10% w/w), gently placed over magnetic stirrer (2000 rpm; 2-4 h). Particles obtained were analyzed for mean particle size by Malvern.
4.5.1.2. Ultrasonically designed particles

Weighed amount of drug (250 mg) was passed through 400-mesh sieve and the sieved drug was added slowly into solution of stabilizer placed on bath sonicator (25°C; 15 min). Suspension was filtered through Whatman filter paper (No. 542, pore size 2.7 µm) to remove large or aggregated particles. The filtrate was then analyzed for mean particle size.

4.5.1.3. Particles production by High pressure homogenization

High pressure homogenization is scalable and has been commercially used for several FDA approved drugs. A slurry feeding stream, usually composed of drug coarse particle and stabilizer, is pressurized with an intensifier pump to 100–2000 bars. The
homogenization pressure used in the reported studies varied from 690 bar (10,000 psi) to 1724 bar (25,000 psi) (James et al., 2008). HPH generally requires shorter processing time, from less than 30 min (James et al., 2008) to a few hours (Bhavna et al., 2009). The processing time depends on the homogenization pressure, which in turn determines the flow rate, number of cycles, solid loading, and amount of materials to be processed. In this experiment, particles obtained ultrasonically were passed through high-pressure homogenizer (HPH) at a pressure of 10000-15000 psi and samples were collected at different cycles (1-5 cycles). Samples collected at different homogenization cycle were analyzed for particle size by Malvern.

4.5.1.4. Particle production by probe sonicator

The probe sonicator was used for the production of inhalable particles. The device operates at a fixed wavelength of 20 kHz and is capable of inducing a maximum power output of 250 W. A 500 mL jacketed glass sonoreactor was used in these experiments providing control over temperature. A weighed amount of drug (250 mg) was allowed to pass through 400 mesh sieve. The processed drug was then added slowly into the antisolvent containing different stabilizer and simultaneously treated with ultrasonic energy (220-250 W; 10 min). finally, the particles were filtered and analyzed by Malvern for MPS.

4.5.1.5. Antisolvent precipitation technique

A diagrammatic view of antisolvent precipitation method is shown in Fig. 4.5. Mixing a solution with an antisolvent generates supersaturation that subsequently induces nucleation and simultaneous growth by condensation and coagulation (Dalvi & Dave, 2009). The drug must be soluble in the solvent but practically not soluble in the antisolvent. The solvent and antisolvent must also be miscible at the operating conditions. In this process, mixing is a critical factor for controlling the final particle size and size distribution (Yang et al., 2005; Tung et al., 2009; Matteucci et al., 2006). When supersaturation is sufficiently high and mixing is uniform, fast nucleation consumes most of the solute and arrests crystal growth, enabling great control of particle size and distribution (Dalvi & Dave, 2009; Tung et al., 2009). The effect of mixing is expressed as the Damkoehler Number ($D_a$), which is the ratio between the mixing time ($\tau_{\text{mix}}$) to the precipitation time ($\tau_{\text{precipitation}}$). $D_a<1$ (uniform mixing) is required to form small particles with controlled size distribution (Yang et al., 2005;
Tung et al., 2009; Matteucci et al., 2006). A Schematic view for the preparation of submicron drug particles has been illustrated in Fig. 4.5. In this method, the drug candidate was dissolved in water (HPLC grade) and passed through 0.22 µm pore size filter to remove particulate impurities.

In a typical procedure, acetonitrile or ethanol and water were used as solvent and antisolvent of TBS respectively. In brief, 10% TBS dissolved in HPLC grade water was passed through 0.22 µm pore size filter to remove particulates impurities. The obtained solution was added dropwise (0.5 mL min⁻¹) into antisolvent (acetonitrile) slowly containing stabilizer at a constant stirring speed of 1200 rpm on magnetic stirrer for 6 h.

![Fig. 4.5. Diagrammatic representation of antisolvent precipitation process.](image)

(a) Effects of surfactants: Absorbed surfactants on the precipitating drug surface stabilize the particle by steric and electrostatic stabilization. Generally this increases particle growth by condensation and coagulation, and effectively increases precipitation time and decreases $D_a$ (Bhavna et al., 2009).

In quest of an excellent stabilizer, the effects of various stabilizers (PVA, Tween 80, Span 80, Poloxamer and L-leucine) in both type and concentration were investigated. In brief, the particle aggregation behavior was studied in detail for precipitated submicron particles.

(b) Effects of process variables: Lower temperatures generate higher supersaturation that prevents crystal growth and are also favorable for an exothermal precipitation reaction. Finally the effects of the process parameters, such as mixing time, mixing
speed, temperature, homogenization, and flow etc., were investigated. Process variables, such as rotating packed bed, reduces mixing time ($\tau_{\text{mix}}$), yielding lower $D_a$ and enabling good control of size and distribution. Reaction temperature is a critical operating parameter in controlling the particle size and size distribution (Simis et al., 2008).

4.5.2. Optimisation of drying technique

After particle formation, it is necessary to remove residual solvents by means of any suitable drying technique (Hot plate, rotary evaporator, Spray drying, vacuum drying, lyophilization etc.) to get dried inhalable powder less than 1 μm in size for effective lungs deposition.

4.5.2.1. Hot plate

Optimised nanosuspensions were placed over a hot plate for uniform solvent evaporation at a temperature of 60°C to get dried product.

4.5.2.2. Rotary evaporator

Nanosuspension was gently placed in round bottom flask tilted as shown in Fig. 4.6 and allowed to dry under the vacuum condition (5m Torr) at a uniform temperature of 60°C with rotating speed of 100 rpm. A thin film of dried particles will be obtained at the end of operation, as all the intact solvent mixture is evaporated by means of applied vacuum at reduced temperature.

Fig. 4.6. Rotary Evaporator.
4.5.2.3. Spray drying

Spray drying is the most commonly used and extensively studied solvent evaporation method (Fig. 4.7). Solvent in an atomized droplet is thermally removed inside the spray drying chamber. This is a one-step process to produce dry powder and allows control of particle properties such as morphology, density, and surface composition. The typical spray drying process encompasses four fundamental steps: (a) atomization of feed into a spray, (b) spray–air contact, (c) drying of spray, and (d) separation of dried product from the drying air.

![Spray Dryer](image)

Fig. 4.7. Spray Dryer.

Generally depending upon drying operation conditions the typical size of spray dried pharmaceutical particles ranges from 0.5 to 50 \( \mu \text{m} \) (Vehring R, 2008; Yang et al., 2008). In a typical Spray drying procedure, the precipitated solution was dried using a mini-spray dryer (S.M. Scientech, Calcutta, India) with a standard 0.5 mm nozzle. The nanosuspension was fed to the nozzle with a peristaltic pump, atomized by the pressure (60 lb/inch\(^2\)) of compressed air and blown together with a hot air to the chamber where the solvent in the droplets was evaporated. The dry product was then collected in a collection bottle. The drying conditions are detailed in Table 4.3.
### Table 4.3. Spray-drying condition for precipitated solution

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution feed rate</td>
<td>90 mL h(^{-1})</td>
</tr>
<tr>
<td>Inlet air temperature</td>
<td>70°C</td>
</tr>
<tr>
<td>Outlet air temperature</td>
<td>50°C</td>
</tr>
<tr>
<td>Aspirator setting</td>
<td>10</td>
</tr>
<tr>
<td>Air pressure</td>
<td>60 lb/inch(^2)</td>
</tr>
</tbody>
</table>

#### 4.5.2.4. Freeze drying

Lyophilization subjects formulations to two important stresses, freezing and drying, with addition of appropriate cryoprotectants. Nanosuspension was freeze by liquid nitrogen and the remaining liquid nitrogen was allowed to evaporate, and the resulting powder was dried for 48 h in a freeze drier (Labconco Corp., Kansas City, MO, USA). The collector was held at −52°C while the vacuum was 0.004 mbar. To prevent the powder aggregation, the vesicle containing the powder was held at subzero temperature for 7 h. The vesicle was kept at 21°C for the remainder of the 48 h. The powder was subsequently collected and stored in a sealed vial without desiccant at 4°C.

![Fig. 4.8. Lyophilizer](image)
4.5.3. **Formulation characterization**

4.5.3.1. **Particle distribution curve (PDS)**

The particle size of optimised formulation before and after the drying was measured by using Malvern. Particles were suspended in freshly filtered IPA and filled into a disposable cuvette. A properly diluted sample (1:10) was taken into the cuvette for measurements. In order to deaggregate particles, the samples were treated for a short time in an ultrasonic bath before measurement. Each sample was analyzed in triplicate. The standard algorithm of Malvern software based on the Mie theory was used for calculating the volumetric particle size distribution.

4.5.3.2. **Transmission electron microscopy (TEM)**

To prepare sample for TEM analysis, particles were suspended in IPA and ultrasonicated for a minutes to deaggregate particles. A small drop of a suspension was then placed on carbon coated grid covered with nitrocellulose and they were negatively stained with phosphotungstic acid (PTA) for better resolution. Further, they were dried at room temperature. A TOPCON 002B transmission electron microscope was used for analysis. TEM had a point resolution of 0.18 nm at 300 kV in the phase contrast HRTEM imaging mode and was equipped with an energy dispersive X-ray (EDX) spectrometer. Both low-magnification TEM and HRTEM imaging modes were used.

4.5.3.3. **Scanning electron microscopy (SEM)**

The surface morphology and particle dimension were determined by SEM. For sample preparation, gold sputter coating was carried out under reduced pressure in an inert argon gas atmosphere (Agar Sputter Coater P7340) on the dried sample. After sputter coating the sample on the carbon coated grid was examined under scanning electron microscope operated at 15–25 KV and photographs were recorded.

4.5.3.4. **Zeta potential (ZP) and polydispersity index (PI)**

The ZP and PI values were determined by using a Malvern Zeta-Sizer. Isopropyl alcohol was used for the preparation of all dilutions. Samples were diluted in a similar fashion as that described above for the particle size distribution. All measurements were made in triplicate and the mean values and standard deviations were reported.
4.5.3.5. Fourier Transform Infrared Spectrometry (FTIR)
FTIR spectra were recorded in the range of 400–4000 cm$^{-1}$ using a resolution of 4 cm$^{-1}$ and 10 scans, to evaluate the molecular states of micronized and submicronized formulations of drugs. Samples were mixed with potassium bromide (KBr) and were pressed to obtain supporting disks.

4.5.3.6. Differential Scanning Calorimetry (DSC)
The phase transition of plain and submicron TBS was analyzed by differential scanning calorimeter (DSC, Pyris#1, PerkinElmer, USA) at a heating rate of 10$^0$C min$^{-1}$. Also enthalpy study was performed to see the compatibility with excipients used in making of formulation. A dry nitrogen purge of 20 mL min$^{-1}$ was employed in the process. The DSC analysis was conducted over a heating range of 50–400 °C under optimized conditions. Calibration of the instrument with respect to temperature and enthalpy was achieved using high purity standard of indium.

4.5.3.7. Powder X-ray Diffraction (PXRD)
The physical state of submicron TBS powder was studied by means of XRD patterns and compared to plain TBS. Phase identification was conducted using an X-ray powder diffractometer (Shimadzu XRD 6000, Shimadzu, Japan) with Cu Ka radiation at a scanning speed of 0.05º min$^{-1}$.

4.5.3.8. Nuclear Magnetic Resonance (NMR)
The particles were analyzed by NMR as procedure described earlier. In brief, drug samples were dissolved in 200 μL D$_2$O containing 0.1% ethylene glycol and pH was adjusted to 6.6-7.2. Then samples were transferred into 5 mm NMR microtubes and power gated proton decoupled $^{13}$C spectra were obtained on an advance DRX500, 11.7T spectrometer. Relevant carbon signals in the $^{13}$C spectra were identified and integrated using $X$WIN-$NMR$ software. The amounts of $^{13}$C were quantified from the integrals of the peak areas, using ethylene glycol as internal standard.

4.5.3.9. Drug content
Drug content (DC) of TBS was assessed by making dispersion of accurately weighed 10 mg of sample (SµTBS) in 10 mL of methanol. The resulting dispersion was sonicated for 2 hrs for complete dissolution of TBS, and thereafter allowed to centrifuge at 15,000 rpm for 30 min in order to remove insoluble fraction of stabilizers. The amount of TBS in the supernatant was determined by UV spectrometer.
(1500 HS, Shimadzu, Japan) at 281.5 nm. The percentage drug content was calculated as follows:

\[
\% \text{ DC} = \frac{\text{Recovered TBS mass}}{\text{total mass of TBS}} \times 100.
\]

### 4.6. STABILITY STUDY

The study was designed to investigate the influence of carriers on particle size and zeta potential, when exposed to different *in vitro* exposure conditions.

Submicronized formulation containing 250±25 µg of TBS was filled into HPMC capsule shells (Size#3). Twenty five filled capsules from each batch (for each study) were packed in HDPE (high density poly-ethylene) bottles in order to prevent from atmospheric changes (temperature/ humidity) and sealed with aluminum foil and then placed in stability chamber for 12 month at controlled condition (25±2°C; 60±5%RH) and for 6 month at accelerated conditions (40±2°C; 75±5%RH). The samples were stored in environmental simulation chambers for maintaining constant climatic conditions (Stability chamber) (Fig. 4.9). During sampling, one bottle containing 25 capsules was withdrawn at definite time intervals and formulations were examined visually for the evidence of caking and discoloration. The content of the capsule were tested for the drug content (DC), MPS, moisture content (MC), FPD and ED.

![Stability chamber](image-url)
4.7. DRUG RELEASE STUDY

In vitro dissolution study has been implicated for inhalable particles to envisage in vivo particle behavior (absorption, metabolism, and elimination). Paddle, basket, and flow-through cell are official in Pharmacopoeia for oral formulations, but these methods are found insufficient enough for submicron particles because of wetting performance as newly formed surface (Hickey & Crowder, 2007). For inhaled particles, (i) dissolution media: distilled water, phosphate buffer (Hickey & Crowder, 2007), and simulated lung fluid (SLF) (Sinswat et al., 2008); (ii) apparatus: flow-through cell (Newman et al., 2004), standard dissolution apparatus, USP (Sinswat et al., 2008) and a stirring vessel; (iii): operating conditions: sinking, supersaturated condition; powder or sample introduction: (i) particles directly to media, aerosolized form (Yang et al., 2008) have been optimised in literatures for dissolution study.

In our study, the dissolution test was performed by the flow-through dissolution method (USP Apparatus #4). The dissolution was carried out in simulated lung fluid (SLF) pH 7.4; maintained at temperature 37±2ºC with the flow rate 0.2 mL min⁻¹ (Newman et al., 2004) because a simulated lung fluid is a solution that mimics the surfactant fluids released by Type II alveolar cells. The fluid fills the space between alveolar cells and acts to reduce the surface tension of the water in the lungs, facilitating gas exchange (Singh et al., 2010). The collection of sample fractions were carried out at predetermined intervals; the drug content of aliquot fractions was determined by UHPLC/MS method. The amount of drug released was extrapolated from the standard curve for TBS in SLF (pH 7.4).

Preparation of simulated lungs fluid (SLF): Properly weighed samples were mixed to make volume upto 1L: MgCl₂·6H₂O: 0.212 (g/L); NaCl: 6.415 (g/L); CaCl₂·2H₂O: 0.255(g/L); Na₂SO₄: 0.079 (g/L); Na₂HPO₄: 0.148 (g/L); NaHCO₃: 2.703 (g/L); Sodium tartrate: 0.199 (g/L); Trisodium citrate dihydrate: 0.180 (g/L); Sodium lactate 0.175 (g/L); Sodium pyruvate 0.172 (g/L); Glycine 0.118 (g/L).

Ostwald–Freundlich equation is generally consider to explain solubility of small particles (<2 μ), and De Noyes–Whitney equation supports this statement that reduction in particle size creates not only a high saturated solubility, but also a larger surface area for enhanced dissolution (Muller et al., 2004).
4.8. REGIONAL LUNG DEPOSITION STUDY

4.8.1. Radiolabeling protocol

4.8.1.1. Radiolabeling of TBS

TBS was radiolabelled as per standard protocol of INMAS (DRDO, New Delhi). A 50 µL stannous chloride solution (SnCl₂ dissolved in methanol; 1mg mL⁻¹) was added to 3 milligrams of TBS. The activity (⁹⁹mTc Pertechnetate) was added and incubated for 20 min at 60 ºC (Fig. 4.10). The % radiolabeling efficiency was determined by ITLC using acetone as a mobile phase.

4.8.1.2. Stability studies of labeled drug

The stability study of ⁹⁹mTc-TBS was determined in vitro by ITLC. The drugs were radiolabeled by standard protocol and incubated at 37°C for 24 h.

Fig. 4.10. Schematic representation for radiolabeling.

Serum stability of the complex was determined by mixing the complex with human serum and incubated at previously described conditions. ITLC was performed at different time intervals (0, 1, 2, 3, 4, 5, 6, 24 h) to assess the stability of the complex.

4.8.1.3. Radiolabelling validation study

In order to assess whether the radiolabelling process had any effect on the particle size distribution (PSD) of the drug and also to determine the degree to which the radiolabel distribution would reflect the distribution of drug substance, validation experiments were carried out prior to starting clinical investigations. The PSD of the radiolabel drug was determined for each batch before performing clinical studies.
4.8.2. In vitro aerosolization behaviour

The Anderson Cascade Impactor (ACI, Copley Scientific, UK) is usually the method of choice to characterize the quality of the dose emitted from an inhaler. The ACI consists of eight stages and a GF/A filter (Whatman plc, UK) was placed in the final stage of the ACI impactor (Fig. 4.11). The collection plates were sprayed with silicone lubricant (Pro-Power Silicone Lubricant, Premier Farnell plc, UK) and dried for 30 min prior to analysis. The initiation port (IP) and preseparator (PS) were used and attached over stage zero. The PS was filled with 10 ml of 60% methanol (in water). Pharmacopoeias recommend the use of the pre-separator for dry powder inhalers (DPIs) to entrain large particles usually >10 µm (USP, 2005; Mitchell & Nagel, 2003). The ACI was connected to a Critical Flow Controller Model TK2000 (Copley Scientific, UK) to ensure sonic flow and provide the required inhalation flow and volume.

<table>
<thead>
<tr>
<th>Flow rate</th>
<th>60 L min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time/ actuation</td>
<td>5 sec</td>
</tr>
<tr>
<td>Stages (ACI)</td>
<td>Cut-off diameter (µm)</td>
</tr>
<tr>
<td>Zero</td>
<td>5.8</td>
</tr>
<tr>
<td>One</td>
<td>4.7</td>
</tr>
<tr>
<td>Two</td>
<td>3.3</td>
</tr>
<tr>
<td>Three</td>
<td>2.1</td>
</tr>
<tr>
<td>Four</td>
<td>1.1</td>
</tr>
<tr>
<td>Five</td>
<td>0.7</td>
</tr>
<tr>
<td>Six</td>
<td>0.4</td>
</tr>
<tr>
<td>Seven</td>
<td>0.15</td>
</tr>
<tr>
<td>Eight</td>
<td>Filter</td>
</tr>
</tbody>
</table>

The vacuum flow was provided by a HCP5 High Capacity Pump (Copley Scientific, UK). Accurately weighed quantity of plain TBS (commercial drug passed through 400 mesh sieve) and submicron TBS powder were weighed (5 mg each), mixed with 495 mg lactose, passed through 400 mesh sieve and loaded into size 3 cellulose capsules (Chawla et al., 1994). 25 mg of powder blend was carefully weighed and incorporated.
to each #3 cellulose capsule (1 capsule contained TBS: 250 µg; Lactose: 24.75 mg) and aerosolized by means of Rotahaler® (RHL) (Glaxo Wellcome, Inc., NC). Capsule contents were released by twisting the RHL and the system was vacuumed to produce air streams of 60 L min⁻¹ for 5 sec. In all the cases the airflow was kept constant for a certain period of time so that a volume of 4L of air was used for each actuation (USP, 2005). The operating conditions and theoretical cutoff diameters at the flow rate used are mentioned in Table 4.4. For each preparation two actuations were delivered to the impactor.

![Image of Andersen Cascade Impactor (ACI)](image)

**Fig. 4.11.** Andersen Cascade Impactor (ACI).

Each stage is washed and the amount of drug collected in each stage assayed after appropriate dilution. The amount of drug deposited in the mouthpiece of Rotahaler® (M), IP, PS and various impactors were determined by UHPLC as described previously in section “drug content”. Respirable fraction was calculated as the amount deposited in the lower stage as a percentage of the emitted dose (amount emitted into upper and lower stages excluding the amount remaining in the device). Samples were analysed in triplicate. Statistical analysis was carried out using Minitabe statistical software (Version 13.1). Fine Particle Fraction (FPF), Geometrical Standard Deviation (GSD) and Total Emitted Dose (ED) were further calculated.
In this study, fine particle fraction (FPF) was defined as the fraction of loaded powder that was collected on plates 2–8. GSD is defined by the ratio of the diameters of particles from aerosols corresponding to 84% and 50% on the cumulative distribution curve of the weights of particles. To calculate the GSD, a nonlinear least squares analysis with a log-normal function was used. The emitted dose was calculated as the amount of loaded powder minus the amount collected in the ACI.

4.9. **IN VIVO PULMONARY EFFECTS**

4.9.1. **Fabrication and validation of inhalation apparatus**

In order to deliver optimised SµTBS to investigational rodents, an inhalation apparatus was engineered (Sharma et al., 2001), and conditions were optimised for better delivery by conducting preliminary trials based on loaded amount and fluidisation time (Fig. 4.12). In brief, the delivery chamber consisted of a 15 mL plastic centrifuge tube having an even hole of 0.5 cm in diameter on its wall at a distance of about 2.5 cm from the lip. A fine tubing of 2 mm internal diameter was gently inserted into the centrifuge tube from the top of the taper to a clearance of about 2.5-5 cm from cap’s inner surface. The tubing was connected to a pipette sucker rubber bulb, which was manually triggered to fluidize the bed of submicronized powder blend. Complete dispersion of powder bed was achieved with very low volumes of high velocity air coming out from inlet end of the tubing to form the desired aerosol. Pre-weighed sample for inhalation were gently put in centre of the cap which acts as a receptacle and thereafter fastened (screw tight) to the body. The sterile cotton wool plug (200 mg in weight) was held over the outer orifice of the centrifuge tube.

![Fig. 4.12. Design of In House Inhalation Apparatus](image)
The dose dispensed from the in-house inhalation apparatus under comparable conditions of airflow was determined by collecting submicronized TBS on cotton plug held at delivery outlet when the apparatus was actuated with different amount of sample loaded into the centrifuge tube. For the system validation, the fabricated apparatus was primed with submicronized DPI by fluidizing two doses and discarding the residue. This procedure led to adsorption of significant amounts of DPI on the walls of the apparatus, and increased the amount as well as the reproducibility of dose delivered as compared to unprimed apparatus (Calleja et al., 2004).

4.9.2. Optimisation of dose & delivery

The rubber bulb of the apparatus was actuated with the pressure from thumb and index finger 30 times (Rate: 1 actuation/sec) with the different amounts of sample placed in the cap. The quantity of powder get deposited on the cotton wall plug at the outlet orifice was estimated by UHPLC/MS. Different amounts of SµTBS particles (5–40 mg) were taken in inhalation apparatus to optimise best dose level suited for the study. Also, the time of fluidisation was studied (15–90 sec; n=3) at constant amount of 25 mg for SµTBS, to ensure optimum exposure duration desired for further study. To observe the quantity available for animal at mouth piece region during the exposure, a sheaf of cotton wool was used to completely occlude the delivery port. The sheaf surface exposed to the fluidised SµTBS remained flush with the inner wall of the tube and the area of the sheaf exposed to the aerosol was the quite same in each determination. Finally, the powder laden cotton wool plug was extracted for TBS in water and analyzed by our validated UHPLC-ESI-qTOF/MS method.

<table>
<thead>
<tr>
<th>Table 4.5. Experimental design for the in-house exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPI amount for fluidization</td>
</tr>
<tr>
<td>Exposure Period</td>
</tr>
<tr>
<td>Air flow</td>
</tr>
<tr>
<td>Rat per experiment</td>
</tr>
</tbody>
</table>
4.9.3. Lungs delivery of particles by In-House Apparatus

The protocol to perform animal study was approved by Hamdard University’s Institutional Animal Ethics Committee. Animals handling was fully complied with our institutional policies. Wistar rats (200–250 g, 8–10 weeks old) provided from Central Animal House, Hamdard University were kept in an environmentally controlled room (Temperature: 25±2 °C, humidity: 60±5%, 12 h dark–light cycle) for at least 1 week before the experiments. Animals were allowed free access to standard pelleted diet (Ashirwad Industries, Chandigarh) and water, but were fasted overnight before the day of the experiment. For exposure, the cap of the centrifuge tube was loaded with sample and tightened to the main body. Rats were anaesthetized (5 mg kg⁻¹ xylazine combined with 50 mg kg⁻¹ ketamine HCl injected intraperitoneally) and restrained with their snout placed against the peripheral aperture of the apparatus (mouth piece) and the bulb was gently actuated to fluidise the sample (Fig. 4.13). The total aerosol concentration in the exposure atmosphere was determined by collecting aerosol from the breathing zone of the animals onto preweighed cotton plug at the end of exposure. Dose delivered to different compartments namely, alveolar macrophages and systemic circulation was determined by using UHPLC/MS.

4.9.4. Lungs pharmacokinetics

Rats were exsanguinated by transecting the aorta, and thereafter the diaphragm was incised. The trachea was exposed and gently cannulated to suck out the BALF present there (Qamar & Sultana, 2008).

![Fig. 4.13. Inhalation delivery to rats by in house apparatus](image-url)
In brief, aliquots of 3 mL/chilled PBS (0.05 mM EDTA) were instilled into lungs after intubating the trachea with a syringe fitted with tracheal cannula. Both lungs were massaged and the fluid withdrawn immediately and collected in centrifuge tube kept on ice. The first lavage was kept aside and successive lavages after the first one were pooled to obtain approximately 10 mL of three samples in sequential manner. The recovered lavage was centrifuged and the supernatant of first lavage and pooled were collected separately. All lavages were pooled and centrifuged (1500 rpm/10 min) to recover airway and lung macrophages. The cell yield from each rat was determined using a haemocytometer.

The supernatant and cell pellet were separately assayed by UHPLC for TBS content. BALF and LH samples were further processed as same discussed earlier in the section “sample preparation protocol for UHPLC/MS” and stored at < 20°C till analysis. Prior to use, all the solutions (Calibration curve standards, QC samples, extracted BALF and LH samples) were withdrawn from storage area and allowed to thaw in wet ice bath and thereafter vortexed to ensure complete mixing of contents.

200 µL aliquot of each samples were taken into glass tube. 50 µL of IS (100 ng mL⁻¹) was added. Further 500 µL of formic acid (5% w/v) was added and vortexed (300 rpm; 5 min). 5 mL of ethyl acetate was added to the solution, then vortexed for 5 min. The supernatant (1 mL) was transferred to glass tubes and it was evaporated to dryness at 50 ºC under a hot stream of nitrogen for 10 min. 500 µL of reconstitution solution (acetonitrile: ammonium acetate buffer; 10: 90, v/v) was then added and vortexing for 10 s at 300 rpm, the solution was transferred into the clean autosampler vials and 10 µL was injected into UHPLC/MS system for analysis.