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Stabilized terbutaline submicron drug aerosol for deep lungs deposition: Drug assay, pulmonokinetics and biodistribution by UHPLC/ESI-q-TOF-MS method

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ABSTRACT

Terbutaline submicron particles (SμTBS) were prepared by nanoprecipitation technique followed by spray drying for deep lungs deposition. Inhalable SμTBS particles were 645.16 nm of diameter with 0.11 μm of MMAD, suggested for better aerosol effects. Both submicron and micron-sized TBS particles were administered in rodents administered via major delivery routes, and their biological effects were compared by using UHPLC/ESI-q-TOF-MS method. TBS was found stable in all exposed conditions with 96.28–99.0% of recovery and <4.34% of accuracy (CV). An inhalation device was designed and validated to deliver medicines to lungs, which was found best at dose level of 25 mg for 30 min of fluidization. Both submicron and micron particles were compared for *in vivo* lung deposition and a 1.67 fold increase in concentration was observed for SμTBS exposed by inhalation. Optimized DPI formulation contained lesser fraction of ultrafine particle (<500 nm) with the major fraction of submicron particles (>500 nm), advocated for better targeting to lungs. UHPLC/ESI-q-TOF-MS confirmed that designed submicron particles has been successfully delivered to the lungs. From tongue to lungs, the landing of pulmonary medicines can be improved by submicronization technology.

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1. Introduction

During a long journey of pulmonary medicines from tongue to lungs, it was felt that particle engineering have significant role in improving aerosol features and airways landing capacity of variety of drug candidates (Couvreur and Vauthier, 1991; Johnson, 1997; Weda et al., 2004). In recent years, submicron drug particles have been utilized in airway disease as an excellent aspirant to improve fluidization and lung deposition (Kreuter, 1991; Sangwan et al., 2001; Shekunov et al., 2007; Weda et al., 2004). Particles less than 1 μm possess unique aerosol and deposition effects due to their increased surface for dissolution and improved fluidization for redispersion of drug (Sangwan et al., 2001; Todoroff and Vanbever, 2011), which enable deep targeting to airways for quick relief from asthma exacerbation. For effective lung deposition, the particle size must be in the range of 0.5–5 μm (Couvreur and Vauthier, 1991; Todoroff and Vanbever, 2011; Weda et al., 2004). Also, submicronized drugs present several advantages for drug delivery to the lung including tailored release, decreased toxicity and deep targeting capabilities (Kreuter, 1991; Sangwan et al., 2001; Shekunov et al., 2007; Todoroff and Vanbever, 2011). Advanced drug delivery implicated submicron therapeutic entities from conventional routes, including oral and intravenous, as well as targeted sites like transdermal/topical, retro-bulbular and tracheobronchial (Couvreur and Vauthier, 1991; Johnson, 1997; Kreuter, 1991; Sangwan et al., 2001; Shekunov et al., 2007; Todoroff and Vanbever, 2011; Weda et al., 2004). A prolonged residence time at the site of absorption would be still beneficial. Microparticles are transported out of the lungs *via* mucociliary movement (Luo et al., 2010), however small particles at submicron (or nano) scale stick onto the mucosal surface for a longer time (Faiyazuddin et al., 2010), and in that way the submicron particles can be used to increase the absorption of antiasthmatic molecules for quick relief.

Terbutaline sulfate (TBS) is a synthetic β_2-adrenoceptor stimulant advocated for the treatment of asthma and related airway conditions by inhalation from an aerosol type device or power inhaler (Persson et al., 1995; Sangwan et al., 2001; Testa, 2004). A plethora of reports are available to estimate TBS in biomatrices, including LC/MS/MS (liquid chromatography–tandem mass spectroscopy) methods (Dickson et al., 2005; Fesser et al., 2005), but these published methods either involve cumbersome sampling steps or are insufficient enough to perform TBS analysis rapid and

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specific. A direct estimation of TBS and its degradation products in bulk and submicronized dry powder inhalers by stability-indicating HPTLC (high performance-thin liquid chromatography) method reported earlier by our group was found to have adequate assay sensitivity (Faiyazuddin et al., 2010). Furthermore, this method was successfully implicated to pharmacokinetic study in biological samples (Faiyazuddin et al., 2011). Since nano-level rapid quantification with the range of samples was not practically feasible with HPTLC, therefore we have adopted a standard hyphenated technology as UHPLC/ESI-q-TOF-MS (ultra high performance liquid chromatography/electron spray ionization/quadrupole-time of flight-mass spectrometry) method, as it utilizes high linear velocities which dramatically improves peak resolution, sensitivity and speed of analysis (Luo et al., 2010; Novákov et al., 2006; Orlovius et al., 2009; Wilson et al., 2005). The use of q-TOF-MS (quadrupole time-of-flight mass spectrometry) offers more possibilities in screening and identification, resulting in valuable fragmentation information (Plumb et al., 2004).

Therefore the present investigations were undertaken to set comparative distinction between micro and SμTBS DPI formulations, stipulated on the basis of biodistribution and pulmonokinetics by UHPLC/MS technology. To deliver dry particles safely to the lungs, we fabricated a cost-effective inhalation device and which was optimized for dose availability and exposure time. Administration of micro and submicron particles *via* i.v., oral and inhalation route was also of interest in assessment of drug distribution in various tissues and biofluids.

2. Materials and methods

2.1. Reagents and samples

TBS (purity >99.5%) was purchased from Netco Ltd., India. HPLC–MS grade acetonitrile (purity: 99.9%) was purchased from Sigma–Aldrich, Germany. MS grade ammonium acetate, ammonium formate and formic acid were commercially obtained from Fluka analytical, Germany. Deionized water was purified using a Milli-Q water purification system (Millipore, Bedfrod, MA, USA). For *in vivo* study the experimental protocol was approved by Hamdard University's Institutional Animal Ethics Committee to carry out animal study. Wistar rats provided from Experimental Central Animal House were kept in an environmentally controlled room for at least 1 week before the experiments on pelletized diet and water *ad libitum*.

2.2. Submicron drug particles

Development of pharmaceuticals for inhalation is a particular challenge, as it involves the preparation of a formulation and the selection of a device for aerosol dispersion (Luo et al., 2010; Novákov et al., 2006; Orlovius et al., 2009; Shaheen et al., 2011). Also, the lungs have lower buffering capacity than other delivery sites (like GIT or blood), which limits the range of excipients that could enhance delivery outcomes (Johnson, 1997; Shekunov et al., 2007). Therefore selection of excipients was strictly followed from GRAS category. Submicron formulation of TBS for effective lung targeting was prepared by antisolvent precipitation technique in which drug solution was added into stabilizer solution under magnetic stirring (Shaheen et al., 2011). Different antisolvent and stabilizer were tested and optimized on the basis of particle size. A schematic view for the preparation of TBS submicron particles (SμTBS) has been illustrated in Fig. 1A. Large sized and aggregated particles were formed when ethanol, acetonitrile and Isopropyl alcohol were used as a precipitation medium. Therefore in a typical procedure, acetonitrile/ethanol (9:1, v/v) 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/ethanol; 9:1, v/v) slowly containing stabilizer (0.25% Tween 80) at a constant stirring speed of 1200 rpm on magnetic stirrer for 6 h. Precipitation was formed immediately upon mixing. Suspension obtained after evaporating the organic solvent was further treated for homogenization (at 600 bars for 3 cycles) and finally spray dried using a Buchi mini drier (SM Scientech SMST, Calcutta) with a standard 0.5 mm nozzle. The suspension was fed to the nozzle with a peristaltic pump, atomized by the force 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. Scanning electron microscopy (Joel Scanning Microscopy, JSM6060 LV, Tokyo, Japan) was used as a tool for selection of submicron particles for better aerosolization study by means of surface contour the morphology characteristics. The mass median aerodynamic diameter (MMAD) for optimized Sμ particles was determined by using Anderson cascade impactor.

2.3. UHPLC/ESI-qTOF/MS

2.3.1. Chromatographic conditions

UHPLC system (Waters ACQUITY UPLC™, Waters Corp., USA) coupled with Waters Q-TOF Premier™ (Micromass MS Technologies, Manchester, UK) mass spectrometer was used for pulmonary studies. BEH C18, 100.0 mm × 2.1 mm; 1.7 μm (ACQUITY UPLC™, Waters Corp., MA, USA) column was used as stationary phase. The mobile phase for UHPLC analysis consisted of acetonitrile–2 mM ammonium acetate (1:9, v/v), which was degassed prior to use. The total chromatographic run time was 3.0 min. The flow-rate was set at 0.25 mL min^{-1} and 10 μL of sample solution was injected in each run. Quantitation was performed using Synapt Mass Spectrometery (Synapt MS) of the transitions of m/z 226.19 → 152.12 for TBS and m/z 260.34 → 183.11 for IS (propanolol) respectively with a scan time of 1.0 min scan time, and 0.02 s inter-scan per transition. The optimal MS parameters were as follows: capillary voltage 3.0 kV, cone voltage 20 V, source temperature 120 °C and desolvation temperature 400 °C. The optimum values for compound-dependent parameters like trap collision energy (Trap CE) was set to 13.2 V, respectively for fragmentation information. Nitrogen was used as the desolvation and cone gas with a flow rate of 500 and 4 L h^{-1}, respectively. Argon was used as the collision gas at a pressure of approximately 5.3×10^{-5} Torr. The accurate mass and composition for the precursor ions and for the fragment ions were collected in multi-channel analysis (MCA) mode were acquired and processed using MassLynx V 4.1 software.

2.3.2. Calibration

A stock solution of TBS (100 μg mL^{-1}) was prepared by dissolving requisite amount in water by sonication method (44 kHz/250 W for 15 min). To prepare calibration standards for plasma, BALF and lungs homogenate (LH); a set of ten non-zero concentrations were prepared for each by 5% aqueous analytes spiking in blank samples of plasma, BALF and LH respectively (50 μL aqueous aliquots to 950 μL blank samples, yielding concentration range from 10 to 1000 ng mL^{-1} for TBS). The final concentrations for each analyte were prepared at concentrations of 10, 20, 50, 100, 250, 500, 750 and 1000 ng mL^{-1}. Quality control samples were prepared at three levels: 800 ng mL^{-1} (HQC, high quality control), 400 ng mL^{-1} (MQC, middle quality control) and 25 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.

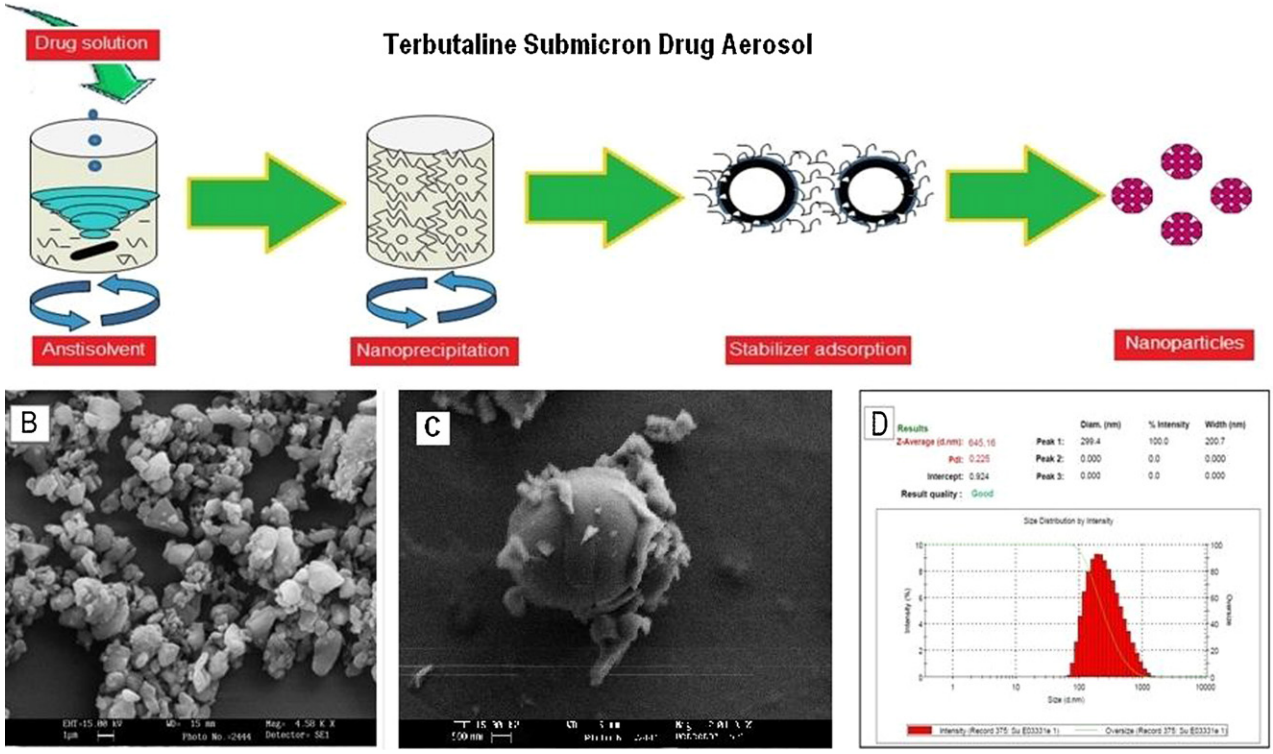

Fig. 1. Respirable particle production: (A) cosolvent nanoprecipitation scheme; and SEM images of: (B) micro-TBS, (C) SμTBS, and (D) Sμ-TBS particle size.

2.3.3. Sample preparation

In a standard operating procedure, the calibration curve standards, QC samples and unknown plasma samples were withdrawn; and 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 °C 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.

2.3.4. Validation

The method was validated as per USFDA guidelines of bioanalytical methods (US Food and Drug Administration, 2011). All analyses were performed in six replicates (n = 6). QC samples (theoretical contents) applied at three different levels as low (LQC: 25 ng), medium (MQC: 400 ng) and high (HQC: 800 ng). The lower limit of quantification (LLOQ) was determined based on the signal-to noise ratio of 10:1. For determining the intra-day accuracy and precision, replicate analysis of plasma samples of TBS was performed on the same day. The inter-day accuracy and precision were assessed by analysis of six precision and accuracy batches on three consecutive validation days. Response factors were assessed by using formula, RF = mean value of {[drug peak height (mV)]/[drug concentration (ng mL^{-1})]}/{[internal standard peak height (mV)]/[internal standard concentration (ng mL^{-1})]}; however recovery was analyzed at various QC levels by using formula, % recovery = mean value of (peak height (mV) obtained from biological sample)/(peak height (mV) obtained from aqueous sample) × 100.

2.3.5. Stability effects

Stability tests of the analytes were designed to cover anticipated conditions of handling of the experimental samples. Therefore stability of TBS in LH, BALF and plasma was evaluated by analyzing six replicates of each at three different QC levels 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 was assessed after one month storage at deep freeze (−80 °C) using six replicates of LQC, MQC and HQC. However, freeze–thaw stability was evaluated for three consecutive freeze–thaw cycles from −20 °C to room temperature. Again six replicates of LQC, MQC and HQC were analyzed after undergoing three freeze–thaw cycles. The bench top stability was determined for 24 h storage in optimized conditions, using six sets each of LQC, MQC 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 for QC samples. After specified storage conditions, samples were processed and analyzed.

2.4. Delivery and dose

In order to deliver optimized SμTBS to investigational rodents, an inhalation apparatus was engineered (Sharma et al., 2001), and conditions were optimized for better delivery by conducting preliminary trials based on loaded amount and fluidization time. The apparatus was designed to fluidize a powder bed within the confines of the centrifuge tube by means of a turbulent air stream. A preweighed amount of SμTBS was placed inside the cap and animal was restrained by placing nares to the exposure unit without touching powder bed. The bulb was then actuated (1 actuation s^{-1}) over the desired period of exposure to fluidize powder bed into air-laden Sμ particulate environment for animal to inhale. Different amounts of SμTBS particles (5–40 mg) were taken in inhalation apparatus to optimize best dose level suited for the study. Also, the time of fluidization was studied (15–90 s; 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 fluidized 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, TBS was extracted from the cotton plug by repeated vortexing with buffer and assayed by UHPLC.

2.5. Animals

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. For inhalational exposure, the cap of the centrifuge tube was loaded with sample and tightened to the main body. Rats were anesthetized (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 fluidize the sample.

2.6. BALF, LH and macrophages

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 and Sultana, 2008). In brief, aliquots of 3 mL of chilled PBS (containing 0.05 mM EDTA) were instilled into lungs after intubating the trachea with a syringe fitted with tracheal cannula. The 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 hemocytometer. 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 "preparation of calibration standard" 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 °C 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.

2.7. Pulmonokinetics

To see the actual deposition of drug in lungs after single dose for both micro as well as Sμ forms by different routes, pulmonary pharmacokinetics was performed at predetermined time intervals (0.5, 1, 2, 4, 8, 12, and 24 h; after exposure; 7 sampling points). A total of 126 Wistar rats were chosen for the study, which were randomly divided into three groups of 42 animals in each set (Gr. 1: i.v.; Gr. 2: oral; Gr. 1: inhalation; $n = 42$). In each group, out of 42 animals, first half will receive micronized form; however the next half will be given submicron sized drug (21: micro, 21: Sμ). The samples for i.v. and oral administration either micro or submicron particle were dispersed in 40% ethanolic PBS by introducing 100 μg-TBS, however for inhalation 25 mg was placed in apparatus for exposure. The i.v. injections were administered through the caudal vein and feeding tube was for oral deliver. Starting at 30 min after administration, sequential sets of three animals analyzed at each of sampling point. Animals were administered ketamine–xylazine for deep anesthesia, and their thoracic cavities were opened. Cardiac puncture to exsanguinate the lungs and BALF were carried out. The trachea was exposed and gently cannulated to suck out the BALF present there by instilling chilled PBS (containing 0.05 mM EDTA) into lungs. The lungs were massaged and the fluid withdrawn immediately and collected in centrifuge tube kept on ice. The recovered BALF was centrifuged (1500 rpm; 10 min), the supernatant was collected and stored at −20 °C till analysis. Immediately after lavage lungs were harvested and processed for TBS estimation by UHPLC.

2.8. Plasma assay

The *in vivo* comparative analysis of micro and submicronized TBS in blood given by different routes were assayed in plasma. The plan of the study was same as depicted in Section 2.7. In short, the blood samples were collected at 0.5, 1, 2, 4, 8, 12, and 24 h of post treatment, using three animals at each point separately for micro and Sμ formulation. After collection, samples were centrifuged for supernatant, then evaporated/reconstituted and finally analyzed by UHPLC. Parameters such C_{max} (maximal peak plasma concentration), AUC_{0-t} (mean area under the plasma concentration–time curve), $T_{0.5}$ (mean elimination half-life) and MRT (mean residence time) were obtained from the data produced.

2.9. Biodistribution

To see the real effect of micro as well as Sμ form of drug in various tissue compartments on introducing by three major routes, the biodistribution study was performed. As same to previous, 30 min after the treatment, animals were sacrificed for plasma, lungs, BALF and macrophages. The lavages were pooled and centrifuged at 1500 rpm to recover airway and lung macrophages. All components were procured separately and get prepared for UHPLC analysis.

2.10. Biostatistics

All computations were done by the software packages Statgraphics plus v5.0 (Manugistics, Rockville, MD) and SAS V9.1 (Cary, NC). All the data were normally distributed. Data are presented as arithmetic mean values of '*n*' observations ± the standard deviation (sd).

3. Results

3.1. Submicron drug particles

Fig. 1A displays the schematic view of Sμ particles production using cosolvent precipitation technique. The drug solution (solvent) was added dropwise to acetonitrile:ethanol (9:1, v/v; antisolvent) containing Tween 80 (stabilizer) and the effect of various process intensifications were optimized. In quest of better particle control various stabilizers with varying ratio were tried (Table 1). The best size before drying was achieved in acetonitrile:ethanol (9:1, v/v) using 0.25% Tween 80 (89.16 ± 13.45 nm). The precipitated nanosuspension was spray dried. Drying was

Table 1
Effect of stabilizer concentration on mean particle size of drug.

Antisolvent	% stabilizer concentration			
	Chitosan	Tween 20	Polyvinyl alcohol	Tween 80
Isopropyl alcohol (IPA)	1–10 (aggregation)	0.25–5 (aggregation)	0.1–5 (no particles)	0.25–5 (aggregation)
Ethanol	1–10 (lumping)	0.25–5 (aggregation)	0.1–5 (aggregation)	0.25–5 (little precipitation)
Acetonitrile (ACN)	1–10 (aggregation)	0.25–5 (aggregation)	0.1–5 (no particles)	0.25–5 (674.30 ± 11.26 nm)
Ethanol:IPA (9:1, v/v)	1–10 (aggregation)	0.25–5 (aggregation)	0.1–5 (aggregation)	0.25–5 (sticky mass)
Acetonitrile:ethanol (9:1, v/v)	1–10 (aggregation)	0.25–5 (aggregation)	0.1–5 (aggregation)	0.25 (**89.16 ± 13.45 nm**)[a] 0.5 (263.19 ± 11.26 nm) 1.0 (535.68 ± 98.10 nm)
Acetonitrile:ethanol (8:2, v/v)	1–10 (aggregation)	0.25–5 (aggregation)	0.1–5 (aggregation)	0.25–5 (>900 nm)

Bold Value denotes the best size achieved for terbutaline was 89.16 nm by using 0.25% of Tween 80 (stabilizer) in 9:1 v/v acetonitrile-ethanol (antisolvent) system.
[a] Best size achieved; remarks/values in parentheses showing mean particle size (nm); drug conc.: 10 mg mL^{-1} in water; titration speed: 0.5 mL min^{-1}; stirring speed: 1200 rpm/4 h; solvent/antisolvent: 0.2.

achieved by feeding the content to nozzle with a peristaltic pump, then atomized by the force of compressed air and blown together with a hot air to the chamber where the solvent in the droplets was evaporated, and finally accumulated in a collection bottle. The optimized drying conditions were as follows: spray flow rate/fed rate 90 mL h^{-1}, aspiration rate (%) of 100, inlet air temperature of 125 °C and outlet air temperature of 73 °C. The mass median aerodynamic diameter (MMAD) was found to be 0.11 μm for optimized formulation suggested having potential for better aerosolization. SEM images of micronized and Sμ sized TBS are shown in Fig. 1B and C. The unprocessed/raw TBS microparticles were 6–10 μm in average diameter (Fig. 1B). The average particle size of SμTBS particle was 645.16 nm (Fig. 1C) with polydispersity index 0.22, suggesting uniform particles (Fig. 1D). It was observed that after the nanosizing, the particles became fine and spherical.

3.2. UHPLC/ESI-q-TOF-MS/MS

3.2.1. Chromatography

Chromatographic separation for TBS was best achieved in acetonitrile:ammonium acetate buffer (10:90, v/v) at flow rate of 0.25 mL min^{-1}. Baseline separation of both TBS and IS (propanolol) was obtained within runtime of 3 min, without any interference. The MS full scan spectra for TBS showed protonated precursor $[M+H]^+$ ions at *m/z* 226.19 and product ion mass spectra at *m/z* 152.12 respectively (Fig. 2A and C). During direct infusion, the mass spectra of IS showed precursor ion peaks at *m/z* 260.34 as $[M+H]^+$ ions and most abundant product ions at *m/z* 183.11 (Fig. 2B and D). Collision energies employed were 13.0 and 16.3 eV for TBS and IS correspondingly. Quantification was performed on main product ions basis and the identical capillary voltage of 3.2 kV was used for

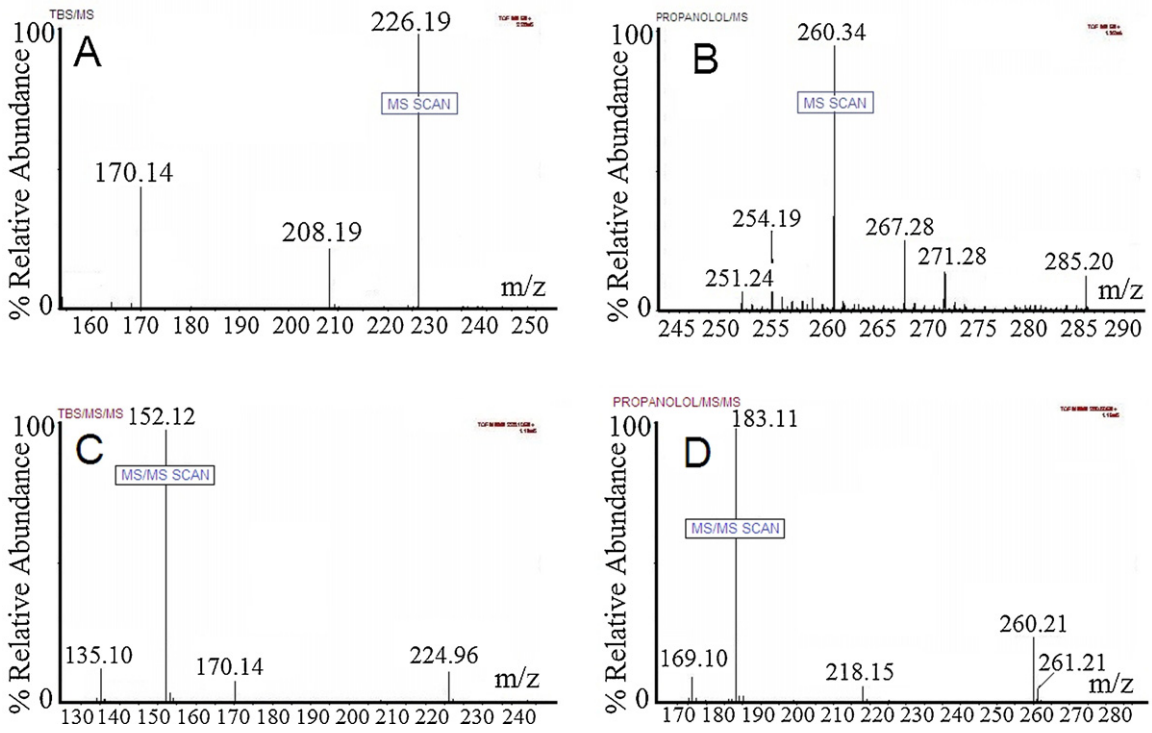

Fig. 2. Mass spectrum of: (A) TBS precursor ion (protonated precursor $[M+H]^+$ ions at *m/z* 226.19); (B) IS precursor ion (protonated precursor $[M+H]^+$ ions at *m/z* 260.34 as); (C) TBS product ion (major fragmentated product ion at *m/z* 152.12); and (D) IS product ion (major fragmented product ions at *m/z* 183.11) showing fragmentation transitions.

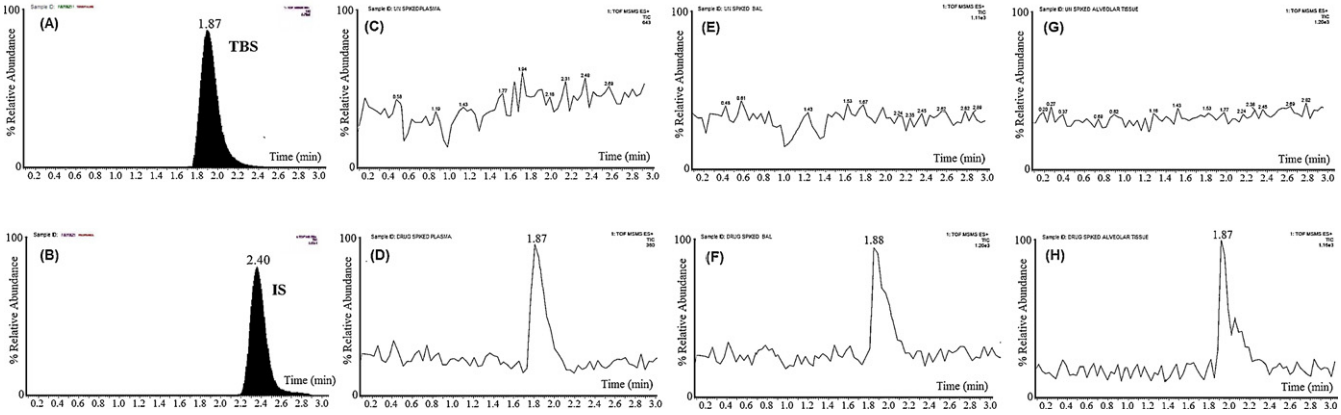

Fig. 3. Typical chromatograms obtained from: (A) extracted TBS; (B) extracted IS; (C) blank or unspiked plasma; (D) TBS spiked plasma; (E) blank BALF; (F) TBS spiked BALF; (G) blank LH; and (H) TBS spiked LH.

monitoring the precursor ions. The liquid–liquid extraction procedures (LLE) were used to prepare TBS samples in biomatrices for our study. The use of Waters ACQUITY UPLC™ BEH C18 column with 1.7 μm particle size helped in separation and elution of both analytes, TBS and IS at 1.87 (±0.05) and 2.40 (±0.08) min, respectively (Fig. 3A and B). Typical chromatograms obtained for blank plasma, BALF and lungs homogenate sample (extracted and reconstituted) have been shown in Fig. 3C and E&G respectively. Fig. 3 illustrates detail of retention time and chromatogram in different biomatrices. TBS eluted at 1.87 min in plasma (Fig. 3D) and LH (Fig. 3H); and at 1.88 min in BALF respectively.

3.2.2. Validation

The mean recovery ($n = 6$) of TBS from tissue homogenates, BALF and plasma relative to that from distilled water is listed in Table 2. Relative recovery of TBS was more than 73% except for LH samples (<53%). This could be a result of binding and/or adsorption of TBS by the endogenous compounds in alveolar tissues. For rat plasma, LH and BALF, linearity was investigated in the ranges 1–1000 ng mL^{-1} for TBS. Representative chromatograms of extracted blank plasma, BALF and LH fortified with TBS demonstrate the selectivity of the method. The recovery of internal standard was 100.1% (data not shown).

Inter-day and intra-day precision and accuracy of the method were determined by analysis of QC samples containing the three analytes at three different concentrations. The results obtained are listed in Table 2. The percentage accuracy (%CV) observed for intra-batch samples were ranged from 94.66 to 98.77 (<4.34) for LH, 91.20–98.95 (<4.91) for BALF, and 91.50 to 98.72 (<5.29) for plasma. However, the percentage accuracy (%CV) observed for inter-batch samples were ranged from 92.67 to 97.95 (<4.51) for LH, 91.90 to 98.07 (<4.79) for BALF, and 91.80 to 98.90 (<5.12) for plasma respectively. The intra-batch response factor (%CV) was ranged from 0.109 to 0.181 (<7.19); however for inter-batch sample it was ranged from 0.098 to 0.198 (<7.13) in different biomatrices.

3.3. Stability effects

Table 3 summarizes the data obtained for stability experiments, advocating the stable life of TBS in all storage conditions

Table 2
Validation data of TBS in biomatrices.

Matrix	Theoretical[a]	Intra-batch precision				Inter-batch precision			
		Observed[b]	%Accuracy[c] (%CV)[d]	RF[e] (%CV)[d]	%Recovery[f] (%CV)[d]	Observed[b]	%Accuracy[c] (%CV)[d]	RF[e] (%CV)[d]	%Recovery[f] (%CV)[d]
LH	1.5[g]	1.42 ± 0.01	94.66 (4.34)	0.125 (6.32)	78.2 (7.35)	1.39 ± 0.03	92.67 (4.51)	0.119 (7.26)	53.71 (5.32)
	25	24.12 ± 2.09	96.33 (3.23)	0.157 (7.19)	73.5 (4.27)	23.961 ± 0.88	95.10 (3.75)	0.161 (5.59)	59.90 (7.64)
	400	394.05 ± 4.75	98.20 (3.17)	0.148 (6.03)	81.7 (10.1)	392.37 ± 5.23	97.67 (3.21)	0.167 (3.69)	58.63(3.88)
	800	787.12 ± 2.14	98.71 (3.09)	0.162 (5.44)	85.4 (10.0)	779.53 ± 6.85	97.95 (3.19)	0.198 (1.37)	66.92 (11.5)
BALF	2.0[g]	1.87 ± 0.05	93.50 (4.68)	0.128 (6.14)	79.2 (6.81)	1.85 ± 0.02	92.51 (4.79)	0.098 (3.91)	81.32 (7.81)
	25	22.94 ± 1.95	91.20 (4.91)	0.149 (7.11)	83.8 (6.62)	22.71 ± 0.88	91.90 (3.40)	0.151 (3.09)	80.51 (6.91)
	400	393.17 ± 4.35	98.63 (3.15)	0.166 (4.69)	93.6 (8.16)	390.38 ± 7.45	98.07 (3.30)	0.173 (7.13)	90.53(3.37)
	800	789.65 ± 6.49	98.95 (2.99)	0.170 (5.57)	92.3 (5.04)	778.95 ± 2.01	97.89 (3.75)	0.180 (2.98)	91.2 (7.03)
Plasma	2.0[h]	1.89 ± 0.01	94.50 (3.40)	0.109 (6.14)	77.19 (7.81)	1.91 ± 0.02	95.50 (5.12)	0.118 (5.97)	79.61 (6.48)
	25	22.48 ± 0.24	91.50 (5.24)	0.112 (7.11)	85.18 (5.36)	22.96 ± 0.31	91.80 (4.29)	0.145 (4.09)	87.05 (6.91)
	400	392.17 ± 6.43	98.43 (4.09)	0.141 (5.29)	90.06 (5.16)	394.13 ± 3.75	98.82 (3.97)	0.162 (4.13)	89.11 (5.01)
	800	787.25 ± 10.64	98.72 (3.89)	0.181 (4.15)	93.15 (4.10)	789.05 ± 9.21	98.90 (2.81)	0.183 (3.88)	92.51 (4.03)

Values (mean ± sd) are derived from 6 replicates ($n = 6$).

[a] Theoretical concentrations applied as low, medium and high QC samples (25, 400 and 800 ng).

[b] Concentration observed.

[c] Mean value of [(mean observed concentration)/(theoretical concentration)] × 100.

[d] Values in parentheses are coefficients of variation (CV, %; mean, $n = 6$).

[e] Response factor: mean value of {[drug peak height (mV)]/[drug concentration (ng mL^{-1})]}/{[internal standard peak height (mV)]/[internal standard concentration (ng mL^{-1})]}.

[f] Mean value of (peak height (mV) obtained from biological sample)/(peak height (mV) obtained from aqueous sample) × 100.

[g] Lower limit of quantification (LLoQ) for lung tissue homogenate.

[h] Lower limit of quantification (LLoQ) for plasma.

Table 3
Stability data of TBS in biomatrices.

Exposure condition	LQC (25 ng)			MQC (400 ng)			HQC (800 ng)		
	LH	BALF	Plasma	LH	BALF	Plasma	LH	BALF	Plasma
Long term stability (storage condition: −80 °C for 30 days)									
Initial	24.49 ± 0.27	24.74 ± 0.31	24.88 ± 0.71	386.34 ± 7.03	390.08 ± 8.15	389.71 ± 6.03	769.36 ± 12.27	781.25 ± 10.37	777.53 ± 13.22
1 month	23.58 ± 0.40 (96.28%)[a]	24.33 ± 0.16 (98.35%)	23.35 ± 0.65 (97.26%)	372.97 ± 8.73 (96.54%)	375.89 ± 9.10 (97.10%)	373.50 ± 5.19 (96.68%)	748.62 ± 10.65 (97.29%)	768.13 ± 11.05 (98.66%)	764.08 ± 12.16 (98.62%)
Freeze–thaw stability (cycles: −80 °C to 25 °C)									
Cycle 0	24.82 ± 0.17	24.91 ± 0.08	24.86 ± 0.15	383.78 ± 10.25	386.50 ± 9.94	387.98 ± 9.11	779.16 ± 12.25	778.42 ± 10.02	789.86 ± 7.25
Cycle 1	24.70 ± 0.23 (98.77%)	24.78 ± 0.15 (98.68%)	24.75 ± 0.14 (98.88%)	379.35 ± 9.34 (99.08%)	381.93 ± 8.29 (99.06%)	379.61 ± 10.03 (98.28%)	771.75 ± 10.34 (99.24%)	764.01 ± 11.09 (98.52%)	777.20 ± 10.83 (98.72%)
Cycle 2	24.68 ± 0.28 (98.57%)	24.75 ± 0.18 (98.38%)	24.72 ± 0.20 (98.41%)	375.22 ± 8.48 (98.31%)	377.72 ± 10.48 (98.19%)	374.38 ± 9.89 (97.21%)	763.61 ± 13.48 (98.41%)	759.32 ± 10.17 (98.04%)	771.32 ± 14.08 (98.12%)
Cycle 3	24.62 ± 0.27 (97.96%)	24.73 ± 0.21 (98.18%)	24.61 ± 0.19 (98.27%)	372.56 ± 7.79 (97.68%)	375.61 ± 10.79 (97.76%)	372.61 ± 12.10 (96.85%)	760.53 ± 11.09 (98.09%)	747.71 ± 14.39 (96.86%)	765.89 ± 15.74 (97.57%)
Bench top stability (storage condition: room temperature; 25 °C for 24 h)									
0 h	24.86 ± 0.25	24.89 ± 0.09	24.92 ± 0.03	389.22 ± 8.12	389.86 ± 8.19	390.32 ± 3.35	783.71 ± 10.25	787.13 ± 8.57	789.25 ± 6.02
24 h	24.65 ± 0.34 (98.88%)	24.68 ± 0.17 (98.85%)	24.78 ± 0.07 (99.59%)	381.69 ± 10.34 (98.46%)	383.19 ± 10.23 (98.63%)	385.61 ± 10.03 (99.03%)	774.07 ± 13.34 (99.02%)	778.37 ± 18.34 (99.11%)	781.09 ± 9.23 (99.17%)
Post processing stability (storage condition: in autosampler; 10 °C for 24 h)									
0 h	24.89 ± 0.03	24.91 ± 0.02	24.93 ± 0.01	390.93 ± 8.01	393.72 ± 7.09	391.88 ± 7.12	782.19 ± 10.11	785.42 ± 10.02	789.93 ± 8.06
24 h	24.68 ± 0.10 (98.88%)	24.80 ± 0.05 (98.89%)	24.82 ± 0.02 (98.89%)	384.82 ± 0.02 (98.77%)	389.19 ± 10.12 (99.08%)	385.90 ± 8.05 (98.78%)	773.23 ± 12.25 (99.08%)	777.86 ± 11.13 (99.23%)	783.05 ± 10.17 (99.30%)

Values (mean ± sd) are derived from 6 replicates.
[a] Values in parenthesis represent % analyte concentration relative to time zero. Theoretical contents applied as low, medium and high QC samples (25, 400 and 800 ng).

(long-term, freeze–thaw, bench-top and post processing stability). The recovery of the analytes relative to that at time zero is reviewed. In long-term stability (one month of storage) the TBS was recovered as 96.28–98.66% in all biomatrices. However after three cycles of freeze–thaw, the TBS recovery was appeared in the range of 96.86–98.27% at all QC levels, suggesting the drug was stable even after abrupt handling. During bench-top stability the recovery of TBS was attained from 98.46 to 99.30%. In this exposure, plasma samples withhold the maximum stability (99.03–99.59%) at all the QC level as compared to other biofluids. In all the samples TBS recovery was good (>98.77%), when they were subjected for post-processing stability evaluation (98.77–99.30%).

3.4. Delivery and dose

To aerosolize the powder bed for experiment, an inhalation apparatus was designed for animals (Fig. 4A). Results obtained by trials taken to optimize the dose size and duration of fluidization are presented in Fig. 4B and C. Fig. 4B represents a review of available dose for inhalation when different charged amounts of SμTBS were charged for 30 s of exposure. The doses taken for aerosolization were ranged from 5 to 40 mg; and the amounts received at delivery port were 0.78–3.09 mg, which is <7% of original. A proportionate increase in linear fashion can be noticed for the dose received at delivery port and the charged samples. Moreover, a little fluctuation was observed at 25 mg of dose level (available dose: 2.53 mg, 10.2%), but after that the dose availability at the port became almost jammed (around 2.80 mg), probably due to the saturation of the exposed surface during fluidized state.

Similarly, to see the effect of duration of exposure for amount received at delivery port, another trial was taken and the obtained results are depicted in Fig. 4C. According to figure, during the first 15 s of exposure the amount (1.35 mg; 5.4%) reached at the delivery port was not sufficient enough to inhale submicron particles, but a two fold increase in received amount (2.52 mg; 10.12%) was observed when the same amount was fluidized for 30 s. However, an increase in exposure time from 30 s to 90 s laid to a mere increase in amount (10.72–12.48%). Since no any drastic change was observed after 30 s, therefore we have selected a dose level of 25 mg for 30 s for the study.

3.5. BALF, LH and macrophages

BALF was taken out at different time points after administration of inhalation or oral doses, or intravenous injections to animal in order to investigate the lung concentrations of both forms of TBS. Alveolar macrophages recovered by BALF were assayed to establish intracellular concentrations. As shown in Fig. 5A and B, the intravenous administration of particles resulted in deferred attainment of TBS concentrations in the lung lumen. However, the inhalation resulted in immediate peak concentrations sooner than oral route, since the engineered particles were deposited deeper in the lungs as such. When both micronized and submicron particles compared in lungs, a 1.67 fold increase in drug concentration was observed for SμTBS given by inhalation. In case BALF, again the inhalation route was emerged as best to maintain an enriched drug level for lung transfer. In macrophages, only inhalation route showed holding capacity of TBS particles, which was superior for Sμ sized particles (Fig. 6).

3.6. Pulmonokinetics

The first time ever, a detailed pharmacokinetics has been presented here by our group for micro/Sμ-sized drug administered by three major routes (i.v., oral and inhalation) to just check the actual fate of Sμ particles *in vivo*. Fig. 5A and B illustrates the particle

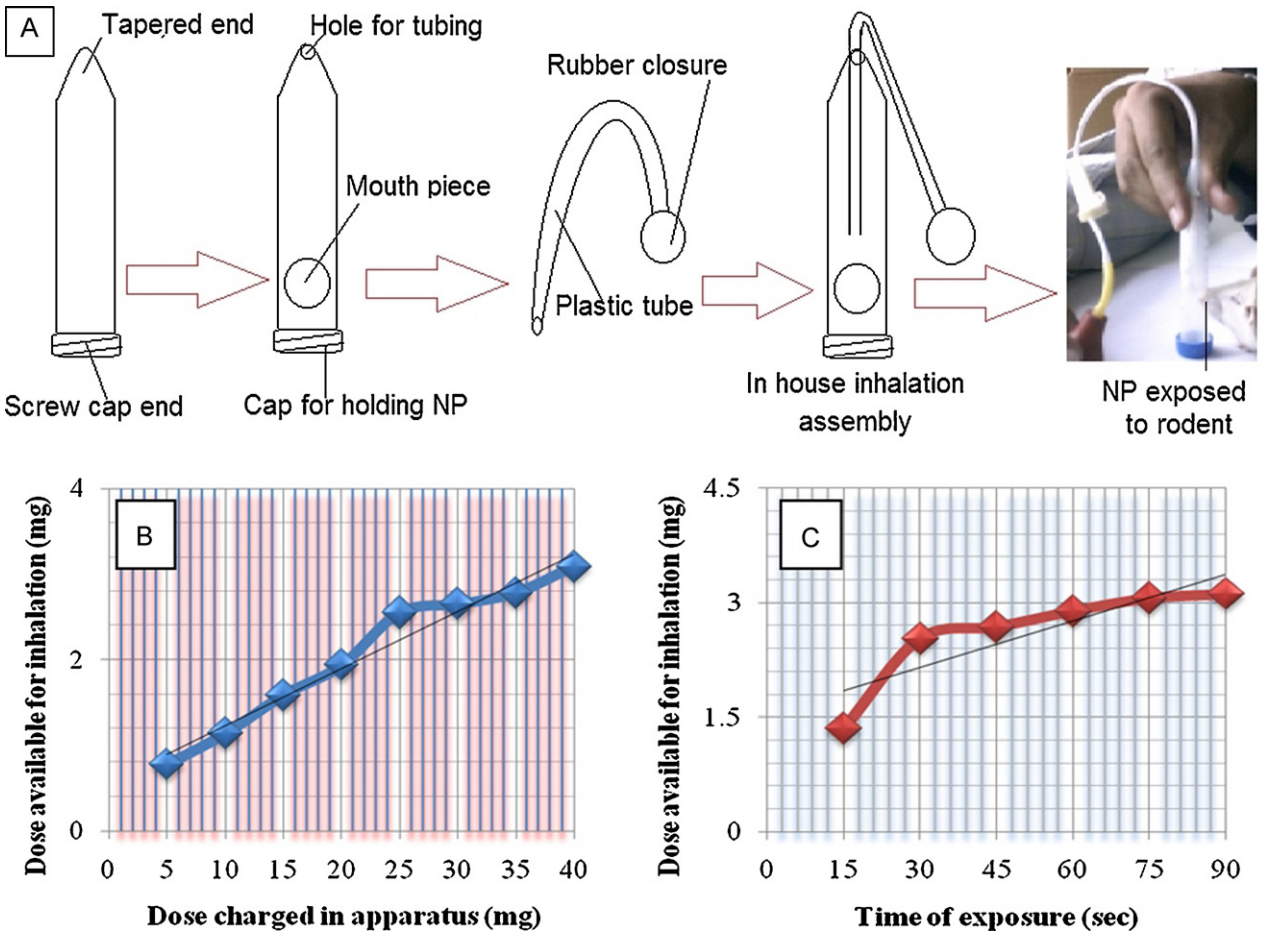

Fig. 4. Fabrication and validation of inhalation device: (A) apparatus design for powder fluidization; and effect of (B) charged amount (dose available at delivery port *versus* dose charged for fluidization), and (C) exposure time (dose available at delivery port *versus* time of exposure) on fate of aerosolized powder.

deposition in lungs and particles to be engulfed present in BALF. For micronized TBS, the maximum drug concentration in lungs was attained in inhalation after 4 h of treatment [23,365 ng (4 h, post inhalation) > 21,916 ng (6 h, post i.v. injection) > 19,965 ng (4 h, post oral treatment)], however for SμTBS, an amplified concentration of drug was observed at 2 h post inhalation [38,967 ng (2 h, post inhalation) > 27,078 ng (4 h, post i.v. injection) > 23,089 ng (4 h, post oral treatment)]. TBS analyzed in BALF was highest in inhalation for both the particles, which act as reserve drug for additional incorporation to lung.

3.7. Plasma assay

The plasma concentration *versus* time profiles of micro as well as submicron forms are shown in Fig. 5C and D. Table 4 summarizes pharmacokinetic parameters calculated by non-compartmental analysis to the concentration–time data. The model selected for intravenous administration was a single-intravenous-bolus model, while an extravascular-bolus model was used to fit data emerging from oral and inhalation experiments. In general, inhalation resulted in sustained drug concentrations in the plasma compared to intravenous injection. The most pronounced effects observed were the enhancement of plasma half-life ($T_{0.5}$), and the mean residence time in both micro and Sμ-sized particles. Maximal plasma concentrations (C_{max}) resulting from intravenous administration were greater than those observed after oral and inhalation dose in both forms.

3.8. Biodistribution

Distributions of TBS were studied in plasma, LH, BALF and macrophages, and the data acquired are presented in Fig. 6. After 30 min of the treatment (i.v., oral and inhalation), the sample or specimen was collected, processed and analyzed. SμTBS *via* inhalation reached maximum to lungs, while plasma concentration of TBS was highest *via* injection.

4. Discussion

The UHPLC/ESI-q-TOF-MS technique emerged as a gizmo to estimate the actual nano-content in biomatrices with all kind of delivery system for either for local or systemic effect. TBS submicronization was achieved by cosolvent nanoprecipitation technique (Shaheen et al., 2011). Preliminary studies revealed that choice and concentration of stabilizer had a marked influence on particle size. Tween 80 was found best in attaining the minimum size. Nanosuspension drying was the major concern to obtain submicron particles without any further growth; therefore various drying techniques were demonstrated. The spray drying method was found to have better capacity than others to hold particles in nano-range. Submicron particles obtained for TBS were 645.16 nm in diameter (PI: 0.22) and when subjected to *in vitro* lung deposition study, the MMAD was found to be 0.11 μm. SEM images showed the texture and contour of Sμ particles, which further showed better aerosolization prospective.

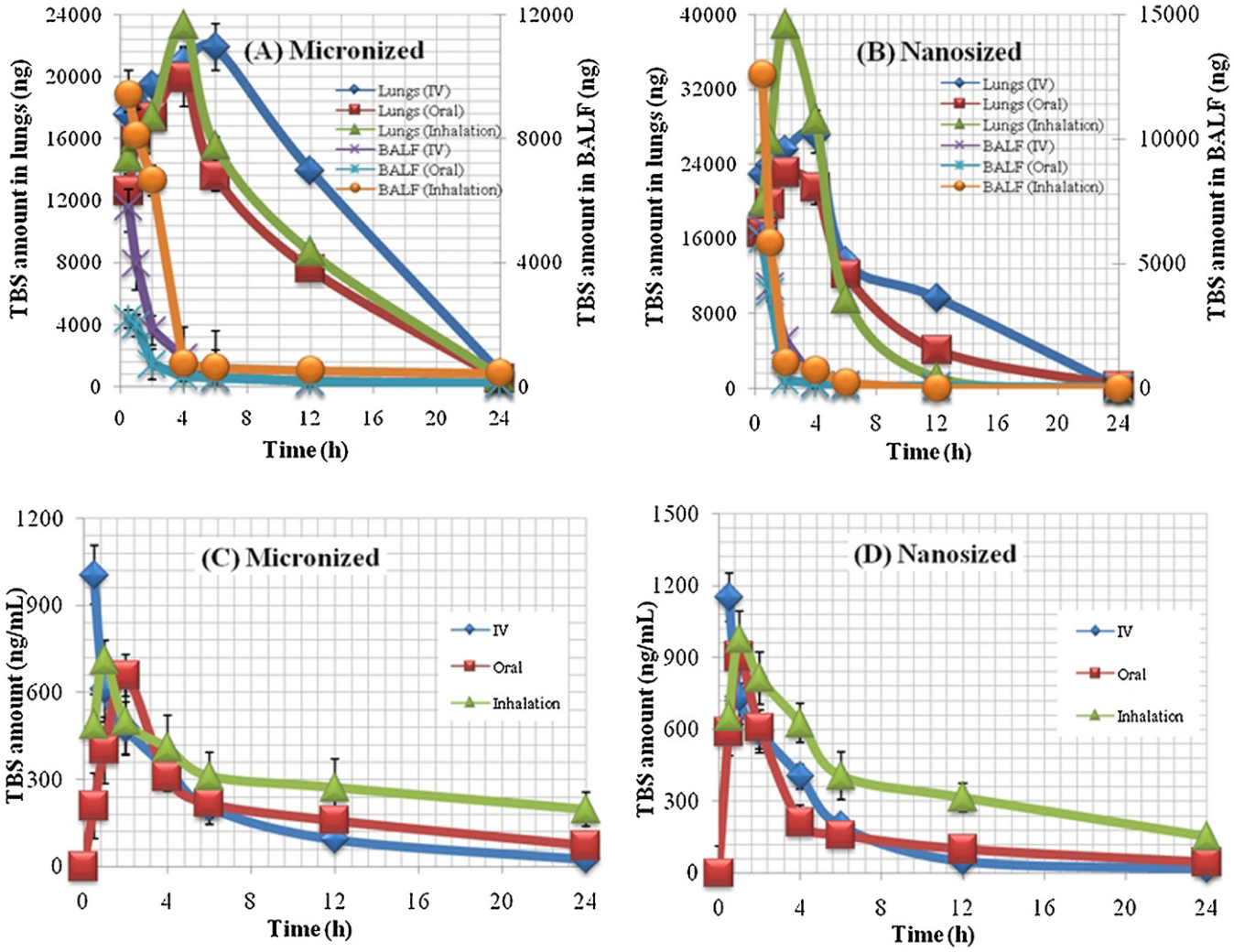

Fig. 5. Pulmonary pharmacokinetics of: (A) micro and (B) SμTBS; and plasma assay of: (C) micronized TBS, and (D) SμTBS, given *via* three major routes.

As the analysis reported on UHPLC is about seven times faster than HPLC (Dickson et al., 2005; Wilson et al., 2005), and q-TOF-MS offers better quality data in terms of increased detection limits and chromatographic resolution with greater sensitivity in biologicals (Plumb et al., 2004; Wilson et al., 2005). Therefore a UHPLC/ESI-q-TOF-MS method was developed and validated for TBS as bulk and formulations in different biomatrices. The separation was best achieved on ACQUITY UPLC™ BEH C18 (100.0 mm × 2.1 mm;

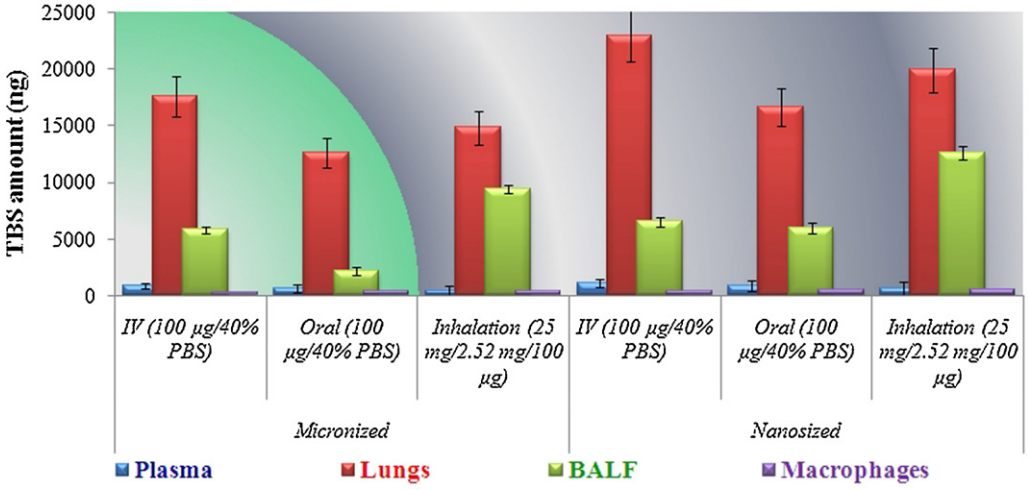

Fig. 6. Biodistribution study (micro *versus* SμTBS).

Table 4
Delivery effects on pharmacokinetics: micro *versus* SμTBS.

Parameters	i.v.		Oral		Inhalation	
	Micro	Nano	Micro	Nano	Micro	Nano
C_{max} (ng mL^{-1})	1005.17 ± 49.06	1149.35 ± 64.33	657.73 ± 58.00	905.15 ± 86.14	713.36 ± 0.98	978.67 ± 105.30
AUC_{0-t} [(ng mL^{-1})/h]	3342.42 ± 97.48	5125.81 ± 139.75	4450.53 ± 125.86	3855.21 ± 152.07	6950.11 ± 217.26	10,178.34 ± 392.67
$T_{0.5}$ (h)	1.64 ± 0.21	2.01 ± 0.33	3.67 ± 0.71	3.89 ± 1.04	3.62 ± 0.84	5.06 ± 1.46
MRT (h)	3.15 ± 0.49	3.78 ± 0.56	5.08 ± 0.89	6.33 ± 1.17	5.92 ± 1.00	8.13 ± 1.96

AUC_{0-t}, area under the plasma conc.–time curve; C_{max}, maximal plasma concentration at time zero; $T_{0.5}$, mean elimination half-life; and MRT, mean residence time. Data are represented as mean ± sd (n = 3).

1.7 μm) in isocratic mode with 2 mM ammonium acetate and acetonitrile in 9:1 (v/v) have flow rate of 0.25 mL min^{-1}. The recovery of the analytes was optimized using liquid–liquid extraction technique (LLE) in ethyl acetate. The total run time was 3.0 min and the elution of TBS occurred at 1.85 ± 0.05 min.

Accuracy for intra- and inter-batches in terms of %CV was ranged 2.99–5.24 (<5.5). Low %CV suggested, the developed method is accurate. The response factor (%CV) obtained for intra- and inter-batches of analytes in different biomatrices was 1.37–7.26 (<7.5). The tiny value advocated that mobile phase used for the study have potential to analyze TBS in plasma, BALF and LH samples. During stability analysis, TBS was found stable in stability conditions. As all parameters appear in acceptable limits, therefore only this UHPLC method was applied for further *in vivo* studies. The dose available at delivery port of inhalation apparatus was estimated by UHPLC, and the proportionate augmentation in amount was observed for charged dose and exposure time (Fig. 4B and C). Loading 25 mg of charged dose, a 2.52 mg dose was available at delivery port after 30 min of exposure, which was further assayed by UHPLC reaching about 100 μg in nares of animal. This was the reason for selection of 100 μg of dose in all routes (i.v., oral and inhalation).

Inhalation delivery of Sμ particles were expected to generate a pharmacokinetic ensemble, in which compartment-wise distribution of the drug payload was affected by drug release from submicron drug particles, partitioning, and diffusion of the released drugs from the lung lumen to the bloodstream and subsequent tissue accumulation.

5. Conclusion and outlook

UHPLC/ESI-q-TOF-MS/MS emerged as a fantastic tool for pulmonary pharmacokinetics, which provided a cascade of important information regarding *in vivo* deposition and pulmonary fate of submicron sized medicine. From the research it is clear that a DPI formulation containing fractions of particles *i.e.*, ultrafine submicron (particle < 500 nm) and submicron (1.5 μm < particle > 500 nm) particles had dual nature. Ultrafine particles are therefore advised when rapid action is needed (asthma attack) because of their significant reduction in particle size which elevates many fold increase in exposed surface area, making drug candidate better for rapid dissolution. However submicron (>500 nm) particles are advised when prophylaxis is required (management of asthma). But in our opinion the mixture of ultrafine and fine submicron particles is still beneficial in both conditions.

Conflict of interest

Authors have no conflict of interest in this paper. The financial grant for this work was given by DRDO, New Delhi, India.

Animal study

Hamdard Institutional Ethics Committee (JH/FP/0049/2011). Approval for the animal study was given by IAEC (Institutional Animal Ethical Committee).

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References

Couvreur, P., Vauthier, C., 1991. Polyalkylcyanoacrylate nanoparticles as drug carrier: present state and perspectives. J. Control. Release 17, 187–198.
Dickson, L.C., Mac Neil, J.D., Lee, S., Fesser, A.C., 2005. Determination of beta-agonist residues in bovine urine using liquid chromatography–tandem mass spectrometry. JAOAC Int. 88, 46–56.
Faiyazuddin, M., Ahmad, S., Iqbal, Z., Talegaonkar, S., Ahmad, F.J., Bhatnagar, A., Khar, R.K., 2010. Stability indicating HPTLC method for determination of terbutaline sulfate in bulk and from submicronized dry powder inhalers. Anal. Sci. 26, 1–5.
Faiyazuddin, M., Rauf, A., Ahmad, N., Ahmad, S., Iqbal, Z., Talegaonkar, S., Ahmad, F.J., Bhatnagar, A., Khar, R.K., 2011. A validated HPTLC method for determination of terbutaline sulfate in biological samples: Application to pharmacokinetic study. Saudi Pharm. J. 19, 185–191.
Fesser, A.C., Dickson, L.C., Mac Neil, J.D., Patterson, J.R., Lee, S., Gedir, R., 2005. Determination of beta-agonists in liver and retina by liquid chromatography–tandem mass spectrometry. JAOAC Int. 88, 56–61.
Johnson, K.A., 1997. Preparation of peptide and protein powders for inhalation. Adv. Drug Deliv. Rev. 26, 29–40.
Kreuter, J., 1991. Nanoparticle-based drug delivery system. J. Control. Release 16, 169–176.
Luo, W., Zhu, L., Deng, J., Liu, A., Guo, B., Tan, W., et al., 2010. Simultaneous analysis of bambuterol and its active metabolite terbutaline enantiomers in rat plasma by chiral liquid chromatography–tandem mass spectrometry. J. Pharm. Biomed. Anal. 52, 227–231.
Novákov, L., Matysov, L., Solich, P., 2006. Advantages of application of UPLC in pharmaceutical analysis. Talanta 68, 908–918.
Orlovius, A.K., Guddat, S., Parr, M.K., Kohler, M., Gütschow, M., Thevis, M., Schnzer, W., 2009. Terbutaline sulfoconjugate: characterization and urinary excretion monitored by LC/ESI-MS/MS. Drug Test. Anal. 1, 554–568.
Persson, G., Baas, A., Knight, A., Larsen, B., Olsson, H., 1995. One month treatment with the once daily oral beta 2-agonist bambuterol in asthmatic patients. Eur. Respir. J. 8, 34–39.
Plumb, R., Castro-Perez, J., Gragner, J., Beattie, I., Joncour, K., Wright, A., 2004. Ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 19, 2331–2337.
Qamar, W., Sultana, S., 2008. Farnesol ameliorates massive inflammation, oxidative stress and lung injury induced by intratracheal instillation of cigarette smoke extract in rats: an initial step in lung chemoprevention. Chem. Biol. Interact. 76, 79–87.
Sangwan, S., Agosti, J.M., Bauer, L.A., Otulana, B.A., Morishige, R.J., Cipolla, D.C., et al., 2001. Aerosolized protein delivery in asthma: gamma camera analysis of regional deposition and perfusion. J. Aerosol Med. 14, 95–185.
Shaheen, S., Talegaonkar, S., Ali, R., Mittal, G., Bhatnagar, A., Ahmad, F.J., 2011. Formulation development and optimization of alpha ketoglutarate nanoparticles for cyanide poisoning. Powder Technol. 211, 1–9.

Sharma, R., Saxena, D., Dwivedi, A.K., Misra, A., 2001. Inhalable microparticles containing drug combinations to target alveolar macrophages for treatment of pulmonary tuberculosis. Pharm. Res. 18, 1405–1410.

Shekunov, B.Y., Chattopadhyay, P., Tong, H.H.Y., Chow, A.H.L., 2007. Particle size analysis in pharmaceutics: principles, methods and applications. Pharm. Res. 24, 203–226.

Testa, B., 2004. Prodrug research: futile or fertile? Biochem. Pharmacol. 68, 2097–2106.

Todoroff, J., Vanbever, R., 2011. Fate of nanomedicines in the lungs. Curr. Opin. Colloid Interface Sci. 16, 246–254.

US Food and Drug Administration, 2011. Guidance for Industry. Bioanalytical Method Validation., http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf.

Weda, M., Zanen, P., de Boer, A.H., Barends, D.M., Frijlink, H.W., 2004. An investigation into the predictive value of cascade impactor results for side effects of inhaled Salbutamol. Int. J. Pharm. 287, 79–87.

Wilson, I.D., Nicholson, J.K., Castro-Perez, J., Granger, J.H., Johnson, K.A., Smith, B.W., et al., 2005. High resolution ultra performance liquid chromatography coupled to q-TOF mass spectrometry as a tool for differential metabolic pathway profiling in functional genomic studies. J. Proteome Res. 4, 591–598.