ABSTRACT
Budesonide (BUD) exhibits a very low bioavailability to lungs which makes it less favourable as an inhalational dosage form. Developed-Nanoparticles (NPs) [coated with chitosan (CS)] i.e. BUD-NPs are intended to enhance lungs-BUD bioavailability, aerosolization, lungs deposition as well as pharmacokinetic profile for BUD-NPs. BUD-NPs were developed through single-emulsification-solvent-evaporation technique. Characterisation of BUD-NPs was done for particle size, zeta potential, size distribution, encapsulation efficiency, and in vitro drug-release. A particle size (196.4 ± 10.05 nm) with smooth and spherical shape along with zeta potential (11.8 ± 0.91 mV) and drug-content (44.64 ± 2.91 %w/w) was observed. Ultra-high-performance-liquid-chromatography-mass spectroscopy (UHPLC-MS/MS) study was successfully applied for comparative effects of BUD-NPs lungs bioavailability via major delivery routes, and their biological effects. The NPs i.e. BUD-NPs revealed more bioavailability and in vivo lung deposition in animal model as compared to oral (3.0-times-higher) and i.v. (2.0-times-higher). BUD 0.75 min and 431.61/323.16 m/z whereas Fluticasone (IS) 1.16 min and 501.42/313.31 m/z, elution time and transition respectively. The CS-approach was successfully designed and safely delivered BUD to the lungs without causing any risk. BUD-NPs did not cause any toxicity, it showed safety and have no obvious toxic-effects on the rat’s lungs and does not produce any mortality followed by no abnormal findings in the treated-rats.

KEYWORDS
Budesonide; BUD-NPs; UHPLC-MS/MS; Inhalational Drug Delivery for Asthma; Comparative Pulmokinetics

Introduction
Inhaled corticosteroids are considered a first line treatment during asthma whereby a pronounced effect is observed in terms of alleviating the inflammatory mediators [1], with a subsequent decrease in morbidity and mortality [2]. Pulmonary dosage form plays a key role in the treatment of lung disease such as corticosteroids in chronic obstructive pulmonary diseases (COPD) to improve the quality of life [3]. Pulmonary dosage form is getting more attention due to its improved product stability and efficacy [4,5]. Likewise, strategies using systemic treatments via lungs are also well known because of enhanced blood-supplied to surface area...
with avoidance of first-pass metabolism [6,7]. However, poor drug deposition, shorter drug action with high dose frequency, still remains a major challenge in this delivery system [7,8].

In last few years, significant effort has been devoted to expand nanotechnology for drug delivery, as it offers a potential means of improving the delivery of small drug molecule and macromolecules (proteins, peptides or genes) to the tissue of interest [9]. The emerging active pharmaceutical moieties with low solubility in water forces researcher to find a better solution for clinical application of these drugs in market [10]. Nanoparticles, whether crystalline or amorphous, suggest an interesting way of formulating medicines containing poor water solubility [11]. By presenting drugs at the nanoscale, dissolution can be rapid and as a result the bioavailability of poorly soluble drugs can be significantly improved [12,13]. Formulation in the form of dry powder inhalable (DPI) offers more advantages of reducing dose frequency, less side effects and an overall better patient compliance [14]. Nanoparticles (NPs) have also gained considerable attention as a carrier for pulmonary delivery due to the ability to penetrate into airway mucosa, while avoiding macrophages and mucociliary clearance. They provide some other advantages, including increase of drug solubility, high drug loading capacity, sustained release property which reduces frequency of dosing and shortens treatment period. NPs also have wider distribution in the lung because of the higher surface area to mass ratio [15]. Chitosan, a natural biological polymer called as being biodegradable with biocompatible has been studied frequently for the delivery of various medicines and chemotherapeutic agents [16–20]. Chitosan was used as carriers to evaluate the pulmonary delivery of so many drugs like isoniazid [21], ciprofloxacin [22], gentamicin [23], heparin [24] etc. The most important applications of nanoparticles/nanobiocomposites based on chitosan in wound dressing, pulmonary drug delivery, tissue engineering, and biosensors are increasing [25–27] with a firm belief that these substances are biocompatible and biodegradable without comprehensive any type of supporting data. Chitosan based micro/nanoparticles have been extensively examined as a carrier for lung drug delivery [25,28,29]. Polymeric nanoparticles composed of poly lactide-coglycolic acid (PLGA) have received a great attention for delivery of a wide variety of therapeutic agents due to its advantages such as biocompatibility, biodegradability, drug release control, drug targeting and very low toxicity compared to other polymers and more stability when it compared with liposomes and other drug delivery systems in the biological surroundings [30,31].

Currently, due to a broad use of CS in protein [32,33] and metal adsorption field [34], much interest has been developed for using CS. CS and modifications of the surface of NPs with CS gives more different advantages: controlled and sustained release of drug delivery systems followed by mucoadhesive nature have been enhanced the drug absorption and prolongation of drug release [16], reduced and controlled the burst release of drug due to CS coating as well as increased retention and permeation of NPs due to affinity between positively charged CS and negatively charged membrane [35,36]. In this way, we have prolonged the retention time of nanoparticles deposition in the deep lungs followed by a controlled release with enhancing the bioavailability of drug and more therapeutic effect.

Budesonide (BUD) is a practically water insoluble glucocorticoid, Biopharmaceutical Classification System (BCS) Class II, used for management of asthma. Hydrophobic nature of the drug is an important obstacle in its clinical use that causes low solubility and absorption for the drug. To improve its solubility and absorption, CS-coated-PLGA-nanoparticles have been suggested [16,18,20,37]. BUD possesses significant anti-inflammatory activity from topical to systemic level however, it is only administered via [14,38]. As discussed the flaws in inhalational delivery system, BUD also needs an attention for a delivery system which may increase its dissolution and solubility at the level of lungs. Nanoparticles do offer the advantages of solubility and dissolution enhancer and in addition the powder nature of nanoparticles increases the adhesiveness at mucosal level where large amount of drug is available for absorption [39].

For BUD plasma analysis a number of literature reports are available where BUD have been determined individually as well as simultaneously. However, none of the studies have reported a sensitive analytical method for BUD detection in lungs tissues at picogram level [40–44]. Current research study is to develope and validates a sensitive, efficient method with low retention time for BUD for the first time using UHPLC-MS/MS.

The aim of study is to evaluate the aerosolization effect for BUD nanoparticles prepared through single emulsification technique. A novel CS-coated-BUD-loaded-PLGA-NPs (BUD-NPs) will be prepared and optimized with the use of their comparative pharmacoekinetics study via delivery of BUD-NPs from different routes (iv, oral, and inhalational) in wistar rats. The quantification of BUD in lungs will be carried out via a newly developed bioanalytical UHPLC–ESI–Q–TOF–MS/MS method. Finally, the toxicity study will be performed which is an important parameter for the safety of newly developed NPs.

Materials and methods

Materials

Budesonide and Fluticasone propionate (≥99%) (Jubilant Chemsys Ltd. Noida, Uttar Pradesh, India), dichloromethane (DCM) (Qualigens Chemical, India), polyvinyl alcohol (PVA, mol. Wt 25,000) (Sigma, St Louis, MO, USA), LC-MS grade acetonitrile, ammonium acetate and formate, methanol from Sigma, glacial acetic acid and chitosan (IOL Chemical Ltd., Mumbai, India). Milipore system was used to purify water (Bedford, MA, USA).

Cs-PLGA-NPs preparation

A previously reported study for single emulsification preparation was used to prepare BUD-loaded-PLGA-NPs [45]. DCM was used to dissolve PLGA i.e. 10 ml of DCM was added to 200 mg PLGA, under sonication (10 min) in an ice-immersed water bath with 10 mg BUD was dissolved in this solvent.
system consisting of 10 ml dichloromethane. During the next step, 18 ml of an aqueous phase (2% PVA + 0.5% CS stabilized in 4% acetic acid; pH 5.0) was added to previously prepared drug-loaded-polymer solution in a dropwise manner and at the same time emulsified using a probe sonicator. The resulting formulation was transferred to a magnetic stirrer (at room temperature for 24 h using 350 rpm) for solvent evaporation. The formulation was centrifuged further with the help of ultracentrifugation (15,000 rpm 30 min at 4 °C); supernatant collected and washed three times for removal of stabilizers and finally the amount of un-trapped drug was analyzed. The centrifugation resulted pellet was re-dispersed in 0.2% mannitol (cryoprotectant) containing Milli-Q water as well as freeze dried for 24 h using a lyophilizer [19].

**Nps properties (size, distribution, zeta potential)**
Dynamic light scattering system (DLS) with a DTS nano-software was utilized for different properties (size, distribution, zeta potential) of the developed NPs.

**Morphology of NPs (shape, surface texture)**
TEM technique was used for determination of NPs morphology. A paraffin sheet (copper-grid covered) was used to spread a drop of nanosuspension over it (60 s), followed by the placement of copper grid in phosphotungstate drop (5 s) and processed via TEM after air drying.

SEM technique was used for NPs texture determination. The sample was made to spread and stick, using SCD-020-Blazers-sputter-coater-unit, over the surface of a double-sided conductive tap. Following a pre-maintenance of the system (100 s at 50 mA) with an Argon gas, the analysis was performed.

**Drug loading capacity (LC) and entrapment efficiency (EE)**
The loaded and entrapped drug was determined with the help of ultracentrifugation (15,000 rpm 30 min at 4 °C) and the free fraction of BUD was found with the help of UHPLC-MS/MS using chromatographic conditions; mobile phase of ACN:2mM ammonium acetate i.e. 65:35% v/v with a FR of 0.250 ml/min. The following formulae were applied for DLC and DEE estimation [46];

\[
LE\% = \left(\frac{\text{BUD}_{\text{total}} - \text{BUD}_{\text{free}}}{\text{BUD}_{\text{total}}}\right) \times 100
\]

\[
DL\% = \left(\frac{\text{BUD (entrapped amount)}}{\text{Total weight (NPs)}}\right) \times 100
\]

The yield was determined with the help of following formula;

\[
\%\text{Yield} = \frac{W_1}{W_2} \times 100
\]

\[W_1 = \text{recovered dried NPs; } W_2 = \text{total weight of staring material (dry basis)}\]

**Dsc (differential scanning calorimetry) analysis**
A DSC pane of automated DSC-214 Polyma was applied to place ad seal the sample properly. Further analysis was performed with DSC, maintaining constant nitrogen purging in an environment of 20–400 °C at a rate of 10°K min⁻¹.

**Ft–IR with ATR**
Functional groups of the compounds with their chemical structure and composition were characterized through FT–IR with ATR (NICOLET i550 FT–IR). The IR spectrum for BUD, PLGA, PVA, Chitosan, Physical Mixture, and freeze-dried BUD-NPs were determined by an attenuated total reflectance (ATR, wavenumber 4000–400 cm⁻¹). Pure BUD, PLGA, PVA, Chitosan, Physical Mixture as well as freeze-dried NPs (BUD-NPs) were directly analyzed without any special preparation.

**In vitro release modeling**
For release study, the dialysis bag procedure was applied. An equal amount (2 mg) of BUD for BUD-S, BUD–loaded–PLGA–NPs, and also optimized CS–coated–BUD–loaded–PLGA–NPs (BUD-NPs), suspended in 5 ml dissolution media was placed in a dialysis bag. The dialysis bag was immersed in a dissolution medium (50 ml) of PBS (phosphate buffered solution) as reported [19,20,46–48]. For assessing in vitro release pattern an incubator shaker was used whereas for evaluation of dissolution parameters, a pH 7.4 was maintained for 48 h. Finally, at a pre-selected time points of 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 48 h, the samples were collected and analyzed with the help of an in-house developed biochemical method.

**Uplc/ESI-Q-TOF-MS/MS method development and validation (MDMV)**

**Chromatographic conditions**
The chromatographic conditions used during in-house biochemical MDMV consisted of; a filtered and degassed mobile phase of ACN: 2 mM AA (65:35% v/v) over C18 column (100 × 2.1 mm; 1.7 μm) with a FR of 0.250 ml/min and IV of 10 μL. The total run time for chromatography was 2.0 min. The MS conditions used were; scan time (1.0 min), scan/transition (0.020 s), cone and capillary voltage of 20 and 3.0 kV respectively, source and desolation temperature of 150 and 450 °C, respectively. The trap-collision energy (Trap-CE) for fragmentation was optimized at 23.6 eV whereas Nitrogen was used as a cone gas (4 L/hr) and for desolation (500 L/hr). For collision of the molecules, Argon gas was used at a rate of 5.3 × 10⁻² Torr. For BUD and IS i.e. Fluticasone propionate, the quantitation was done at m/z transition of 431.61 → 323.16 and 501.42 → 313.31, respectively. MCA (multi-channel analysis) mode was used to acquire an accurate mass and composition for precursor ions and its fragments. The MassLynx V 4.1 software was used to process the data [49–51].

**Calibration**
For stock solution, BUD (1.0 mg mL⁻¹) was dissolved in methanol with sonication. Calibration standards were
prepared for lungs and plasma homogenate where eight different concentrations were prepared by spiking aqueous analyte (2%) in blank plasma and lung samples i.e. aqueous analyte (20 μL)+blank samples (980 μL). The concentration range for BUD was 1–1000 ng/mL with final analyte concentration of each sample as 1.0, 2.0, 25.0, 208.0, 420.0, 640.0, 850.0 and 1000.0 ng/mL. The QC (quality control) samples at three level were prepared as; HQC (high quality control, 800.0 ng/mL), MQC (middle quality control, 400.0 ng/mL) LQC (low quality control, 2.90 ng/mL). Methanol and water (50:50) v/v was used to prepare an IS (internal standard) working solution (100 ng/mL). The solutions were preserved at 2–8 °C, till use.

Sample preparation
For sample preparation, CC standards, QC and unknown plasma samples were withdrawn (350 μL aliquot) in glass tubes, IS (50.0 μL) and 5% w/v orthophosphoric acid (500 μL) was added while vortexed at the end (5 min, 300 rpm). In the next phase, 5 ml ethyl acetate (60): methanol (40) was added and vortexed again (5 min). The supernatant (1 ml) was obtained and placed in another glass tube for evaporation under hot Nitrogen steam (50 °C for 10 min), till complete dryness. Finally, mobile phase (550 μL) was added for reconstitution of the sample, vortexed (300rpm for 10 s), transferred to clean vials and subjected (10 μL) to UHPLC/MS analysis.

Validation
For validation analysis six replicates (n=6) as well as three QC level samples (HQC, MQC, LQC) were used according to USFDA guidelines [49]. Based on recommended signal-to-noise-ratio of 10:1, LLOQ (lower limit of quantification) was also determined. BUD-plasma samples were replicated on the same day for intra-day accuracy and precision whereas six batches of accuracy and precision on three consecutive days were analyzed for inter-day precision and accuracy.

The following formula was applied to assess response factors (RF);

\[
RF = \frac{\text{mean value of } \left( \frac{\text{drug peak height (mV)}}{\text{drug concentration (ng/mL)}} \right) \left( \frac{\text{internal standard peak height (mV)}}{\text{internal standard concentration (ng/mL)}} \right)}{\text{mean value of } \left( \frac{\text{drug peak height (mV)}}{\text{drug concentration (ng/mL)}} \right) \left( \frac{\text{internal standard peak height (mV)}}{\text{internal standard concentration (ng/mL)}} \right)}
\]

The % recovery (at different QC levels) was determined as;

\[
\% \text{ recovery} = \frac{\text{mean value of } \left( \frac{\text{peak height (mV) obtained from biological sample}}{\text{peak height (mV) obtained from aqueous sample}} \right)}{\text{peak height (mV) obtained from aqueous sample}} \times 100.
\]

Stability effects
The time and temperature factors were observed for the effect on drug stability. Six replicates of QC samples (HQC, MQC, and LQC) were studied for BUD in plasma and lung homogenate.

The formula used was;

\[
\text{Stability (\%)} = \text{mean corrected response of stability stock/mean response of comparison stock} \times 100
\]

For long term stability, six replicates of QC samples stored at deep freeze (−80.0 °C) for one month were studied. For freeze thaw stability, samples were observed for consecutive three cycles of freeze-thaw i.e. −20.0 °C to room temperature. For bench top stability, the QC samples in six replicates were stored for 24 h using optimized conditions and quantified compared to freshly-spiked CC standards. For short-term stability, QC samples were exposed to temperature of 10.0 °C for 24 h in an autosampler.

Delivery and dose
For optimized dose delivery of prepared NPs, rodents were used to produce data with the help of an inhalational apparatus as mentioned by Sharma et al. [52]. The apparatus was used previously optimized conditions i.e. based on the loaded drug amount and fluidization time. The powder is fluidized within centrifuged tubes with the help of a turbulent air steam, according to Faiyazuddin et al. [50]. A preweighed amount of BUD-NPs was kept in the cap whereas animals were placed under control (nares exposed to the unit without touching the powder). The animal inhaled the fluidixed powder by actuating (1 actuation/second) the bulb for required period of exposure. Different amounts of BUD-NPs (5.0–40.0 mg) were used in inhalation apparatus in order to find an optimum dose for the study. The time of fluidization was also studied (15–90 s; n=3) at constant amount of 25.0 mg for BUD-NPs, to ensure optimum exposure duration desired for further study. During exposure, cotton wool sheaf was used to occlude completely the delivery port. It helps determine the amount available at mouth region of the animal. The area of sheaf which was exposed to fluidixed BUD-NPs stayed flush from inner wall of tube whereas sheaf area exposed to aerosol remained same. At the end, a repeated vortex process using buffer was applied in order to extract BUD from cotton and analyzed with the help of developed bioanalytical method.

In vivo study
A proper ethical approval was sorted for the study as approved from animal ethical committee of Hamdard University. The animals (8–10 weeks) weighing 200–250 g, were kept in a controlled environment of humidity (60 ±5%) and temperature (25 ±2 °C) for one week. To perform inhalational study, cap of centrifuge tube was loaded with sample and made tighten to the main body. The rats were anaesthetized using a combination of xylazine (5.0 mg kg⁻¹) and ketamine HCl (50.0 mg kg⁻¹) intraperitoneally. The anaesthetized animals were restrained and snouts were made against peripheral aperture of inhalational apparatus whereas, the bulb of the instrument was actuated in order to fluidize the powder sample.
In this study, optimized BUD-NPs have been used for a comparative pharmacokinetics study via delivery of BUD-NPs from different routes (i.v., oral, and inhalational) in Wistar rats. Based on the results, we are able to know the best route for lungs targeting drug delivery. For pulmonokinetics and actual drug deposition of BUD-NPs, rats were divided into three groups ($6 \times 3 = 18$). The study was carried out at seven pre-selected time points of 0.25, 0.50, 1, 2, 4, 8, 12 and 24 h. Eighty-one Wistar rats in three groups ($27 \times 3 = 81$) were randomly distributed as; Group-I: i.v., Group-II: oral, Group-III: inhalation. The samples for i.v. and oral administration BUD-NPs were dispersed in 4.0% ethanolic PBS by introducing 100.0 mg-BUD, however, for inhalation 25 mg was placed in apparatus for exposure. For bioanalytical determination of BUD in lungs and plasma, three animals at each time point were sacrificed to obtain plasma and lung homogenate.

**Pulmonokinetics**

In this study, optimized BUD-NPs have been used for their comparative pharmacokinetics study via delivery of BUD-NPs from different routes (i.v., oral, and inhalational) in Wistar rats. On the basis of results, we are able to know the best route for lungs targeting drug delivery. For pulmonokinetics and actual drug deposition of BUD-NPs, rats were divided into three groups ($6 \times 3 = 18$). The study was carried out at seven pre-selected time points of 0.25, 0.50, 1, 2, 4, 8, 12 and 24 h. Eighty-one Wistar rats in three groups ($27 \times 3 = 81$) were randomly distributed as; Group-I: i.v., Group-II: oral, Group-III: inhalation. The samples for i.v. and oral administration BUD-NPs were dispersed in 4.0% ethanolic PBS by introducing 100.0 $\mu$g-BUD, however, for inhalation 25 mg was placed in apparatus for exposure. For bioanalytical determination of BUD in lungs and plasma, three animals at each time point were sacrificed to obtain plasma and lung homogenate.

**In vivo histopathological examination of BUD-NPs toxicity**

Wistar rats were selected for BUD-NPs toxicity evaluation as this animal model provided a large surface area for inhalation of BUD-NPs via inhalation apparatus reported by Faiyazuddin et al., 2012; Sharma et al., 2001 [50,52] and to accurately determine the particle toxicity on the lungs as per the method reported by Bao et al., 2015; Menon et al., 2017 [53,54]. Following a seven days treatment, the rats were sacrificed; lungs were extracted intact and inflated by tracheal instillation of 4.0% paraformaldehyde at an airway pressure of 25.0 cm water. Tissue specimens were then embedded in paraffin, sectioned and stained with haematoxylin and eosin stain.

**Statistical analysis**

The data was expressed as mean ± standard deviation (SD). For data comparison ANOVA with $p < .05$ was applied as a statistical tool. The software used for analysis was GraphPad v 3.0 (San Diego, CA, USA).

**Result and discussion**

**Preparation and characterization of BUD-NPs (CS-BUD-PLGA-NPs)**

Literature reports suggest an ideal size of <250 nm for NPs preparation [30,55–60]. Herein, the size for NPs observed was...
196.4 ± 10.05 nm with a PDI of 0.264 ± 0.006 which represent a very proper size for NPs (Figure 1(A)). The zeta potential (11.8 ± 0.91) observed was suggested to be the property of amine as seen over CS surface (Figure 1(B)). Furthermore, the results observed for BUD %loading and entrapment efficiency (5.09 ± 0.08 and 76.41 ± 4.64, respectively), process yield (89.06 ± 4.08%) and drug content (44.64 ± 2.91 μg/mg) are in-line with studies reported [20,46,61]. For further confirmation of NPs properties, SEM and TEM analysis was performed where NPs with smooth and spherical surface in a size range of 150–250 nm were observed (Figure 1(C,D)).

The entrapment of BUD with more efficiency and controlled release property is due to application of biodegradable polymers in the study. These polymers possesses additional properties of being safe in terms of toxicity, eliminates through normal metabolic pathways and are biocompatible [20,46,61]. Though PLGA (synthetic polymer) have enormous applications in biological procedures [62], its more permeability does necessitate the need for a coating material. Chitosan (CS), a biodegradable polymer exhibits prolonged release phenomenon and is used extensively for lungs delivery system of drugs. In addition, PVA is applied as a stabilizer (0.50–10%) for PLGA because of its penetration for PLGA. The polarity of organic solvent also plays an important role for PVA-vinyl acetate interpenetration [20,30,46,56–58,61]. Present study focussed to prepare BUD-NPs using single emulsion phenomenon [18] where PLGA and CS polymers

![Figure 2. DSC thermograms of pure DAUN, PLGA, PVA, and chitosan; physical mixtures of BUD, PLGA, PVA, CS; and freeze-dried BUD-loaded-CS-coated-PLGA-NPs.](image)

![Figure 3. Different FT–IR spectra with an ATR attachment of BUD, PLGA, PVA, chitosan, physical mixture, and freeze-dried CS-coated-BUD–PLGA-NPs.](image)
were tried along PVA at various concentration. In addition, optimization of sonication time was performed in order to see the effect on PDI, loading, particle size and entrapment efficiency. However, the NPs size of <250 nm is an appropriate size with an ability to diffuse into lungs mucus more easily [56,57,59]. The other conditions for the adopted formulation resulted NPs with zeta potential in a range of –20 to +20. Depending upon the site of deposition, NPs with different charges are very essential as they have to face interaction from a variety of fluids and cell components such as mucus, macrophages, epithelial cells, negatively-charged biofilm etc. available alongside respiratory tract. PLGA-NPs due to optimum shape, size and surface attached ligand [48,56,57,59] and PVA-NPs with properties of high permeability, rheological properties, solubility and wide compatibility are extensively applied for drug targeting during inhalational delivery systems [63,64].

Recently CS (chitosan) got wide attention due to its enormous applications in protein and metal field [17,32,34]. The presence of CS and the modification of NPs surface by CS offers additional advantages of mucoadhesiveness and prolonged drug release which delays drug release by avoiding burst effect and enhancing its absorption [18] along with more permeation and retention of NPs (due to attraction between negatively charged membrane and positively charged chitosan) as reported [61].

Chitosan is a natural polymer that is known as biodegradable and biocompatible and has no toxicity and it can be bound with therapeutic drugs and made into nanoparticles. Lung targeting by inhalational targeted drug delivery system

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**Figure 4.** In vitro release profile of BUD-S, BUD–loaded–PLGA–NPs and CS–BUD–PLGA–NPs performed by using dialysis bag method, revealing sustained release pattern of BUD–PLGA–NPs and CS–BUD–PLGA–NPs (mean ± SD, n = 3).

**Figure 5.** Mass spectrum of (A) budesonide parent ion (protonated precursor [M-H]⁺ ions at m/z 431.61) and (B) Budesonide product ion (major fragmented product ion at m/z 323.16) showing fragmentation transitions.
via chitosan nanoparticles have been replaced intravenous and oral delivery with a non-invasive, lower dose, high bioavailability, avoids first pass metabolism and better uptake. This is the main reason for the coating of chitosan based PLGA-NPs is also because it has mucoadhesive properties which means it sticks to the lining of the lung cells and it has been found to improve the absorption of therapeutic agents by opening the junctions between cells of the lung lining to allow the drug to target lungs cells [58,65,66].

**DSC analysis**

DSC technique resulted endothermic peaks at 259.6 °C (BUD), 53.2 °C; 361.2 °C for PLGA, 329.6 °C for PVA and exothermic (316.8 °C) peaks for CS (Figure 2). The physical mixture of drug and polymer exhibited an intense peak for drug however, no peak was observed for NPs. The results prove the stability of formulation with good incorporation of the drug in polymer [20,67].

**Analysis by ATR based FT-IR**

The characteristic peaks and stretching assignments were observed as: pure BUD at 3403.6 (O–H), 2907.78 (C–H), 1719.21 (C=O), 1664.76 (C=C), and 892.53 cm⁻¹ (C–H) i.e. aromatic ring. For PLGA (C=C)-stretching was observed at 1746.34 cm⁻¹. PVA showed characteristics peaks 3248.6, 2934.8, 1642.8, 1415.4, 1326.2, 1137.7, 1082.9, 916.1, cm⁻¹. The IR peaks stretching assignment for CS was: 3299.27 (O–H), 1660.21 (NH₂) and 1081.23 cm⁻¹ (C–O). The developed BUD-NPs revealed spectra with assignment of 3404.5 (O–H) and 3234.3 cm⁻¹ (C–H). The FT-IR spectra of the (Figure 3), show peaks at cm⁻¹ due to vibration and at due to C–H stretching vibration for the BUD, for CS exhibits 3313.5 (O–H) (stretching), 1748.9 (C=O) (stretching), 1272.6 (C–O). Lack of any interaction was observed among BUD and the polymers (CS-PLGA-NPs) applied. Drug encapsulation in nanoparticulate system was evident from a slight shift of BUD-NPs peaks (Figure 3).

**Bud in vitro release study**

The pattern of drug release was observed in 1 h and 48 h as 83.58 ± 4.83% (BUD-S), 64.52 ± 4.59% (BUD-loaded–PLGA–NPs) and 79.52 ± 5.16% (BUD-NPs) as shown in Figure 4. A biphasic release phenomenon was observed for BUD-NPs where 39.55 ± 3.58% of drug release was observed in first 2 h (burst release) followed by sustained release. The burst release is suggested to be the effect of enhanced solubility in dissolution media along with a fast release for drugs from NPs surface. On the other hand, the sustained release happened due to dense embedding of drug and presence of drug more towards the centre of NPs. In addition, the polymer was a hydrophobic in nature which restricted the inward flow or penetration for the dissolution media. This behaviour may help extend drug therapeutic effect.

**Bioanalytical method development and validation by UPLC/ESI-Q-TOF-MS/MS**

**Instrument chromatographic conditions**

The chromatographic conditions optimized for BUD separation consisted of ACN (65%): 2 mM ammonium acetate (35%) v/v and flow rate of 0.25 ml/min, where a separation of BUD and IS was obtained in 2 min runtime. MS-scan for BUD resulted a protonated precursor ion [M+H]⁺ at m/z 431.61 with a product ion at m/z 323.16 (Figure 5(A,B)). During identification of the mass spectra of IS by directly infused and showed precursor ion peaks at m/z 501.42 as [M⁺H] ions and most intense product ions at m/z 313.31 (Figure 6(A,B)). Collision energies (eV) employed were 23.6 (BUD) and 19.45 (IS) whereas the main product ion was used as a base for quantification. LLE (liquid–liquid extraction) procedures were applied for BUD samples preparation. The column used (C18; 1.7 µm, 2.1 × 100 mm) resulted a retention time of 0.75 ± 0.02 min. Typical chromatograms showed in Figure 7 for Blank Lung Homogenate Extracted [7A], Extracted Blank Plasma [7B], Extracted Lung Homogenate Budesonide [7C], Plasma Extracted Budesonide, [7D], Extracted Lung Homogenate Fluticasone [7E], and Extracted Plasma Fluticasone [7F] (IS). Retention time and
details regarding chromatogram in various biomatrices is shown in Figure 7. BUD eluted at 0.75 min in plasma (Figure 7(D)) and Lungs Homogenate (Figure 7(C)); and IS at 1.16 min, respectively.

Bioanalytical method validation
Table 1 represents recovery (n = 6) of BUD in plasma as well as lungs samples as compared to distilled water used. A comparative recovery of ≥79% was observed for TBS which suggests a proper adsorption or binding for BUD at the level of alveolar tissue. Linearity within the range of 1–1000 ng/mL was obtained for BUD in rat plasma as well as lung homogenate. Selectivity of the method is evident from chromatograms of blank plasma and LH when fortified with BUD. A recovery of 99.99% was observed for IS.

The QC level samples at three different concentrations were used to calculate Inter and intra-day accuracy and precision (Table 1). The accuracy (%) and precision (% CV) for intra-batch showed a range of 96.04–98.62% and 2.27–3.25% for lungs homogenate; and 95.37–98.97% and 2.32–3.10% for plasma. The inter-batch precision and accuracy (%) was observed from 96.04–98.28% and 2.27–3.37% for lungs homogenate and 94.06–98.62% and 2.46–3.36% for plasma, respectively. The %CV observed was; intra-batch (2.27–3.25%) (<3.5) and inter-batch (2.37–3.36%) (<3.5).

Stability effects. The stability of BUD at all storage conditions was evaluated (Table 2). The BUD recovery observed was 94.47–98.58% (long term stability) and 90.56–98.90% (three freeze-thaw cycles). The results suggest the drug stability even after abrupt handling. Similarly, 96.86–98.21% and 95.42–99.54% recovery were observed for bench-top and post processing stability. All the data for stability was observed in the acceptance range as of US–FDA, 2018 [49] and as per previous reports [16,19,37,61,67].

Furthermore, the in-house developed and validated bioanalytical method (UHPLC–ESI–Q–TOF–MS/MS) bears the properties of being sensitive even up to pictogram level, economic, sensitive and shorter (<2.0 min). This is a first time a highly sensitive and robust bioanalytical method developed and successfully validated. Interesting results were obtained following the application of the method in PKs studies.

Delivery and dose
Inhalational apparatus was utilized for powder aerosolization and delivery of CS-BUD-PLGA-NPs as per the previously reported method [50] for the animals. The trials results noted for dose optimization and duration of fluidization are presented in Figure 8(A,B). Figure 8(A) represents a review of available dose for inhalation when different amounts of CS-BUD-PLGA-NPs were charged (30 s). The aerosolization dose (5–40 mg) and dose received at the level of delivery port (0.65–2.94 mg) is <7.5% of original. For the charged samples and dose received at delivery port, a proportionate linear increase was observed. In addition, at dose level of 25 mg a slight fluctuation was observed (available dose: 2.45 mg, 9.8%), however availability of dose at port was observed jammed i.e. almost at 2.76 mg which is suggested due to exposed saturation during the process of fluidization.

Another trial was conducted in order to see duration of exposure effect Vs amount received at the level of port
Table 1. Validation: precision and accuracy data for budesonide in lungs homogenate and plasma.

| Biomatrix       | Quality controls samples | Theoretical concentration (ng mL⁻¹ or ng g⁻¹) | Observed concentration (ng mL⁻¹ or ng g⁻¹) ± S.D. | Accuracy (%) | Precision (%) | Recovery (%) |
|-----------------|--------------------------|-----------------------------------------------|--------------------------------------------------|--------------|---------------|--------------|
| Lungs Homogenate| LOQQC                    | 1.01                                          | 0.97 ± 0.022 (96.04) ± 2.27                         |              |               |              |
|                 | LQC                      | 2.90                                          | 2.86 ± 0.039 (98.62) ± 3.25                         |              |               |              |
|                 | MQC                      | 400.0                                         | 386.36 ± 8.89 (96.59) ± 2.30                         |              |               |              |
|                 | HQC                      | 800.0                                         | 786.64 ± 17.92 (98.58) ± 2.27                        |              |               |              |
| Plasma          | LOQQC                    | 1.01                                          | 0.98 ± 0.029 (97.03) ± 2.96                         |              |               |              |
|                 | LQC                      | 2.90                                          | 2.87 ± 0.089 (98.97) ± 3.10                         |              |               |              |
|                 | MQC                      | 400.0                                         | 381.48 ± 9.25 (95.37) ± 2.42                         |              |               |              |
|                 | HQC                      | 800.0                                         | 782.53 ± 18.16 (97.82) ± 2.32                        |              |               |              |

Values (mean ± SD) are derived from 6 replicates; *Accuracy (%) = mean value of (mean observed concentration)/theoretical concentration) × 100; **Precision (%) = coefficient of variance (percentage) = standard deviation divided by mean concentration found × 100; *Recovery (%) = mean value of (peak height (mV) obtained from extracted biological sample)/peak height (mV) obtained from aqueous sample) × 100.

Table 2. Validation: stability data for budesonide in lungs homogenate and plasma.

| Exposure condition | Lungs homogenate | Plasma | Lungs homogenate | Plasma | Lungs homogenate | Plasma | Lungs homogenate | Plasma |
|--------------------|------------------|--------|------------------|--------|------------------|--------|------------------|--------|
| LOQC (2.900 ng/mL or ng g⁻¹) |                   |        |                   |        |                   |        |                   |        |
| Previous day       | 2.86 ± 0.06 (97.90%) | 2.81 ± 0.027 (98.62%) | 389.46 ± 8.66 | 385.49 ± 9.02 | 979.51 ± 26.48 | 981.01 ± 27.09 |
| 30th day           | 2.80 ± 0.05 (97.00%) | 2.77 ± 0.038 (98.58%) | 367.94 ± 9.46 | 365.47 ± 8.41 | 928.64 ± 27.01 | 931.67 ± 28.47 |
| Freeze–thaw stress|                   |        |                   |        |                   |        |                   |        |
| Pre-cycle          | 2.87 ± 0.06 | 2.86 ± 0.022 | 388.94 ± 8.75 | 390.18 ± 8.42 | 980.24 ± 26.51 | 979.06 ± 27.11 |
| First cycle        | 2.76 ± 0.08 (96.17%) | 2.74 ± 0.021 (95.80%) | 371.64 ± 9.09 | 375.66 ± 9.17 | 966.35 ± 27.05 | 968.31 ± 30.02 |
| Second cycle       | 2.69 ± 0.06 (97.33%) | 2.67 ± 0.028 (93.36%) | 364.71 ± 8.84 | 364.56 ± 7.86 | 949.26 ± 28.16 | 943.24 ± 29.16 |
| Third cycle        | 2.63 ± 0.07 (91.64%) | 2.59 ± 0.029 (90.96%) | 355.46 ± 8.96 | 358.44 ± 8.68 | 926.18 ± 28.06 | 921.09 ± 26.08 |
| Bench top stability|                   |        |                   |        |                   |        |                   |        |
| Recovery (ng)      | 2.85 ± 0.032 | 2.80 ± 0.021 | 385.61 ± 7.86 | 386.01 ± 8.06 | 979.02 ± 26.33 | 980.02 ± 26.16 |
| 24hr               | 2.73 ± 0.039 (95.79%) | 2.73 ± 0.022 (98.21%) | 374.69 ± 8.34 | 369.46 ± 9.44 | 955.46 ± 24.18 | 949.27 ± 25.46 |
| Post processing    |                   |        |                   |        |                   |        |                   |        |
| recovery (ng)      | 2.84 ± 0.033 | 2.84 ± 0.036 | 383.45 ± 7.86 | 376.98 ± 8.51 | 980.06 ± 25.83 | 976.47 ± 26.14 |
| 4hr                | 2.71 ± 0.034 (95.42%) | 2.72 ± 0.0238 (95.77%) | 370.49 ± 8.64 | 367.48 ± 8.10 | 968.28 ± 26.58 | 957.15 ± 27.05 |

Values (mean ± SD) are derived from six replicates. Figures in parenthesis represent analyte concentration (%) relative to time zero. Theoretical contents: LOQC: 2.900 ng mL⁻¹; MQC: 400.0 ng mL⁻¹; and HQC: 1000.0 ng mL⁻¹.
As evident from the figure, during the first 15 s of exposure, the dose amount i.e. 1.33 mg (5.32%) at the site of port was not enough to inhale the NPs however, fluidization (30 s) of the same amount resulted a two-fold increase in the dose i.e. 2.45 mg (9.8%). Increase in exposure time (30–90 s) had a very minute effect in increasing the amount i.e. 9.8–11.76%. As no changes were observed even after 30 s, thus the dose level selected was from 25 mg for 30 s.

**Pulmonokinetics**

The study reports for the first time, an in-depth and detailed PKs for BUD-NPs where the NPs were administered via the major routes of oral, i.v. and inhalation in order to follow the in vivo fate for developed NPs. The deposition of NPs in lungs and its engulfment is presented in Figure 9 and Table 3. A Cmax (maximum concentration of drug) for NPs in lungs was observed at 0.50 h [716.34 ± 29.66 ng/g] (inhalation; \( p < .001 \)) > at 1.00 h [519.34 ± 23.89 ng/g] (i.v.; \( p < .01 \)) > at 2.00 h [268.76 ± 18.74 ng/g] (oral). Lungs concentration of BUD was highest by inhalation route for BUD-NPs (Figure 9, Table 3).

**In vivo histopathological examination of BUD-NPs toxicity**

The histological micrographs of lung specimens collected from rat's post seven-days of exposure to 0.90% saline and treated with BUD-NPs by inhalation are depicted in Figure 10. There was no apparent histopathologic changes in the lungs tissues (Figure 10(B)) as compared to normal saline treated (control

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*Figure 8.* Fabrication and validation of inhalation device: (A) charged amount (dose available at delivery port versus dose charged for fluidisation), and (B) exposure time (dose available at delivery port versus time of exposure) on fate of aerosolized powder.

*Figure 9.* Pulmonary pharmacokinetic parameters study of budesonide in wistar rats after 10 mg/kg single dose of CS–BUD–PLGA–NPs (intravenous), CS–BUD–PLGA–NPs (oral), and CS–BUD–PLGA–NPs (inhalational). Significantly high AUC was achieved with CS–BUD–PLGA–NPs (inhalational) \( p < .001 \), mean ± SD, \( n = 6 \).
BUD-NPs did not cause any toxicity. These results indicate that BUD-NPs are safe and have no obvious toxic effects on the rat’s lungs. Exposure of above mentioned particles at mentioned dose does not produce any mortality and also and no abnormal findings in the treated rats with BUD-NPs CS–BUD–PLGA–NPs were observed [54,68].

Conclusion
In the proposed research study, biologically biodegradable BUD-NPs with EE of 76.41 ± 4.64% were prepared. A bioanalytical method with high sensitivity (picogram level) and efficiency was developed and validated in order to evaluate the PKs as well as PDs parameters for BUD. The lungs deposition study (in vivo) for developed BUD-NPs confirmed the high adsorption, deep deposition and penetration for BUD at lung level. BUD-NPs showed a highest value of Cmax and AUC by inhalational route which resulted enhanced drug availability at alveolar level as compared to other both routes of drug administration (i.v. and oral). Thus, current formulation may be used effectively as a novel, safe, non-invasive, and effective therapy for targeting of lungs and their treatment of Asthma.

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No potential conflict of interest was reported by the author(s).

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