8.1. Summary

- The aim of the research work was to formulate a nanostructured drug delivery system of steroidal drugs as a means of delivering dose of steroid consistently and reliably for local effects to achieve better management of alveolar pathologies.

- For this research work fluticasone propionate (FP) and ciclesonide (CIC) are selected because these glucocorticoids have potent anti-inflammatory activity and very poor water solubility (0.51mg/L & 1.57mg/L respectively). Both of these displays a high degree of binding to lung tissue (Davies and Feddah, 2003; Fuller et al., 1995; Wu et al., 2009). Micronized FP and CIC are routinely given by inhalation route for management of alveolar pathologies (Murnane et al., 2009). However, micronized dry powder inhalation therapy, including FP and CIC has some limitations like poor and sub-therapeutic concentration of inhaled drug in the target regions of secondary and tertiary bronchi and lungs (Liu et al., 2011; Newman et al., 1981; Newman and Wilding, 1998). Micronized FP and CIC dry powder inhaler (DPI) has the tendency to remain in the oropharyngeal region, which can be swallowed later through the mucociliary route into the gastrointestinal tract, resulting in almost 100% inactivation by the liver. Thus leaving behind an option to increase the lung deposition for better therapeutics.

- The authentication of FP and CIC was done on the basis of organoleptic properties, FTIR, melting point and mass spectrum and it was found that both samples were authentic. After this the physicochemical characterization such as solubility, loss on drying, XRD, DSC and SEM was done.

- UPLC-MS/MS method was developed and validated for the quantification of FP and CIC in bulk and formulation. The developed methods shown good selectivity, accuracy, precision and coefficient of variation (CV<0.1). For the method development of FP WATERS® Aquity® UPLC system and WATERS® Synapt mass spectrometry QTOF system was used. Acetonitrile: 2mM ammonium acetate (65:35) was selected as mobile phase and elution was performed in isocratic manner. Flow rate of mobile phase was 0.2ml/min. Ionization of the eluted FP was done by electrospray ionization method in positive ion mode. The MS/MS transition was selected from 501.2→293.16. The retention time of the FP was found to be 2.75±0.5 min. The LOD and LOQ of FP was found to be 0.375ng/mL and 1.138ng/mL. CIC UPLC-MS/MS method was developed using WATERS® Aquity® UPLC system and WATERS® TQD ms system. (Methanol with 0.1% formic acid): 10mM ammonium acetate (95:05) was selected as mobile phase
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and elution was performed in isocratic manner. Flow rate of mobile phase was 0.25ml/min. Ionization of the eluted CIC was done by electrospray ionization method in positive ion mode. The MS/MS transition was selected from 541.53→147.01. The retention time of the CIC was found to be 0.91±0.2 min. The LOD and LOQ of CIC was found to be 0.341ng/mL and 1.03ng/mL.

- Nanosized particles of FP were prepared in the optimum range using liquid antisolvent precipitation method followed by freeze drying. Acetone, poloxamer F127 and water selected as solvent, stabilizer and antisolvent respectively. A box Behnken statistical design of 17 runs containing central points was made considering drug concentration (from 5mg/mL to 10mg/mL), stabilizer concentration (4.5% to 7.5%) and solvent antisolvent volume ratio (0.05 – 0.10) as independent variables. Particle size was chosen as dependent variable. All the response observed in 17 runs were found to be best fitted with quadratic model. The model F value was significant while “Lack of Fit F-value” was not significant (p<0.1780). The “Pred R–Squared” (0.9834) was in reasonable agreement with “Adj R–Squared” (0.9966) with good adequate precision (Signal to noise ratio = 55.226)

Nanosized particles of CIC were prepared in the optimum range using high shear homogenization followed by freeze drying. Dichloromethane, PVA and water selected as organic phase, stabilizer and aqueous phase respectively. A box Behnken statistical design of 29 runs containing central points was made considering drug concentration (from 5mg/mL to 10mg/mL), homogenization speed (15000rpm – 25000rpm), stabilizer concentration (1.0% to 5.0%) and solvent antisolvent volume ratio (0.05 – 0.10) as independent variables. Particle size was chosen as dependent variable. All the response observed in 29 runs were found to be best fitted with quadratic model. The model F value was significant while “Lack of Fit F-value” was not significant (p<0.0575). The “Pred R–Squared” (0.8024) was in reasonable agreement with “Adj R–Squared” (0.9258) with good adequate precision (Signal to noise ratio = 18.434).

- Both FP and CIC were radiolabeled using $^{99m}$Tc and their radiolabeling efficiency was optimized in terms of amount of SnCl2 required and incubation time. In case of FP maximum radiolabeling efficiency (98.86±4.1) was obtained by adding 100µg SnCl2 after 30min of incubation. For CIC the optimized values for SnCl2 and incubation time were 80µg and 40min respectively. These conditions yielded 99.82±4.34% labelling efficiency of CIC.
The radiolabeling stability of FP and CIC was checked in saline, serum and artificial lung fluid up to 24 Hrs. the radiolabeling stability of FP was found to be 97.34±1.9%, 92.1±1.5% and 97.16±1.6% in saline, serum and artificial lung fluid respectively. The radiolabeled CIC was found to be more stable in saline (97.19±2.4%) and artificial lung fluid (97.00±1.4%) as compared to serum (94.3±1.7%).

- The carrier system was comprised of coarse lactose (ML001) and fine lactose (ML006). To optimize their ratio in vitro aerosolization experiment was performed using Andersen cascade impactor. It was found that ML001:ML006 (75:25) provide best aerosolization performance (p<0.01) with formulation R15. Thus this ratio has been selected as carrier for rest of the experimentation.

- With the selected carrier system different formulations of FP and CIC were prepared using particles obtained from the Box Behnken statistical design and their respiratory fractions (RF) were compared with their parent micronized particles (Micro FP and Micro CIC). Nano FP (formulation R5) exhibited significant increase in RF (p<0.001) as compared to micro FP. The RF values of nano FP and micro FP were found to be 60.3±2.41 and 16.4±0.66 respectively. Similarly formulation A11 provided best RF (p<0.001) for CIC. Nano CIC exhibited a significant increase in the respiratory fraction (RF) (69.1±4.93) as compared to micro CIC (24.7±1.96).

- Best formulations of FP (R5) and CIC (A11) were compared with their parent micronized particles in terms of FTIR, DSC, XRD, particle size, SEM, biodistribution in rats and lung deposition in human using gamma scintigraphy.

- Both micro FP and nano FP showed similar stretchings in FTIR except nano FP showed additional stretchings of O-H and C-O-C groups of poloxamer at 3137.67cm⁻¹ and 1035.24cm⁻¹ respectively. These stretchings may be due to the presence of Poloxamer F127.

- The FTIR spectra of micro CIC and nano CIC showed no additional peak. Only slight differences in the peaks positions of micro CIC and nano CIC were observed and that may be due to the processing of nano-CIC.

- The DSC spectrum of both micro FP and nano FP were almost overlapping except the nano FP showed a small endothermic peak at 54.2°C indicating the presence of poloxamer F127. The DSC spectrum of micro CIC showed a peak at 212.7°C while nano-CIC showed peak at 211.9°C. This slight difference in the peaks may be due to processing of nanoparticles.
Summary and conclusion

- The peak intensity in XRD spectrum of both nano FP and nano CIC has been decreased as compared to their parent micro FP and micro CIC. This is indicative of increase in amorphous content. The size reduction process has the possibility to increase the amorphous content.

- The particle size of nano FP was found to be 290.5nm with poly dispersity index of 0.2 showing mono-modal size distribution while the particle size range of micro FP was in the range of 10-20µm. Similarly nano CIC particles were of 310.0nm and micro CIC particles were in the range of 5-10µm. The results of particle size were further validated by SEM micrographs of FP and CIC (both nano and micro).

- Biodistribution studies in rats confirmed that nanoparticles have better lung penetration as compared to their microparticles. Nanoparticles of both drugs (FP and CIC) have found better lung deposition (p<0.001) and poor uptake in the stomach (p<0.001)

- To assess the lung deposition of nano FP and nano CIC and to compare their deposition pattern with their parent counterparts (micro FP and micro CIC) gamma scintigraphy has been used. Radiolabelled formulations prepared and given to the healthy human volunteers. Their images taken under gamma camera and region of interest were drawn to divide lungs into central, middle and peripheral regions as described by Biddiscombe et. al. The central Vs peripheral ratios (C/P) and peripheral Vs central (P/C) ratios were calculated. An increased value of C/P is indicative of deposition in upper part of lung while increased value of P/C is indicative of deposition in lower lungs or peripheral part of lungs. Both nanoformulations (nano FP and nano CIC) showed better lung deposition as compared to their parent counterpart (micro FP and micro CIC).

- Nano FP and nano CIC dry powder inhaler capsules were subjected to high temperature and humidity (40±2°C, 65±5%RH) conditions for 90 days to check their stability. After 90 days of exposure to high temperature and humidity it was found that respiratory fraction of nano FP decreased from 60.30±2.41% to 48.61±2.60% and mass median aerodynamic diameter increased from 3.87±0.5µm to 4.06±0.7µm; similar effects were seen with nano CIC. The respiratory fraction of nano CIC decreased from 69.10±3.07% to 49.76±2.79% and mass median aerodynamic diameter increased from 2.81±0.7µm to 3.66±0.4µm. All this indicates that prepared nanoparticles are prone to aggregation which resulted in decreased respiratory fraction as compared to their freshly prepared DPI capsules.
Summary and conclusion

- The acute lung toxicity studies showed nano FP and nano CIC are safe in rats up to the dose of 582.5µg/Kg and 233.3µg/Kg. The higher doses may show some potential side effects.

- We got the DCGI approval (F. No. 12-183/10-DC dated 31/08/2012) for the efficacy testing of nano FP only. The efficacy study of nano FP showed no significant differences in FVC and FEV₁ of the patients when compared with the treatment of micro FP. The difference in oxygen saturation was also not significant. But from the data it is clearly visible that the nano FP has more potential to maintain the blood oxygen saturation in stressed condition. The changes in heart rate showed significant difference between standard and test medications. The test medication (nano-FP) showed significantly (p<0.01 at time points 3Hrs and 4Hrs) better potential in maintaining heart rate normal as compared to standard medication.
8.2. Conclusion
Our findings along with the already established role of FP and CIC as a therapeutic option in medical management of various respiratory disorders suggest that

- Nano-formulations of both drugs (FP & CIC) were developed successfully.

- Computational modelling (Box Behenken statistical design) is a powerful tool for process optimization. It helped in faster interpretation of data in a better way.

- Liquid antisolvent (LAS) precipitation method coupled with ultrasound or high shear homogenization and stabilizers is very much efficient in producing drug nano particles.

- Use of appropriate ternary mixture increases the aerosolization performance of an inhalable formulation.

- Nanosized particles achieved better lung deposition for both FP and CIC as compared to their micronized particles. The differences in respiratory fraction, fine particle dose and mass median aerodynamic diameter are statistically significant.

- Nano DPI technology has the potential to be used as an inhalation based approach for reducing lung inflammatory responses associated with inhalation of toxic irritant gases such as ammonia chlorine, hot smoke etc.

- Use of properly validated radiometric method is very helpful in Andersen cascade impactor based in vitro aerosolization behaviour assessment. The method is less time consuming. Although the method is related to indirect estimation but the close agreement with analytical (LCMS/MS) method suggests it can be used for data collection.

- Gamma scintigraphy is a sophisticated, non-invasive technique. It helped a lot in authenticating the in vitro results in healthy human volunteers. The human scintigraphy images proved the better lung deposition of nano formulations as compared to micro formulations.
• Gamma scintigraphy is a powerful tool and helped very much in authentication of intratracheal instillation procedure.

• Toxicity studies revealed no significant morphological, haematological and biochemical changes in treated groups when compared with control for both drugs (FP and CIC). No significant evidence of damage to the lung parenchyma was observed in histological studies.

• Clinical studies of micro FP and nano FP showed no significant differences in lung function (FVC and FEV1) while nano FP formulation was comparatively more helpful in achieving good oxygen saturation and better therapeutics in maintaining the heart rate low during the walk test. This data indicates that better lung deposition resulted in better therapeutic performance because the lung functions generally describes the upper respiratory tract while oxygen saturation and pulse rate are associated with lower respiratory tract.

• Apart from increasing pulmonary drug deposition and probably enhancing the therapeutic potential of FP in treatment of asthma, COPD and other such respiratory diseases, this nano-formulation has primarily been developed as a potential prophylactic/therapeutic option against acute lung injuries caused due to inhalation of toxic irritant gases such as ammonia, chlorine, hot smoke and burning plastic fumes. Exposure to these irritant gases and inhaled particles predominantly affect the airways, causing tracheitis, bronchitis, and other inflammatory responses similar to those seen in conventional respiratory disorders (do Pico, 1995; Makarovsky et al., 2008; White and Martin, 2010).

• Nano-inhalation may be developed as a preventive or therapeutic approach for members of rescue teams as well as victims of industrial accidents and/or chemical terrorism involving toxic gas exposure.