PREPARATION OF HUMAN SERUM ALBUMIN MICROAGGREGATES LABELLED WITH $^{99}$m$^\text{Tc}$ TO EXAMINE THE NASAL DEPOSITION AND CLEARANCE OF SUSPENDED PHARMACEUTICAL FORMULATIONS

by

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A thesis submitted in conformity with the requirements for the Degree of Master of Science, Graduate Department of Pharmaceutical Sciences, in the University of Toronto

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PREPARATION OF HUMAN SERUM ALBUMIN MICROAGGREGATES LABELLED WITH $^{99m}$Tc TO EXAMINE THE NASAL DEPOSITION AND CLEARANCE OF SUSPENDED PHARMACEUTICAL FORMULATIONS

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Abstract

In order to simulate the intranasal drug distribution of budesonide suspension aerosol, a tracer particle having a size distribution similar to this parent pharmaceutical was developed. This particle can be easily labelled with the $\gamma$-emitting radionuclide technetium-$99m$, and permits the study of deposition and subsequent movement of the formulation. Microaggregated human serum albumin (HSA) particles of about 2 - 3 microns in size were prepared by denaturing human serum albumin with a stabilizer and surfactant in a boiling water bath. By carefully controlling the time of thermal denaturation, microaggregates with the desired particle size distribution were prepared. Microaggregates were labelled with Tc-$99m$ by adding diluted stannous chloride to reduce the $^{99m}$Tc(VII) to $^{99m}$Tc(V) and subsequently bind the$^{99m}$ Tc to the albumin. Polycarbonate membrane filters (Nuclepore) were utilized to perform particle size analysis by differential filtration. In addition, a quantitative, non-invasive three-dimensional image of human serum albumin microaggregates was obtained by using the laser confocal scanning microscope (Bio-Rad 600). The aerodynamic size distribution of microaggregates suspended in distilled water were verified by a single-particle, laser sizing system based on the time-of-flight data. Fifty percent of dried, aerosolized microaggregate particles were determined to be smaller than 2.040 microns in aerodynamic size, compared to 2.099 microns of the parent pharmaceutical. The characteristics of the tracer particles in an aqueous vehicle after nebulization was studied with an Andersen Impactor. Using a versatile shear cell, the diffusion coefficients of $^{99m}$Tc microaggregates and of the parent pharmaceuticals in a viscous vehicle were compared.
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TABLE OF CONTENTS

ABSTRACT

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION
  1.1 Objective of study
  1.2 Thermal denaturation of human serum albumin
  1.3 Labelling human serum albumin microaggregates with $^{99m}$Tc
  1.4 Intranasal drug delivery system
  1.5 Anatomy of the nose
  1.6 Deposition and clearance of particles from intranasal aerosol therapy
  1.7 Analysis of particle dimensions

2.0 METHODS

2.1 Preparation of microaggregated human serum albumin

2.2 Determination of albumin residue concentration

  2.2.i Biuret reagent preparation
  2.2.ii Human serum albumin standard preparation

2.3 Labelling the microaggregates with sodium pertechnetate

2.4 Microaggregates particle size determination by serial filtrations

2.5 Particle sizing using the optical sizing Aerosizer
LIST OF TABLES

Table 1. Absorbance of standard human serum albumin and of supernatant at 540nm 30

Table 2. Experimental data on serial filtrations of $^{99\text{m}}\text{Tc}$ labelled human serum albumin microaggregates (prepared by thermal denaturation for various durations) using Nuclepore Polycarbonate membrane filters 33

Table 3. Cumulative percentage of microaggregates in a size range of 0 - 5 microns 34

Table 4. A diffusion rate comparison between human serum albumin microaggregates and budesonide in a viscous vehicle 45
LIST OF FIGURES

Figure 1. A picture of the optical sizing system: Aerosizer 26

Figure 2. The Pari Master 27

Figure 3. The Andersen Impactor 28

Figure 4. Standard curve of spectrophotometric assay on budesonide 31

Figure 5. Radiochromatogram scan of bound $^{99m}$Tc human serum albumin microaggregates 32

Figure 6. Radiochromatogram scan of free pertechnetate 32

Figure 7. A size distribution plot of three batches of 4 minute denatured human serum albumin microaggregates after serial filtration 37

Figure 8. The aerodynamic size distribution plot on aerosolized budesonide droplets and dried budesonide particles using Aerosizer 38

Figure 9. The aerodynamic size distribution plot on human serum albumin microaggregates obtained after various durations of thermal denaturation 39

Figure 10. A comparison on size distribution of microaggregates to that of budesonide using the Aerosizer 40

Figure 11. Aerodynamic size distribution of $^{99m}$Tc human serum albumin microaggregates determined by Andersen Impactor 41

Figure 12. Aerodynamic size distribution of aerosolized Pulmicort Nebuamp® (0.5 mg/mL) using Andersen Impactor 42

Figure 13. Aerodynamic droplet size distribution of a premixed solution of $^{99m}$Tc microaggregates and Pulmicort Nebuamp® (from three trials) 43

Figure 14. Average aerodynamic droplet size distributions of $^{99m}$Tc microaggregates and Pulmicort Nebuamp® 44

Figure 15. The best straight line describing the relationship between the diffusion coefficients of $^{99m}$Tc microaggregates and that of budesonide 46
Figure 16. A three dimensional image on microaggregates using laser confocal scanning microscope
1.0 Introduction

Microparticles like microspheres, microcapsules and macroaggregates, in the size range of 0.1 - 1000 microns, are widely used in biomedical diagnostic agents and as controlled-release and targeted drug delivery. Microparticles with a wide range of compositions and morphological characteristics, are produced from different natural or synthetic starting materials and by a large number of polymerization and microencapsulation techniques. These particulate materials can then be chemical, fluorescent, magnetic or radioactively labelled, and be observed in vitro and in vivo by monitoring under the appropriate sensing device. Human serum albumin particles are especially useful in biomedical applications because of their advantage of being metabolized easily and being non-antigenic to humans. In contrast, inert plastic particles are not biodegradable, will remain indefinitely in the body, and may only be used in experimental animals.

Human serum albumin particles may be labelled with $^{99m}$Tc. Radiolabelled human serum macroaggregates have often been used to investigate altered circulatory states in the human. $^{99m}$Tc labelled human serum albumin macroaggregates (10 - 90 microns) have been used extensively as routine diagnostic nuclear medical imaging procedures for the analysis of regional lung perfusion. $^{99m}$Tc labelled human serum albumin macroaggregates can be used to detect early regional pulmonary ischemia from emboli without infarction and other diseases which obstruct pulmonary arterial blood flow before signs of these lesions are recognizable in routine chest x ray films. $^{99m}$Tc labelled macroaggregates are normally trapped throughout the pulmonary capillary bed. An area of decreased radioactivity in the lung scan indicates that impaired arterial circulation exists in the region. The macroaggregates are then excreted from the lungs, following the splitting of particles into smaller sizes, which are able to pass through the
capillaries. Human serum albumin macroaggregates are prepared from heat-denatured human serum albumin by either boiling in a water bath or autoclaving with subsequent centrifugation. A lack of uniformity in shape and stability in size distribution sometimes causes macroaggregates to have uneven distribution throughout the lung and produces "hot spot" artifacts 3,4.

$^{99m}$Tc labelled microspheres (15 - 30 microns), with rigid spherical shape and narrower size distribution, were developed in 1968 for lung imaging 4. Since the radiolabelled microspheres fundamentally measured regional blood flow, they could also be used to assess the changes in distribution of coronary blood flow after a variety of pharmacological and mechanical interventions 5. Liu et al. (1986) gave a small dose of intracardiac injection of $^{99m}$Tc and $^{111}$In radiolabelled microspheres to five pigs and successfully imaged them with single photon emission tomography for in vivo myocardial localization and relative quantitative distribution of the microspheres 5. An intracarotid injection of radiolabelled microspheres for brain scintigraphy provided a method of determining relative regional cerebral blood flow 6. Etani et al. (1982) performed cerebral perfusion imaging with $^{99m}$Tc and $^{111}$In labelled human serum microspheres in 15 patients with unilateral occlusion of the internal carotid artery 6. With this method, positive diagnosis of internal carotid artery occlusion was definitely made in eight patients, suspended in six and missed in one. Furthermore, microspheres were utilized in renal microcirculation studies to provide the index of the viability of preserved kidneys before transplantation 7. After preserving the canine kidneys for 5, 24, 48 and 72 hours, Anaise et al. (1984) perfused the kidneys with 50,000 $^{99m}$Tc labelled microspheres 20 microns in size and then imaged them using a gamma camera 7. An excellent correlation with the renal cortical activity before transplant and the function of the same kidney after transplantation was observed. Albumin microspheres may
be prepared by suspension crosslinking which starts with the formation of small droplets of aqueous albumin in an immiscible phase (oil phase), hardening of the small droplets by covalent cross-linking, and the recovery of the resulting cross-linked particles. The average size of the albumin droplets in the oil phase at the steady state size equilibrium is determined by the parameters of apparatus design, viscosity of the two immiscible phases and speed of mixing. The albumin droplets are then converted to the hydrated microspheres at the approximate same size. Albumin droplets are hardened by covalent cross-linking, either thermally or by the addition of a chemical cross-linking agent. Self cross-linking between functional groups on the polypeptide side chains is usually carried out by heat treatment at above 50°C. The use of a condensing agent such as dimethylaminopropylethylcarbodiimide also leads to protein cross-linking at or below room temperature. It is important to realize that protein concentration, temperature, concentration of the cross-linking agents and the duration of the cross-linking reaction control the extent of protein cross-linking. Microsphere preparation by these methods requires time-consuming washing in organic solvents to remove the oil residue. Moreover, it is not easy to control and reproduce all the parameters in determining albumin droplet sizes on a laboratory scale.

1.1 Objective of study

The objective of this research was to develop biodegradable, easily prepared and 99mTc radiolabelled human serum albumin microparticles which can simulate the deposition pattern of an aerosolized parent pharmaceutical deposited in the nose. The parent pharmaceutical cannot be 99mTc radiolabelled directly due to a lack of electron donor groups. Distribution and short-term clearance studies will be possible by detecting and analyzing the radioactive marker. In order to be a useful
marker, the microparticles are designed to have a size distribution similar to that of the parent pharmaceutical. The active medicinal ingredient budesonide delivered in the Rhinocort Aqua® nasal spray or by the Pulmicort Nebuamp® was chosen as the parent pharmaceutical. Human serum albumin microaggregates with a narrow size range of 2 - 5 microns were prepared by thermally denaturing human serum albumin in a boiling water bath and recovered after centrifuging three times. The human serum albumin microaggregates were then radiolabelled and proposed as the marker.

1.2 Thermal denaturation of human serum albumin

Human serum albumin is a fairly stable hydrated macromolecule consisting of a single folded polypeptide chain with about 50% of α-helix 15,16. The helical folding of albumin is maintained by intramolecular hydrogen bonds and is limited by the disulphide bridges 15. Under thermal heating, the albumin chain starts to unfold with a partial loss of α-helix. This configurational change can still be reversed with the removal of heat. However, if the albumin solution is further heated to higher temperature, the α-helix unfolding will become partially irreversible. At a temperature of 60 °C or more, Wetzel et al. (1980) found an increased unfolding of the pocket containing the free -SH group of cysteine-34 which was available for intermolecular disulphide bridges 15. Albumin aggregation initiates with the formation of intermolecular -S-S- exchanges and continues with the subsequent hydrogen bonding between independent molecules 17. The albumin aggregates will be finally stabilized as an extended gel configuration, the b-pleated form, after cooling back to 20 °C. The molecular form of albumin was found to retain its native configuration in the range of pH 4 - 8 15. With the use of CD (circular dichroism) spectrum as a means to estimate the relative proportions of secondary structures in proteins, Wetzel et al. (1980) found the unfolding of human
serum albumin would be reversible up to a temperature of 68 °C. The albumin samples heated up to 80° C would be subject to an irreversible denaturation of about 40%. In this study, human serum albumin microaggregates were prepared by denaturing the albumin in a boiling water bath for a period of three to seven minutes at a pH of 7.0.

1.3 Labelling human serum albumin microaggregates with $^{99m}$Tc

$^{99m}$Tc is readily available in a sterile, carrier-free state from a $^{99}$Mo - $^{99m}$Tc generator. The $^{99}$Mo radionuclide has a half-life of 67 hours, and decays by β− emission to metastable state $^{99m}$Tc (87%) and to the ground state $^{99}$Tc (13%) \(^1\). The $^{99}$Mo - $^{99m}$Tc generator is a glass column loaded with alumina Al₂O₃. The $^{99}$Mo radioactivity is adsorbed on alumina in the chemical form MoO₄²⁻ (molybdate) and in various amounts. $^{99m}$Tc will grow by the decay of adsorbed $^{99}$Mo according to the equation \(^3\)

\[
(D_{Tc-99m})_t = \lambda\beta_{Tc-99m} N\beta_{Tc-99m} = [\lambda\beta_{Tc-99m} (D_{Mo-99})_t] (e^{-\lambda\beta_{Mo-99} t} - e^{-\lambda\beta_{Tc-99m} t}) / (\lambda\beta_{Tc-99m} - \lambda\beta_{Mo-99})
\]

where

- \(D\) is the disintegration rate,
- \(N\) is the number of radionuclides,
- \(\lambda = 0.693 / t_{1/2}\)

until its maximum activity is reached after approximately four half-lives of $^{99m}$Tc. At equilibrium and thereafter, the $^{99m}$Tc radioactivity follows the half-life of $^{99}$Mo. The $^{99m}$Tc radionuclide is eluted out of the column with 0.9% normal saline as sodium pertechnetate (Na$^{99m}$TcO₄). The pertechnetate ion, $^{99m}$TcO₄⁻, is chemically nonreactive and cannot label any compound by direct addition. Prior to the $^{99m}$Tc labelling of many complexes, a reduction of $^{99m}$Tc from the 7+ state to a lower oxidation state is required \(^2, 3, 18\).
\[
\text{reduction} \quad \text{ligand (L)}
\]

\[
{^{99}\text{TcO}_4^-} \quad \Rightarrow \left[{^{99}\text{TcO}_m(\text{H}_2\text{O})_n}\right]^{p+} \quad \Rightarrow \left[{^{99}\text{TcO}_m(\text{H}_2\text{O})_n}\right]^{p+}L_x
\]

Tc (VII) \quad Tc (III, IV, V)

\[m = 1-3; \quad n = 0-2; \quad p = 0-2; \quad x = 1-6\]

Ligand = -COO\(^-\), -NH\(_2\), -NH, -OH, etc.

Various reductants such as stannous chloride, stannous citrate, stannous tartrate, concentrated HCl, borohydride sodium dithionite, and ferrous sulfate have been utilized in \(^{99}\text{Tc}\)-labelling \(^3\). The nature of reductant used in the formation of any technetium complex can have a significant effect on the biodistribution of the resultant complex \(^1\)\(^8\). The choice of reducing agent influences the oxidation state of technetium, and if a mixture of complexes is formed, the proportion of the radioactive components of that mixture \(^1\)\(^8\). The reduced \(^{99}\text{Tc}\) is then covalently bound to various chelating agents which can donate lone pairs of electrons.

The amount of unbound, pertechnetate ion left after a radiopharmaceutical preparation is within acceptable limits. However, if oxygen is accidentally introduced into the vial during labelling, the stannous ions will be oxidized before its availability for reducing \(^{99}\text{Tc}\). This results in an increase in free \(^{99}\text{TcO}_4^-\) in \(^{99}\text{Tc}\) radiopharmaceuticals \(^3\)\(^1\)\(^8\). In addition, ionizing radiation from high activity of \(^{99}\text{Tc}\) in the presence of oxygen, may decompose water molecules to give hydrogen peroxide and other free radicals. Once all the stannous ions have been consumed, the free radicals will interact with \(^{99}\text{Tc}\)-chelates producing free \(^{99}\text{TcO}_4^-\). Therefore the kit vials are flushed with nitrogen to maintain an inert gas atmosphere.

Reducant stannous ions may undergo hydrolysis in aqueous solution to form insoluble colloids. These colloids are then bound to the reduced \(^{99}\text{Tc}\) and reduces the yield of \(^{99}\text{Tc}\).
complexes $^{3,18}$. Antioxidants, such as ascorbic acid and gentisic acid, may be added to prevent the hydrolysis of stannous ions $^{3,18}$. On the other hand, reduced $^{99m}$Tc may react with water to form hydrolyzed species $^{99m}$Tc$\text{O}_2^-$, $^{99m}$Tc$^{2+}$, and $^{99m}$TcOOH$^-$. The hydrolysis competes with the chelation process and decreases the yield of the desired $^{99m}$Tc labelled complex. The hydrolysis of reduced $^{99m}$Tc can be avoided by adding sufficient ligand. The ratio of the ligand to stannous ions should be large enough to ensure complete $^{99m}$Tc binding $^3$. All the hydrolyzed radiochemical impurities mentioned above may result in poor quality imaging because of insufficient localization in target organs and the high background activity from the surrounding tissues. Therefore, quality control tests like precipitation, paper, thin-layer, and gel chromatography, paper and gel electrophoresis, ion exchange, solvent extraction, and high performance liquid chromatography, are employed to detect and determine the radiochemical impurities after radiolabelling.

Albumin microspheres (20-30 microns) have been successfully labelled with $^{99m}$Tc by electrolysis, reductants ferric chloride, stannous chloride and sodium thiosulfate $^8$. During electrolysis, a mixture of 1N HCl, sodium pertechnetate and microspheres is transferred to an electrolysis vial using zirconium wire electrodes. In Mayron and Kaplan (1975) comparison of these four techniques for labelling microspheres $^8$, the maximum binding efficiency obtained by electrolysis was 74%, by ferric chloride was 91%, by stannous ions was 92%, and by thiosulfate was 83%. Stannous chloride was chosen as the reducing agent for $^{99m}$Tc labelling of human serum albumin microaggregates in this study because of its solubility, stability, low toxicity and effectiveness at room temperature $^{18}$. The oxidation state of technetium in $^{99m}$Tc labelled albumin was suggested to be $5^+$ by polarographic measurements and iodometric titrations $^3$. 
1.4 Intranasal drug delivery systems

Medicinal preparations are administered into the nasal cavity as topical treatments for nasal diseases. Allergic rhinitis, chronic sinusitis, nasal polyps, and other related conditions may be treated by the topical application of vasoconstrictors, antihistamines, anticholinergic agents, topical corticosteroids, and cromolyn sodium. The nasal cavity has also been used as a drug absorption site because of its large surface area, the highly vascularized mucosa and its venous blood passing directly into the systemic circulation without first-pass metabolism in the liver. The intranasal drug delivery systems may provide a convenient means of administration for systemically acting drugs which cannot be given orally and for which the only alternative is parenteral administration.

Antidiuretic hormone and gonadotropin releasing hormone analogues are commercially available as nasal sprays, and insulin, glucagon and growth hormone are at present undergoing clinical trials. The parent intranasal pharmaceutical in this study, budesonide, is available topically as an aqueous aerosol from a metered-dose pump spray, or as a micronized powder from a pressurized canister in the market.

1.5 Anatomy of the nose

The nasal cavity consists of a 5 cm high and 10 cm long dual chamber. The total surface area of both nasal cavities is about 150 cm² and the total volume is about 15 mL. The apparent external nose surrounds the nostrils and one-third of the nasal cavity. Approximately 1.5 cm from the nose entrance is the narrowest portion of the entire airway, the internal ostium, with a cross-sectional area of about 0.3 cm² on each side. The slit-like nasal cavity is limited by the septal wall, and the lateral wall, dominated by inferior, middle and superior turbinate, and with orifices of the nasolacrimal duct and the paranasal sinuses. The anterior one-third of the nasal cavity is covered by a squamous and
transitional epithelium and the upper part of the cavity by an olfactory epithelium. Ciliated cells are found behind the front edge of the inferior turbinate, the posterior part of the nasal cavity, and the paranasal sinuses. The density of ciliated cells is inversely proportional to the linear velocity of inspiratory air in the nasal cavity.

The material deposited onto the vascularized and ciliated posterior nasal passage (starting from the middle part of inferior turbinate) will be cleared away either by absorption or by mucociliary action at approximately 6 mm/min. On the other hand, clearance of deposited drug on the anterior, non-ciliated region of nasal cavity are retained for long periods unless they are dragged into the ciliated region by contiguous mucus. Therefore in nasal aerosol clearance studies, a biphasic clearance curve will be obtained with an initial fast removal followed by a prolonged second phase reflecting the slow removal of material from the non-ciliated region.

1.6 Deposition and clearance of particles from intranasal aerosol therapy

The quantity of aerosolized drug deposited in the nasal passage and the distribution pattern of the deposited drug are of great importance simply because therapy or prophylaxis can be successful only if sufficient drug is deposited at the designated site. The efficacy of intranasal drug delivery system is influenced by the subsequent rate of clearance. Our body's defence mechanism, mucociliary clearance, may remove the deposited drug before it can either act topically or be absorbed. The physical mechanisms operated on aerosolized particles to move them across streamlines of air and deposit towards the surface of the respiratory tract include gravitational sedimentation, inertial impaction, Brownian diffusion and electrostatic forces. Particles with aerodynamic diameters from 0.5 - 2.0 microns predominantly deposit in the respiratory tract by sedimentation. All particles with density greater than that of air experience a downward force due
to gravity. For particles with diameters smaller than 0.5 microns, diffusion is found to be the predominant mechanism and most deposition takes place in the distal airways. Particle diffusion is caused by random collision with air molecules. As an electrically charged particle approaches a surface in the airways, it may deposit onto the opposite induced charged surface. Even when an uncharged particle approaches a neutral surface, electrostatic Van der Waal's force may cause an attraction between the two. Inertial impaction is the most important deposition mechanism for particles with an aerodynamic diameter larger than 2 microns in the nasal cavity. This is due to the anatomical configuration of the nose. An inertial impaction of an aerosol particle is caused by a sudden change in the direction of flow. The deposition site of a specific particle is also highly dependent on the particle's aerodynamic characteristics, the subject's breathing pattern, the geometry of the respiratory tract, and the flow and mixing pattern of the aerosol within the respiratory tract.

The distribution of nasal aerosols deposited onto the walls of the nasal cavity, and their subsequent rates of clearance into the pharynx have been studied extensively. The approaches employed included the direct observation of fluorescent particles, the imaging of radiopaque and radioactive materials. Several particle deposition studies used radiolabelling techniques since they could provide non-invasive *in-vivo* measurement with high sensitivity and resolution. Most drugs are composed of the elements hydrogen, carbon, nitrogen and oxygen, therefore $^3$H, $^{14}$C (pure $^\beta^-$ particle emitters) can be easily incorporated into the drug by a simple isotope substitution. However, the $^\beta$ particles from these radionuclides cannot be detected externally and merely contribute to the radiation dose because their range in tissue is a few millimetres. They may be used, however, in deposition studies in animals where dissection of tissue is possible. Drugs labelled
with γ emitting radionuclides could be measured by an external detector. An ideal radiolabel for deposition studies should emit γ but no β⁻ radiation. The physical half-life of the radionuclide should be long enough to perform the study but without causing any unnecessary radiation exposure. The γ radiation emitted should be of a high enough energy to penetrate tissue but without causing any appreciable attenuation.¹⁹ The radionuclide ⁹⁹ᵐ Tc is often chosen for radioaerosol studies because of its favourable half-life (6 hours), suitable γ photon energy (140 KeV), absence of β particle emissions, and it is readily available.

The most common detecting instrument used for a radioaerosol deposition study is the gamma camera. This instrument consists of a collimator, sodium-iodide detector, photomultiplier tube, preamplifier, pulse height analyzer, x, y positioning circuit, and a display or storage system.¹ A lead collimator with various holes of different size and shape is attached to the sodium iodide detector in order to prevent the radiation outside the field of view from reaching the detector. Gamma photons emitted from a radionuclide interact in the sodium iodide detector and light photons are emitted (about 1 light photon per approximately 30 eV). The photons then strike the photocathode of a photomultiplier tube and a pulse is generated at the end of the photomultiplier tube. The pulse is amplified by a preamplifier and then by a linear amplifier. A pulse height analyzer sorts out the amplified pulses according to the desired energy of the gamma radiation and sends the pulse into the computer.¹

However, it is usually not possible to directly label a nasal therapeutic agent with ⁹⁹ᵐ Tc. The ligands would be required to donate a pair of electrons to form co-ordinate covalent bonds with the oxotechnetium. The parent pharmaceutical budesonide, used in this study, lacks electron donor groups such as -COO⁻, -OH⁻, -NH₂ and -SH, and cannot be chemically labelled with ⁹⁹ᵐ Tc. In
aerosol deposition studies $^{99m}$TcO$_4^-$ is added to the formulation so that it traces the presence of drug substance, although not chemically labelling it. Dashe et al. (1974) used a mixture of isoproterenol solution and $^{99m}$TcO$_4^-$ solution in the nebulizer reservoir for the purpose of studying deposition and clinical efficacy. However, the radionuclide could only act as a marker for the deposition of the droplet in the lung and did not necessarily reflect the actual deposition of the drug contained in the droplet. In addition, the pertechnetate ion might not be cleared from the site of deposition in the same manner as the drug substance. $^{99m}$Tc labelled human serum albumin have been added to the aqueous nasal spray formulation for deposition and subsequent clearance studies because the rate of absorption of the tracer was slow compared with the rate of mucociliary clearance. Newman (1994) studied the initial distribution and subsequent clearance of the components of an intranasal insulin formulation by the addition of $^{99m}$Tc - human serum albumin, so that the droplets from the nasal spray contained both drug and radiolabel. This investigation allowed the initial distribution pattern to be determined, but subsequent scans displayed only the mucociliary clearance of the $^{99m}$Tc - human serum albumin marker rather than the clearance of the drug.

$^{99m}$Tc labelled solid particles such as insoluble human serum albumin particles, iron oxide, polystyrene and Teflon were also used in aerosol deposition and subsequent mucociliary clearance measurements. These particles had the advantage of being insoluble in body fluids, biologically inert and non-toxic. The insoluble Teflon particles, which are not absorbed into the circulation, enables the determination of the extent to which the deposited material was removed by mucociliary action. Newman et al. (1987) used a spinning disc generator to manufacture insoluble particles of Teflon of mass median aerodynamic diameter 3.2 microns labelled with $^{99m}$Tc. The $^{99m}$Tc labelled Teflon particles were then collected and added to placebo pressurized canisters.
identical to those used for budesonide intranasal aerosol (Rhinocort®) together with chlorofluorocarbon propellants and sorbitan trioleate as a surfactant.\textsuperscript{27} \textsuperscript{99m}Tc labelled solid particles were designed to replace the drug particles in an aerosol, but it was difficult to reproduce the size spectrum of the drug particle found in the actual formulation. Therefore, it was possible that the radiolabelled particles might not be distributed and cleared in the same way as the parent pharmaceutical. In contrast, this investigation demonstrates that human serum albumin microaggregates with a distinct size distribution were produced by carefully controlling the duration of thermal denaturation. The non-antigenic, biodegradable and readily \textsuperscript{99m}Tc labelled human serum albumin microaggregates may be more useful in simulating a parent pharmaceutical during \textit{in vivo} intranasal deposition and clearance studies when compared to the scintigraphic studies using pertechnetate, \textsuperscript{99m}Tc labelled human serum albumin or Teflon particles.

1.7 Analysis of particle dimensions

A major factor governing the deposition site of an aerosol is the size of the inspired particle or droplet. Since the budesonide particles in the parent formulation were found to have a size distribution range of 2 - 3 microns,\textsuperscript{28} preliminary serial filtrations (through Nuclepore polycarbonate membrane filters) were performed to select a batch of human serum albumin microaggregates with similar sizes. A quantitative, non-invasive and virtually free from out-of-focus blur three-dimensional image of human serum albumin microaggregates, is obtained by using the laser confocal scanning microscope (Bio-Rad 600). The confocal scanning microscope is capable of imaging a plane deep inside a droplet specimen, without appreciable interference from the intervening and deeper lying layers. Aerodynamic diameters of aerosolized microaggregates and budesonide, which combine the characteristics of size, shape and density,\textsuperscript{29} are then experimentally
determined by the methods of optical sizing and inertial separation. The methods for sizing therapeutic aerosols all have limitations, and the results are dependent on the aerosols presented to the sizing instrument by the sampling system\textsuperscript{19}. Therefore the particle size range measured, the size resolution, and the concentration of particles that can be sampled by the system, are the main features to be considered when choosing a sizing system\textsuperscript{30}. Single particle optical counter utilizes the signals produced by the aerosolized particles to measure their velocity as they cross the laser beams\textsuperscript{30}. Aerosizer is used as the optical sizing systems to measure aerodynamic diameters of microaggregates and budesonide directly within seconds. Andersen impactor size-fractionate aerosol particles aerodynamically according to their inertia, which increases with size. Aerodynamic sizes of droplets containing budesonide or $^{99m}$Tc human serum albumin microaggregates generated by PARI LL nebulizer system (a more detailed description of PARI LL nebulized system can be found in the method section) were assessed by this inertial separation device. Inertial impaction is useful tool for biopharmaceutical assessment of a dosage form in vitro by measuring the drug mass distribution among the various aerodynamic size ranges of the aerosol\textsuperscript{29}. Diffusion may be used to investigate a number of important molecular characteristics such as molecular size, weight, interaction with solvent\textsuperscript{31}. A free diffusion method using a versatile shear cell was employed in this study to compare the diffusion rate of $^{99m}$Tc labelled microaggregates and budesonide in a viscous vehicle. The result could be used to predict the difference in movement rate between $^{99m}$Tc labelled microaggregates and budesonide particles in the mucus blanket after deposition in the nostril.
2.0 Methods

2.1 Preparation of microaggregated human serum albumin:

Human serum albumin microaggregates were prepared by denaturing human serum albumin at the boiling temperature. In order to avoid frothing, 0.80 mL of 25% human serum albumin (IMMUNO, obtained from the blood bank of McMaster University Medical Centre) was slowly added to a sterile 50 mL Erlenmeyer flask containing 33.5 mL of 100 mg/mL stabilizer sodium acetate and 1.35 mL of surfactant 1% Tween 80. A sufficient quantity of 0.9% sodium chloride was then added to make up a volume of 40 mL and a final albumin concentration of 5 mg/mL. The albumin mixture was dispensed into test tubes and placed into a boiling water bath for various periods of time (3, 4, 5, 6, and 7 minutes). The denaturation was then stopped by cooling the test tube in cold water with vigorous shaking. Microaggregates were extracted from the resulting solution after several centrifugations and washings with 0.9% sodium chloride solution. Human serum albumin microaggregates suspended in saline were stored at 4 °C refrigeration to retain particle size stability.

2.2 Determination of albumin residue concentration

The Biuret assay was used as an indirect way to check the concentration of soluble albumin residue remaining in the microaggregates preparation. Cupric ions in the alkaline Biuret solution chelate with the amino groups on the soluble albumin, resulting in a colour change to violet. After centrifugation, all the undenatured soluble albumin should be dissolved in the supernatant with the heavier microaggregates staying at the bottom. If the albumin concentration in the supernatant after several centrifugations was found to be lower than 0.25 mg/mL (within 5% of the starting albumin concentration of 5 mg/mL), it could be assumed that the microaggregates extracted were free of
albumin contamination.

2.2.i Biuret reagent preparation:

A concentration of 0.1 N carbonate-free sodium hydroxide solution was prepared by dissolving 4.0 grams of sodium hydroxide pellets (from a previously unopened bottle) in 1 litre of deionized water in a volumetric flask. Cupric sulfate (0.15 gram) and potassium sodium tartrate (0.60 gram) were then dissolved in 50 mL of water. The resulting solution was then diluted to a total volume of 100 mL with the 0.1 N carbonate-free sodium hydroxide solution to prepare the Biuret reagent. Biuret reagent was kept in a closed polyethylene bottle at room temperature up to six months.

2.2.ii Human serum albumin standards preparation:

Human serum albumin standard solutions in concentrations of 2.5 mg/mL and 5.0 mg/mL were prepared. A volume of 0.5 mL human serum albumin standard was added into three test tubes containing 0.5 mL of deionized water and 4.0 mL of Biuret reagent. The tubes were allowed to stand at room temperature for 30 minutes for colour development. A triplet of test tubes using the supernatant after several centrifugations instead of the human serum albumin standards were also prepared. The UV-Visible Spectrophotometer was set to a wavelength of 540 nm and blanked with the Biuret reagent only. The absorbance of each of the standard series and the supernatant test series were recorded, relative to a reference cell filled with deionized water.

2.3 Labelling the microaggregates with ⁹⁹ᵐTc sodium pertechnetate

Human serum albumin microaggregates were labelled with ⁹⁹ᵐTc by a stannous chloride reduction technique. ⁹⁹ᵐTc was obtained in the form of ⁹⁹ᵐTc sodium pertechnetate from the Nuclear Pharmacy of the Hamilton Health Science Corporation. ⁹⁹ᵐTc-sodium pertechnetate was added to a vial flushed with nitrogen to avoid oxidation of stannous ion to stannic ion.
Microaggregates were then slowly added to the pertechnetate with stirring to prevent large clumps from forming. In order to bind with the microaggregates, Tc(VII) has to be reduced to Tc(V). A volume of 0.2 mL stannous chloride in a concentration of 2 mg/mL was added to reduce an amount of 6 mCi pertechnetate. Gentisic acid (0.04 mg) was also added as an antioxidant. The radiochemical purity of $^{99m}$Tc-HSA microaggregates was determined by descending paper chromatography using Whatman #1 strip as the stationary phase and 85% methanol as the mobile phase. During this chromatographic process, electrostatic forces of the stationary phase tend to retard various components, while the mobile phase carries them along. This effect and different solubilities of various components in a solvent cause the individual component to move at different speeds and to appear at different distances along the paper strip. For this radiochemical purity study, one microlitre of the $^{99m}$Tc-HSA microaggregates was spotted at the origin of the paper strip and allowed to develop for 1-2 hours. The percentage of unbound pertechnetate was obtained by reading the strip under the NSCAN radiochromatogram scanner, which had an automatic integrator device, and plotted the radioactivity versus the distance of the strip.

2.4 Microaggregates particle size determination by a serial filtration

The size of microaggregates was too small to be determined using a conventional light microscope and haemocytometer grid. A serial filtration was performed by passing the $^{99m}$Tc labelled microaggregates in normal saline through Nuclepore polycarbonate membrane filters with 2.0, 3.0 and 5.0 μm pore sizes. The Nuclepore membrane filter was placed in between two plastic filter holders with the shiny surface facing upwards. The amount of radioactivity retained on each filter or passed into the filtrates determined the filtration efficiency. Membrane filters and filtrates were analyzed using a Capintec ionization chamber. $^{99m}$Tc microaggregates prepared from the same
batch of cold microaggregates were passed through three sets of Nuclepore polycarbonate membrane filters. This permitted an analysis of the consistency of the size distribution of microaggregates from the same batch.

2.5 Particle sizing using the optical sizing Aerosizer

The Aerosizer (Figure 1), a single particle counter, is capable of measuring the aerodynamic size of particles in a range of 0.2 to 70 microns. The particles may be in a dry powder form, or suspended in a gas, or sprayed as a liquid suspension (the size of particles from a liquified spray are determined after the water droplets quickly evaporate in the air-flow). The Aerosizer measures particle size by expanding an air-particle suspension through a nozzle into a partial vacuum. If the pressure differential across the nozzle is large enough, the air flow will increase until the air velocity at the nozzle exit is approximately the velocity of sound. Particles in the air-particle mist are accelerated by the drag forces generated by the accelerating air stream. Particles in very small sizes are accelerated to near the sonic velocity, while the large particles are accelerated less due to their greater mass.

The general form of the Force Equation is given by:

\[ C_d (\pi d^2 / 4) \rho_a [(v_a - v_p)^2]/2 = 1/6 \pi d^3 \rho_p \frac{dv}{dt} \]

where \( C_d \) = Drag coefficient
\( \rho_a \) = density of air
\( v_a \) = velocity of air
\( d \) = particle diameter
\( \rho_p \) = density of particle
\( v_p \) = velocity of particle

The \( C_d (\pi d^2 / 4) \) term relates the drag coefficient and projected area of the particle to the force while the \( \rho_a [(v_a - v_p)^2]/2 \) term relates the air density and differential velocity of the air and particle to the
force applied on the particle. The right side of the equation is simply the particle volume multiplied by the density \( \frac{1}{6} \pi d^3 \rho_p \) to get the particle mass and the particle acceleration \( dv/dt \).

The time-of-flight of a single particle is measured as the particle crosses two laser beams generated in the Aerosizer's measurement region. When a particle passes through each laser beam, it scatters light which is detected and converted into electrical signals by two photomultiplier tubes. The electronic signal from the photomultiplier monitoring the first laser beam is employed to start a timer. The electronic signal from the photomultiplier watching the second beam is used to read the timer. The electronic signals are differentiated into two levels. A low level threshold is set for high sensitivity which picks up signals from the small particles. On the other hand, large particles may trigger the low level threshold more than once due to afterpulsing and ringing in the photomultiplier signal. A second high level threshold is therefore set for lower sensitivity which will be triggered once by large particle. The API proprietary software inside the Data Acquisition Analysis computer then calculates the particles size distribution from the measured time-of-flight data. Besides the basic number distribution, the relative surface area and volume for the particles in each size range can also be determined. The surface area and volume are calculated by multiplying the number of particles in each size range by the square of the diameter at the centre of that range or the cube of the diameter at the centre of that range. The percentage of the surface area and volume in each size range is determined by dividing the surface area or volume calculated for each size range by the total surface area or volume of all size ranges.

The sizing investigation started with the generation of an aerosol containing "cold" HSA microaggregates in distilled water, by PARI MASTER (Figure 2). The PARI MASTER, including a PARI MASTER Compressor and a PARI LL Nebulizer System, was used to deliver aerosolized
microaggregates and budesonide in this study. The PARI MASTER is an inhalation device controlled by an air flow interrupter for on-command therapy. The aerosols were sprayed into the Aero-Sampler, a collection chamber of the Aerosizer. Immediately after the sampling, a volume distribution plot of aerosolized microaggregates or budesonide droplets was obtained on the screen. An aerodynamic size distribution curve of dehydrated human serum albumin microaggregates particles or budesonide particles would be displayed in the computer about 30 seconds after sampling, after allowing water to evaporate under the air-stream and leaving the dry microaggregates or budesonide suspended in air. The experiment was then repeated for the microaggregates of different size ranges (human serum albumin solution was denatured at boiling temperature for various periods of time).

2.6 Aerosol droplet sizing using Andersen Impactor

A cascade impactor is a sizing device used to measure the aerodynamic size distribution of airborne particles. The impactor simulates the dust collecting characteristics of the human respiratory system, therefore the sampling data can be used to predict the lung penetration by airborne particles \(^{36}\). The inertial impaction analysis by a cascade impactor is especially useful for sizing therapeutic aerosols because the pharmacological effects of a drug depend on the dose fraction reaching the various locations inside the lungs. The Andersen Impactor (Figure 3) consists of eight stages with stage 0 as an orifice stage and stage 8 as a collecting filter. A constant air sample flow of 1 ACFM (28.3 L/min) is provided by a continuous duty vacuum pump. Each impactor stage contains multiple precision drilled orifices at a constant size. When air is drawn through the impactor, multiple jets of air with airborne particles are accelerated at the exit from the last stage and deflected at 90° to flow in a direction parallel to the impaction plate. Depending upon their inertia
or mass, the airborne particles may not get over a change in airflow direction, and therefore, impact onto the plate. The range of particle sizes collected on each stage depends on the jet velocity of the stage, the distance between the orifices and the collection surface, and the cut-off aerodynamic diameter of the previous stage 30. Any airborne particles not collected on the first stage will follow the air stream around the edge of the plate to the next stage (with orifices smaller in size), and so on until the jet velocity is sufficient for impaction 36.

Aerosolized droplets containing $^{99m}\text{Tc}$ human serum albumin microaggregates, generated by the PARI MASTER, were delivered through the Andersen Impactor for a period of 30 seconds. The impactor was then disassembled with the collection stages and filter removed. The aerodynamic size distribution of the aerosols containing $^{99m}\text{Tc}$-HSA microaggregates was obtained by determining the radioactivity retained on each collection plate using the large field of view gamma camera.

Aerosol made up by the Pulmicort® (0.5 mg/mL of budesonide in saline) was then sprayed through the impactor in order to obtain an aerosolized budesonide size distribution in comparison to the aerosols containing $^{99m}\text{Tc}$-HSA microaggregates. A quantitative analysis for budesonide of sufficient sensitivity was difficult to obtain.

First, the USP Assay For Steroids (The United States Pharmacopeia. Twentieth Revision USP XX. United States Pharmacopeial Convention, Inc. 1980) was employed as the method of detecting budesonide. The Rhinocort Aq® was diluted with 60% ethanol to a concentration of 0.02 mg/mL. As budesonide in the Rhinocort Aq® was dissolved in ethanol, the excipient carboxymethylcellulose would be precipitated out of the suspension. After a centrifugation at 3000 rpm for 10 minutes, a known quantity of supernatant containing the dissolved budesonide was mixed with 60% ethanol to a total volume of 25 mL. To each assay sample, 2.0 mL of a solution prepared
by dissolving 50 mg of blue tetrazolium in 10 mL of methanol was added. Then 2.0 mL of a mixture of 1 volume of tetramethylammonium hydroxide and 9 volumes of alcohol was mixed with the sample. The absorbance of each sample mixture was determined at 525 nm against the blank immediately after standing in the dark for 90 minutes. A standard curve of absorbance against budesonide concentration was then plotted (Figure 4).

Since the amount of budesonide deposited onto each collection plate was found to be in a range of μm, the USP XX Steroid Assay was not sensitive enough to display a concentration difference of budesonide on the different collection plates. An HPLC assay (with a sensitivity of 10^{-2} μg/mL) was used to measure the concentration of budesonide retained on each plate after the cascade impactor sizing. This HPLC analysis was performed in the Inflammatory Mediators Laboratory at McMaster University.

The budesonide aerosol collected on each plate was washed off with 2 mL of absolute ethanol (the filter was washed with 5 mL of ethanol). The washing samples were stored at -70°C and 100 μL of each sample was assayed neat using a Waters HPLC system. The column used was a Zorbax ODS (E. I. DuPont), 5 μm (4.6 mm x 25 cm) supplied by Chromatographic Specialties Inc. (Brockville, Ontario, Canada). A pre-mixed mobile phase of 47.5% acetonitrile and 52.5% 0.01 M acetic acid was passed through the column at a rate of 1.0 mL min⁻¹. The absorbance was read with a spectrophotometer at 234 nm. The budesonide isomers gave peaks at 11.3 minutes and 11.0 minutes.

Since each component within an aerosol collected on each stage of the impactor can be analyzed chemically and independently of other aerosolized constituents, an impaction analysis on a premixed solution of ⁹⁹ᵐTc-HSA microaggregates and budesonide was performed. A nebulized
solution of 2 mL of Pulmicort 0.5 mg/mL mixed with 2 mL of $^{99m}$Tc-HSA microaggregates was generated by the PARI MASTER, and was sprayed into the Andersen Impactor for a period of one minute (sprayed for 30 seconds, rested for 1 minute, and then sprayed for another 30 seconds). The quantity of radioactivity retained on each collection plate was determined by the large field of view gamma camera. The amount of budesonide impacted on each plate was determined by HPLC analysis after the radioactivity had decayed.

2.7 Diffusion study using a versatile shear cell

Diffusion is defined as a process of transferring from a region of high concentration to one of the lower concentration, as a result of random Brownian motion. The rate of diffusion is expressed by Fick’s first law:

$$dq = -DA \frac{dc}{dx} \, dt$$

where $dq$ is the quantity of substance diffusing in time $dt$ across an area $A$ under the influence of a concentration gradient $dc/dx$. $D$ is the diffusion coefficient and has the dimensions of area per unit time. If the diffusing particle is assumed to be approximately spherical, the equation suggested by Sutherland and Einstein:

$$D = \frac{(RT)}{(6\pi\eta rN)}$$

in which $D$ is the diffusion coefficient, $R$ is the molar gas constant, $T$ is the absolute temperature, $\eta$ is the viscosity of the solvent, $r$ is the radius of the spherical particle, and $N$ is Avogadro’s number, can be utilized to study a number of particle characteristics such as size, weight and interaction with solvent.

A diffusion cell based on shearing principle was built as an analytical technique to monitor the free diffusion rates of $^{99m}$Tc- HSA microaggregates and budesonide particles in a pharmaceutical
placebo vehicle. In a versatile shear cell, a solution and a solvent container are kept apart during filling, whereafter they are brought into contact to form an interface allowing subsequent diffusion

Each versatile shear cell consists of an upper and a lower container, 10 mm deep and 5 mm in diameter.\textsuperscript{38,39}

Two cylindrical, stacked Teflon blocks with a common vertical axis of rotation are drilled with cylindrical holes to serve as the "solution" and "solvent" containers. The "solution" container can hold a volume of 0.39 mL while the "solvent" compartment is 0.24 mL in volume. The solution-solvent boundary is then formed by rotating the upper block with respect to the lower one. The top and bottom part of the containers are slightly conical to avoid difficulties of air bubbles. In the present design each dome contains two pairs of "solution"/"solvent" containers drilled symmetrically on diagonals through the centre of rotation. Teflon plungers (Pl) with an O ring (Viton) seal on the lower part are then pressed into the upper block by cups (C) that screw into a dome washer (Dw). The tightening pressure of the cell is exerted by a screw situated on top of the dome shaft (S) and is transferred by a spring washer (Spw) to the dome washer. The upper block is rotated around the common axis between position I (filling) and II (measuring). These positions are set by a cylindrical
pin (P) screwed into the periphery of the upper block, and two adjustable, eccentric pins fixed to the U-bar support. The cell is designed to be filled with very concentrated, viscous solutions and still allow a sharp initial boundary to be formed between a "solution" and a "solvent" container. In order to minimize the effects of any sudden temperature fluctuations, the cell should be placed in a temperature-controlled room or box.

The investigation started by mixing 0.25 mL of previously prepared $^{99m}$Tc-HSA microaggregates with the Rhinocort Aq, to a volume of 1.0 mL ($^{99m}$Tc-HSA microaggregates were centrifuged at 6000 rpm and washed with distilled water to remove stannous chloride residue after labelling). Large clumps were formed when mixing Rhinocort with $^{99m}$Tc-HSA microaggregates containing stannous chloride residue). The resulting mixture was then emptied into the "solution" container of the shear diffusion cell. The "solvent" container was filled up with a placebo supplied by Astra Canada (a vehicle with viscosity similar to the Rhinocort Aq). Diffusion of $^{99m}$Tc-HSA microaggregates and budesonide towards the placebo was initiated by rotating the upper Teflon block to the "measuring" position. The diffusion process was terminated after a predetermined time of five minutes. A sample of 3.0 µL was emptied out from the "solvent" container using a SOCOREX micro-pipette (Terochem Scientific) and assayed in a Auto-Gamma 5000 series gamma counter (Minaxi γ). The rest in the "solvent" container was retained for budesonide HPLC assay. The experiment was repeated for several times in order to determine reproducibility of this shear-cell technique.

The total quantity of diffusing solute Q ($^{99m}$Tc-HSA microaggregates or budesonide) transported across the boundary during time t after forming the initially sharp interface was given by $Q^2 = A^2 (\Delta c)^2 Dt/\pi$
Figure 1. A picture of the optical sizing system: Aerosizer. The Aerosizer is made up with a Sample Presentation Device, a Sensor Unit, a Vacuum pump, and a Data Acquisition and Analysis computer. The Aero-sampler is used to introduce the aerosols into the Aerosizer. The Sensor Unit contains the Source Optics and Detection Optics required to perform the time-of-flight measurement. The Vacuum Pump generates the air flow and pressure necessary for the Sensor Unit and for collecting the analyzed sample to reduce extra operator exposure. The Data Acquisition and Analysis computer controls the Sensor Unit in collecting and analyzing the time-of-flight signals.
where: $A$ is the cross-sectional area of the diffusion components,

$\Delta c$ is the initial concentration difference between the lower and upper compartments,

$D$ is the diffusion coefficient.

2.8 Laser confocal scanning microscopy

In order to visualize the particles, an image was made using a laser confocal scanning microscope. The HSA microaggregates were scanned at the Photonic's Research Ontario (PRO) with the assistance of Dr. K. Zhang. A three-dimensional image was constructed by putting a droplet of HSA microaggregates onto a slide under the laser confocal scanning microscope (Bio-Rad 600). Inside the confocal scanning microscope, light originating from a laser-illuminated aperture was focused on a certain point of the object while the same point was also precisely imaged on a imaging detector aperture. A photomultiplier tube was then used to measure the interaction with each point in the object of the illuminating probe as it scanned through the microaggregates specimen. The light measured was reflected light in response to the probe. A three-dimensional image was built up from a series of these single-point images in a three-dimensional computer array.

The principle of the confocal microscope is that the image of the illuminating aperture and the back-projection of the imaging detector aperture have a common focus on the object. This confocal arrangement ensures that only light emanating from each in-focus point in the specimen is fully passed to the photomultiplier tube. In contrast, reflected light from the illuminated regions of the specimen further from the in-focus plane is defocused at the imaging aperture and thus only a small amount of light from these regions reaches the detector. The effect of these apertures is to eliminate scattered light from other image planes above, below and around the focal point, thus improving the resolution of the microscope.
3.0 Results:

Human serum albumin microaggregates were prepared as a marker to simulate the distribution of the target "parent" pharmaceutical budesonide in this study. Microaggregates were prepared by denaturation of human serum albumin at boiling temperature, and then extracted from the resulting solution after a triplicate of centrifugations and washings. The concentration of soluble albumin residue present in the supernatant after a triplicate of centrifugations was determined by the Biuret assay. The absorbance of the supernatant at 540 nm was found to be -0.010 on average (Table 1). It could be assumed that there was no albumin residue present in the microaggregates products using this test.

<table>
<thead>
<tr>
<th></th>
<th>Standard HSA 2.5mg/mL</th>
<th>Standard HSA 5.0mg/mL</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance I</td>
<td>+0.051</td>
<td>+0.094</td>
<td>-0.010</td>
</tr>
<tr>
<td>Absorbance II</td>
<td>+0.054</td>
<td>+0.099</td>
<td>-0.010</td>
</tr>
<tr>
<td>Absorbance III</td>
<td>+0.053</td>
<td>+0.099</td>
<td>-0.011</td>
</tr>
<tr>
<td>Average</td>
<td>0.053</td>
<td>+0.097</td>
<td>-0.010</td>
</tr>
</tbody>
</table>

Table 1. Absorbance of standard human serum albumin solution in concentrations of 2.5mg/mL and 5.0 mg/mL, and of the supernatant at 540 nm

Human serum albumin microaggregates were radiolabelled with $^{99m}$Tc by using stannous chloride as reductant. The amount of impurity free $^{99m}$Tc pertechnetate was determined by descending paper chromatography using Whatman #1 strip as the stationary phase and 85% methanol as the mobile phase. The radiochromatogram (Figure 5) showed that all of the radioactivity was found staying at the origin of the strip (Rf=0) with no unbound pertechnetate appearing at the solvent front (Rf=1). It was concluded that all of the $^{99m}$Tc present was labelled to microaggregates.
Figure 4. Standard curve of spectrophotometric assay on budesonide extracted from Rhinocort Aq® Assay based on the USP XX Steroid Assay.
Figure 5. Radiochromatogram scan of $^{99m}$Tc-HSA microaggregates.

Figure 6. An example radiochromatogram of unbound, free pertechnetate ($^{99m}$TcO$_4^-$).
Microaggregates prepared by denaturing albumin for various durations of time were passed through a series of Nuclepore membrane filters with 5.0 μm, 3.0 μm and 2.0 μm pore sizes sequentially. The percentage of microaggregates in a particular size range was determined by the amount of radioactivity retained on each membrane filter and passed into the filtrate. The microaggregates obtained after a 4-minute denaturation were chosen as the marker for budesonide in the rest of study because it had the greatest average of 78.82% particles with size in the desire range of 0 to 5 μm (Table 2, Table 3, and Figure 7). The 4-minute denatured ⁹⁹mTc-HSA microaggregates were also found to have a higher percentage of particles within the size range of 2-5 microns compared to that of 3-minute denatured.

<table>
<thead>
<tr>
<th>Particle size (μm)</th>
<th>3-minute thermal denaturation</th>
<th>4-minute thermal denaturation</th>
<th>5-minute thermal denaturation</th>
<th>6-minute thermal denaturation</th>
<th>7-minute thermal denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>21.65</td>
<td>8.11</td>
<td>0.22</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2-3</td>
<td>38.24</td>
<td>24.54</td>
<td>23.88</td>
<td>16.09</td>
<td>23.07</td>
</tr>
<tr>
<td>3-5</td>
<td>18.46</td>
<td>46.17</td>
<td>51.34</td>
<td>56.75</td>
<td>51.25</td>
</tr>
<tr>
<td>&gt; or = 5</td>
<td>21.65</td>
<td>21.18</td>
<td>24.56</td>
<td>27.15</td>
<td>25.68</td>
</tr>
</tbody>
</table>

Table 2. Performing serial filtrations on the ⁹⁹mTc-HSA microaggregates, which were prepared by various durations of thermal denaturation, using Nuclepore Polycarbonate membrane filters.
Table 3. The percentage of four-minute denatured microaggregates at a particular size range was determined by the amount of radioactivity retained on each membrane filter and passed into the filtrate.

<table>
<thead>
<tr>
<th>Particle size (microns)</th>
<th>Trial #1 (%)</th>
<th>Trial #2 (%)</th>
<th>Trial #3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>9.48</td>
<td>7.81</td>
<td>7.04</td>
</tr>
<tr>
<td>2-3</td>
<td>33.98</td>
<td>12.36</td>
<td>27.28</td>
</tr>
<tr>
<td>3-5</td>
<td>34.89</td>
<td>56.88</td>
<td>46.74</td>
</tr>
<tr>
<td>&gt; or = 5</td>
<td>21.65</td>
<td>22.95</td>
<td>18.94</td>
</tr>
</tbody>
</table>

The aerodynamic size of aerosolized budesonide in distilled water was determined by sampling the nebulized solution using the Aerosizer. Figure 8 (Run 21&22), which was provided by the Data Acquisition Analysis computer, showed that 50% of the aerosolized budesonide droplets were smaller than 10.34 μm in size. About 30 seconds after sampling, 50% of aerosolized particles remained in the Aero-Sampler chamber were detected to be smaller than 2.099 μm in size (Figure 8). A drop in aerodynamic size indicated that the actual sizing study of budesonide particles could be performed once the water content inside the aerosols was evaporated.

In addition, human serum albumin microaggregates prepared by denaturing albumin for various durations of time in a boiling water bath were delivered into the Aerosizer for aerodynamic diameter investigation (Figure 9). The aerodynamic size ranges of these microaggregates were found to increase in size gradually when the denaturation times were increased from 3 minutes to 7 minutes. HSA microaggregates, which were prepared from the 4 minute denaturation, possessed an aerodynamic size range most similar to that of the budesonide particles (Figure 10). Fifty percent of dried, aerosolized microaggregates were determined to be smaller than 2.040 μm. Other
microaggregate preparations with different size ranges, illustrated in this experiment, could be used to simulate other pharmaceuticals in distribution and deposition studies.

The Andersen Impactor is a useful apparatus for determining the *in-vitro* assessment of the drug mass or radioactivity distribution among the various aerodynamic size ranges of the aerosols. The MMAD (mass median aerodynamic diameter), the aerodynamic size of the disperse phase between or above which 50% of the drug dose or radioactivity resides was determined in this experiment. The MMAD of aerosolized $^{99m}$Tc microaggregates in saline was found to be about 4.40 $\mu$m (Figure 11). However, it is important to realize that the droplet with a MMAD greater than 4 $\mu$m may contain more than one particle of $^{99m}$Tc microaggregate (the median diameter of dried microaggregate was found to be 2.040 microns in the Aerosizer experiment).

The MMAD of Pulmicort aerosols obtained by the Andersen Impactor was about 9 microns (Figure 12). The non-medical ingredients present in the Pulmicort such as citric acid, disodium edetate, polysorbate 80 and sodium citrate, may contribute to a larger droplet size. As a result a premixed solution of $^{99m}$Tc microaggregates in saline and Pulmicort Nebuamp was delivered through the Andersen Impactor for a more comparable sizing analysis.

The $^{99m}$Tc microaggregate droplet was found to have a similar MMAD in average to the budesonide droplet after passing the premixed $^{99m}$Tc microaggregates and Pulmicort aerosols through the Andersen Impactor three times(Figure 13 & 14). The amount of radioactivity and budesonide collected on the three sets of collecting plates were analyzed by using gamma camera and HPLC assay respectively. In the Aerosizer sizing analysis, the MMAD of the microaggregate was determined to be smaller than that of budesonide particle. This would explain the higher probability of $^{99m}$Tc microaggregate getting into the aerodynamic size range of 0 - 5 microns in the Andersen
A versatile shear diffusion cell was used to determine the rate of diffusion of budesonide and $^{99m}$Tc microaggregate in the Rhinocort Aq® placebo vehicle in the same experiment by performing independent analytical techniques (Table 4). The results of these analysis were compared in two ways. First, the ratio between diffusion coefficients of $^{99m}$Tc microaggregates and budesonide particles was found to be relatively constant at $3.74 \pm 1.95$. Second, using linear regression, the best straight line describing the relationship between diffusion coefficient of $^{99m}$Tc microaggregate and that of budesonide particles was determined to be $y = 0.418x - 2.05$ (Figure 15). The microaggregates were found to diffuse in a faster rate in the vehicle and to have greater diffusion coefficients, compared to that of the budesonide particles. This observation could be explained by the fact that microaggregates had a smaller aerodynamic size distribution. The turbulence initiated in the solution and solvent interfaces when rotating the Teflon blocks, could also explain the variance found in some of the factors.

A three-dimensional image of a human serum albumin microaggregate obtained using a laser confocal scanning microscope is provided in Figure 16. The particle appears to be spherical and to have a diameter of approximately $2 \mu m$. 

Figure 7. A size distribution graph of three preparations of four-minute denatured human serum albumin microaggregates.
Figure 8. The aerodynamic size data of aerosolized budesonide droplets (obtained immediately after sampling into the Aerosizer Runs 21 & 22), and of dried budesonide particles (determined 30 seconds after sampling Runs 23 - 28) were displayed as a volume distribution plot. Fifty percent of the aerosolized budesonide droplets were smaller than 10.34 μm in aerodynamic size. About 30 seconds after sampling, 50% of aerosolized particles remained in the Aero-Sampler chamber were detected to be smaller than 2.099 μm in size.
Figure 9. A plot of distribution against aerodynamic diameter of HSA microaggregates prepared by denaturing the albumin at boiling temperature for different periods of time.

50% of the aerosolized 3 minute denatured HSA microaggregates were smaller than 1.910 \( \mu \text{m} \) in size, 50% of the 4 minute denatured HSA microaggregates were smaller than 2.040 \( \mu \text{m} \) in size, 50% of the 5 minute denatured HSA microaggregates were smaller than 2.728 \( \mu \text{m} \) in size, 50% of the 6 minute denatured HSA microaggregates were smaller than 2.722 \( \mu \text{m} \) in size, 50% of the 7 minute denatured HSA microaggregates were smaller than 2.861 \( \mu \text{m} \) in size.

Run 51&52 = 3 minute denaturation microaggregates, Run 47&48 = 4 minute denaturation microaggregates, Run 43&44 = 5 minute denaturation microaggregates, Run 39&40 = 6 minute denaturation, and Run 35&36 = 7 minute denaturation microaggregates.
Figure 10. HSA microaggregates, which were prepared from the 4 minute denaturation, possessed an aerodynamic size range similar to that of budesonide particles in the volume distribution plot. Runs 1&2, 3&4, 5&6, 7&8 demonstrated the aerodynamic size distributions of the dried and "cold" human serum albumin microaggregates. The size distribution of budesonide particles was displayed by Run 9&10.
Figure 11. Aerodynamic size distribution of aerosolized $^{99m}$Tc-HSA microaggregates was determined using the Andersen Impactor.
Figure 12. Aerodynamic size distribution of aerosolized Pulmicort (0.5mg/mL budesonide) was determined using the Andersen Impactor. Aerosolized Pulmicort droplets with aerodynamic size greater than 10 μm are not included in this plot.
Figure 13. Aerodynamic size distributions of aerosolized $^{99m}$Tc microaggregate droplets and Pulmicort droplets were obtained by passing a premixed $^{99m}$Tc microaggregates and Pulmicort solution through the Andersen Impactor for three times.
Figure 14. Aerodynamic size distributions of aerosolized $^{99m}$Tc microaggregate droplets and Pulmicort droplets (an average result of trial A, B, and C from Figure 13) were obtained by passing a premixed $^{99m}$Tc microaggregates and Pulmicort solution through the Andersen Impactor.

Aerosolized $^{99m}$Tc microaggregates droplets and Pulmicort droplets with aerodynamic size greater than 10 μm are not included in this plot.
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<th>Diffusion time (minutes)</th>
<th>Diffusion coefficient of Tc-99m microaggregates D(Tc-99m MIA)</th>
<th>Diffusion coefficient of budesonide D(budesonide)</th>
<th>Ratio of D(Tc-99m MIA) to D(budesonide)</th>
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Table 4. A comparison of $^{99m}$Tc labelled human serum albumin microaggregates with that of budesonide was performed in a vehicle with viscosity close to Rhinocort Aq.
Figure 15. Correlation between the diffusion coefficients of $^{99m}$Tc microaggregates and that of budesonide was determined to be $y = 0.418x - 2.05$ using linear regression.
Figure 15. A three-dimensional image of human serum albumin microaggregate was obtained by using laser confocal scanning microscope (Bio-Rad 600). The white scale line in the picture is 2 microns in length.
4.0 Discussion:

Medicinal preparations are administered into the nasal cavity as topical treatments for nasal conditions. The efficacy of intranasal drug delivery system is influenced by the deposition site and the subsequent rate of clearance \(^{19, 20, 22}\). Radiolabelling techniques have been widely used in intranasal deposition and clearance studies because they are non-invasive, sensitive, and provide useful \textit{in vivo} data on about the delivery of drug to the target area \(^{19, 20}\). In some studies, \(^{99}\text{Tc}\) pertechnetate ion solution alone had been added to an intranasal formulation in an attempt to trace the deposition of a drug substance, even though it was not chemically linked to it \(^{20, 26}\). However, it is important to realize that the relatively small \(^{99}\text{Tc}\) pertechnetate species may not be cleared from the site of deposition in the same manner as the drug substance \(^{20}\). In a similar manner, \(^{99}\text{Tc}\) labelled human serum albumin \(^{21}\) had also been added to the aqueous nasal spray formulation for deposition and subsequent clearance studies but again subsequent scans displayed only the mucociliary clearance of the \(^{99}\text{Tc}\) - human serum albumin marker rather than the clearance of the drug itself. \(^{99}\text{Tc}\) labelled solid particles would be the most efficacious substitute for the drug particles in the study, but it was necessary to reproduce the particle size distribution and if possible the biodistribution characteristics of the drug particle found in any formulation being studied. In order to attempt to address this challenge, biodegradable, easily prepared \(^{99}\text{Tc}\) radiolabelled human serum albumin microaggregates, having a similar particle size distribution to a parent pharmaceutical (budesonide in Rhinocort Aq), were manufactured to simulate the deposition and clearance. Human serum albumin microaggregates with a narrow size range of 2 - 5 microns were prepared by thermally denaturing human serum albumin in a boiling water bath and recovered after several centrifugations.
Human serum albumin microaggregates were successfully labelled with $^{99m}$Tc. The labelling efficiency was greater than 99% leaving no unbound $^{99m}$Tc pertechnetate in the product. (Figure 5 and 6). After performing the serial filtrations with Nuclepore Polycarbonate membrane filters, an average of 78.32% $^{99m}$Tc microaggregates obtained from the four minute thermal denaturation were found to have an optimum particle size range of 0 - 5 microns (Table 3). The parent pharmaceutical budesonide was reported by Astra Draco to have a particle size range of 0 - 5 microns. We observed a variability in the percentage of microaggregates in the range of 2 - 3 microns (Figure 7). This inconsistency in size distribution could probably be explained by the fact that a large proportion of the $^{99m}$Tc labelled microaggregates might be around 3 microns in size. These microaggregates could barely pass into the filtrate or stay on the filter. The aerodynamic size of "cold" microaggregates were then determined by the Aerosizer and a 50% of aerosolized microaggregates were determined to be smaller than 2.040 microns in size. This finding was confirmed by the three-dimensional laser confocal scanning microscopy performed on the "cold" microaggregates (Figure 15). The Aerosizing analysis also showed that 50% of dried, aerosolized microaggregates were smaller than 2.099 microns. Microaggregates prepared after thermal denaturation in a time frame of 3 to 7 minutes, were also analyzed by the Aerosizer. The result demonstrated that there was a gradual increase in size of microaggregates as the denaturation time was increased (Figure 9). Microaggregates with different particle size distributions could be prepared to simulate other drug particles in deposition and clearance studies by carefully controlling the condition and duration of thermal denaturation. The mass median aerodynamic diameter (MMAD) of aerosolized droplets containing $^{99m}$Tc microaggregates, generated by the PARI LL nebulizer system, was found to be 4.40 microns using
the Andersen Impactor sizing analysis. When a mixture of $^{99m}$Tc microaggregates and budesonide in Pulmicort aerosols was delivered through the Andersen Impactor, the $^{99m}$Tc microaggregates droplets were found to have a similar MMAD as the Pulmicort droplet. The Andersen analysis was used as a biopharmacological assessment of $^{99m}$Tc microaggregates and budesonide in vitro by providing quantitative distribution among the various aerodynamic size ranges of the aerosols. According to the above sizing analysis, the human serum albumin microaggregates are predicted to be successful in simulating the parent pharmaceutical budesonide in an in vivo deposition study using either a spray or a nebulizer.

A free diffusion study was performed using a versatile shear cell to compare the rate of diffusion of $^{99m}$Tc microaggregates and budesonide in a viscous placebo vehicle. A relative ratio of 3.74 (Table 4) in average was noted between the diffusion coefficient of $^{99m}$Tc microaggregates and budesonide particles. The results of a future in vivo clearance study using $^{99m}$Tc microaggregates as the radioactive indicator, should be adjusted in order to obtain an accurate prediction for the clearance rate of the parent pharmaceutical.

Limitations of Particle Size Analysis

Serial filtration through Nuclepore polycarbonate membrane filters were performed on $^{99m}$Tc labelled microaggregates in order to select a batch of microaggregates in a size range similar to that of budesonide particles. The major problem associated with this technique are the possible presence of non-uniform apertures and the "blinding" of the openings by oversized and irregular particles. Dilution of the sample minimised this problem. Nevertheless, the correlation with the other sizing techniques was very close.

The aerodynamic size of aerosolized microaggregate or budesonide was determined to allow
a prediction of its deposition efficiency and clearance rate in the nostril. The two main categories of aerodynamic measurements are based on an optical method using microscopy or light scattering, and on inertial impaction. The Aerosizer, a single particle counter, measured aerodynamic diameter by determining the aerosolized particles' velocities as they cross the laser beams. This optical sizing system measured aerodynamic diameter of the particles directly and rapidly with a high resolution. Another advantage of using the Aerosizer is that only a single puff is needed for each sampling rather than multiple puffs required in most impactors. The detection system of using two photomultipliers monitoring two laser beams and thus obtaining independent start and stop pulses, coupled with cross-correlation techniques, permit a high concentration of up to 100,000 particles per second to be sampled without diluting. However, only part of the aerosol will be sampled as only a fraction of the aerosol is allowed into the laser beam area from the inlet nozzle. Furthermore, the components of the aerosol cannot be differentiated. A particle containing no drug but only surfactant cannot be distinguished from a drug-containing droplet scattering the same amount of light. This limitation was not associated with this experiment since the aerosolized droplet contained only budesonide or microaggregates suspended in distilled water. The assumption that all particles are spherical in shape in the Aerosizer set-up can lead to significant errors when sampling irregularly shaped particles with this system. Particle deformation is another problem as the airflow in the Aerosizer operates at near sonic velocity. Large budesonide or microaggregate particles might undergo fracturing at the nozzle exit due to the drag forces generated by the accelerating air stream.

A cascade impactor was used as a biopharmaceutical assessment tool by separating aerosolized budesonide or microaggregate droplets according to their aerodynamic size. The
aerodynamic diameter of an aerosolized droplet, which determines its deposition location in the respiratory system, will be useful as a prediction of the pharmacological effect of an intranasal delivery system. The resolution of an impactor will depend upon the number of stages in the cascade impactor. The Andersen used in this study provides an advantage of sampling the entire spray from an aerosolized delivery system. The impactor stages allow the analysis of multiple tracers in the spray. After passing a premixed solution of $^{99m}$Tc-HSA microaggregates in 0.5 mg/mL Pulmicort through the Andersen, the amount of budesonide deposited on each stage could be analyzed chemically by HPLC while the quantity of microaggregates collected could be determined by the level of radioactivity on each stage. However, the size distribution of aerosolized droplets measured through the impactor may be underestimated due to a blow-off of droplets from upper to lower stages especially if too much sample is presented. A reduction of material collected on each stage may also result from the loss of sample onto the walls and inter-stage surfaces in the impactor. Since the stages were analyzed rather than weighed in this investigation, small empty droplets containing only surfactant could not contribute any error to the final size distribution.

A versatile shear cell was employed to determine the ratio of diffusion coefficient of $^{99m}$Tc human serum albumin microaggregates to that of budesonide in a viscous Rhinocort Aq placebo vehicle. The diffusion study was simple to perform and could be operated on any stable laboratory desk. The diffusion runs were not time consuming with the HPLC analysis for budesonide being the most tedious task. The mechanical design of the diffusion cell was relatively simple and could be manufactured in standard workshop. Only trace amounts of budesonide ($10^{-2}$ µg/mL) and small quantity of $^{99m}$Tc human serum albumin microaggregates (cpm) were required by the sensitive HPLC analysis and the gamma counter respectively. The diffusion coefficients of budesonide and $^{99m}$Tc
human serum albumin microaggregates could be determined in the same experiment as independent analytical techniques were devised. However, air bubbles might be entrapped during the rotation of Teflon blocks especially when the "solvent" and "solution" containers contained a viscous vehicle. An experimental error may have resulted since a sharp interface could not be created between the "solution" and "solvent" compartments. Mechanical vibrations can be eliminated during boundary-forming process in future diffusion studies by mounting the cell onto a fixed U-bar support.

The laser confocal scanning microscope (Bio-Rad 600) was used in this study to obtain a quantitative, non-invasive three-dimensional image of HSA microaggregates, virtually free from out-of-focus blur. Three-dimensional structural information of an object can be obtained from the confocal microscopy with the object being present in an aqueous environment. In contrast, a specimen needs to be chemically fixed, dehydrated, embedded and finally sectioned during the preparation for electron microscopy. The laser confocal scanning microscope does not allow a real-time imaging of moving objects because it requires a substantial time to capture a complete field. Therefore the microaggregates' images were easily obtained after the droplet specimen had almost been dried out and the microaggregates had stopped moving. The confocal scanning microscope has a limitation on the depth of inspection by the penetration of the light beam and the physical working distance of the objective lens.

Future Directions

Gamma-scintigraphic deposition and clearance studies in animals like rabbits using $^{99m}$Tc microaggregates could be carried out to predict the performance of budesonide in vivo. However, only the early images of $^{99m}$Tc-HSA microaggregates could be used to predict what will be occurring with the budesonide droplets in the nostril. $^{99m}$Tc human serum albumin microaggregates would stay
intact in the nasal secretions and finally be cleared away by mucociliary action. In contrast, budesonide can be dissolved in the mucus layer and diffuse into the circulation. The gamma scintigraphy will be combined with conventional pharmacokinetic assessment in order to obtain a more complete picture of clinical efficacy of a formulation e.g. providing insight into the minimal amount of deposited drug required to produce the maximal clinical effect. The combination of gamma scintigraphy and pharmacokinetic data will be especially useful if the intranasal delivery system is designed for drugs that act systemically. The in vivo gamma scintigraphy will also be valuable in future product development. A series of scintigraphic studies can be performed using an existing delivery device, and the results used to make improvements in device design and in drug delivery optimization.

By carefully controlling the condition and duration of thermal denaturation, microaggregates with different particle size distributions may be prepared to simulate other drug particles in intranasal deposition and clearance studies. Like human serum albumin microspheres and macroaggregates, microaggregates could also be widely used as biomedical diagnostic agents and as part of controlled-release and targeted drug delivery systems.
5.0 Conclusions

1. Thermal denatured $^{99m}$Tc human serum albumin microaggregates with a narrow particle size distribution of 2 to 5 microns were prepared in this study.

2. The aerodynamic particle and aerosolized droplet sizes of human serum albumin microaggregates were determined by serial filtrations, the Aerosizer and the Andersen Impactor, and were found to be similar to that of budesonide. According to the sampling data from the Aerosizer, fifty percent of dried, cold aerosolized microaggregates were determined to be smaller than 2.040 μm in aerodynamic size, compared to 2.099 μm of the budesonide particles. After passing the premixed $^{99m}$Tc microaggregates and Pulmicort® through the Andersen Impactor, the MMAD of $^{99m}$Tc microaggregate droplets was found to be approximately 3.9 μm, compared to 4.2 μm of the Pulmicort® droplets.

3. The human serum albumin microaggregate particles are spherical and may be visualized using the laser confocal scanning microscope.

4. The diffusion coefficient of the $^{99m}$Tc human serum albumin microaggregates was 3-4 times higher than that of the budesonide particles in a viscous placebo vehicle.

5. The $^{99m}$Tc labelled human serum albumin microaggregates may be used to simulate the initial nasal or pulmonary deposition of the parent pharmaceutical.
References:


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38. Sundelof A. A versatile shear cell for diffusion measurements on small sample volumes.


