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Montelukast nasal spray: Formulation development and *in vitro* evaluation

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Abstract:

Objectives: The aim of this study was to evaluate potential development of montelukast sodium (MTS) as a nasal spray.

Materials and methods: The formulations were prepared using hydroxypropyl cellulose (HPC) and carbomer 940 (C940). The prepared formulations were evaluated for clarity, pH, hydrodynamic size, zeta potential, viscosity, contact angle, surface tension, droplet size distribution, muco-adhesiveness, drug release and stability. The suitable formulations were also assessed for their effects on nasal epithelial cells.

Results and discussion: At room temperature (25°C), the formulation containing 0.01% w/v C940 exhibited suitable viscosity and rheological properties. Spray droplets were in ranges of 10-40 µm, which are suitable for nasal administration. The works of adhesion of the formulations containing HPC or C940 were in the range of 0.8 – 4.0 and 0.2 – 27 µJ, respectively. In addition, the formulation containing 0.01% w/v C940 and the 0.5–15µg/50µL concentrations of MTS showed no signs of cytotoxicity and did not open the tight junction of nasal epithelial cells.

Conclusion: The formulated MTS nasal spray is ideal for nasal administration for the treatment of allergic rhinitis. The formulation containing 0.01% w/v C940 had no toxicity nor alteration on the tight junction of nasal epithelial cells.

Keywords: Montelukast, Allergic rhinitis, Nasal sprays, Carbomer 940, Hydroxypropyl cellulose

1. Introduction

Montelukast sodium (MTS) is a leukotriene receptor antagonist (LTRA) that binds with high affinity and selectivity to the CysLT₁ receptor. It is widely prescribed for upper and lower respiratory diseases as well as for the relief of symptoms of allergic rhinitis and the treatment and prophylaxis of asthma attacks. MTS is available in various oral dosage forms such as tablets, chewable tablets, and oral granules. In the management of allergic or perennial rhinitis, it is suggested as 10 mg oral administration once daily (Knorr et al. 2000, Lagos and Marshall 2007, Philip et al. 2009). Although MTS is accepted with a safety profile that is similar in the adult and pediatric populations, MTS has a number of systemic side effects. The side effects of MTS include cough, fever, bronchitis, and adverse effects such as liver dysfunction, agitation, aggression, anxiousness, hallucinations, depression, insomnia, restlessness, and suicidal ideation (Benninger and Waters 2009). Some studies reported reduced systemic side effects of MTS and improved bioavailability by using MTS-loaded nanostructured lipid carriers for oral administration and pulmonary route (Patil-Gadhe et al. 2014, Patil-Gadhe and Pokharkar 2014). However, no study has confirmed the local effects of MTS for allergic rhinitis on nasal epithelial cells. Therefore, a nasal formulation for local delivery in the form of a nasal spray was developed.

The advantages of nasal spray include rapid onset, low dose, low systemic side effects, and avoidance of first-pass metabolism (Illum 2002, Ugwoke et al. 2005, Jadhav et al. 2007). Even though intranasal drug delivery offers many advantages, one of the limitations is the mucociliary clearance mechanism (MCC) (Illum 2002, Ugwoke et al. 2005, Jadhav et al. 2007, Touitou and Illum 2013, Gizurason 2015). The formulation must prolong the residence time within the nasal cavity to enhance the bioavailability of the drug. To achieve this goal, the use of mucoadhesive

polymer-based systems must demonstrate a prolonged contact time with the mucosa. There are some studies that use cellulose derivatives like hydroxypropyl cellulose (HPC) and acrylates copolymer like carbomer940 (C940) as the mucoadhesive in the formulations. These polymers were used in various dosage forms such as powder, liquid and gel for intranasal drug administration. They are widely used in topical formulation drug delivery systems as a mucoadhesive agent because of nontoxic, nonirritant and safe in humans (Chaturvedi et al. 2011). In this work, HPC and C940 were employed as mucoadhesive polymers.

The objective of the study was to prepare MTS nasal sprays using HPC and C940 as the mucoadhesive agents. The formulations were characterized for their physicochemical properties and *in vitro* cytotoxicity on a nasal epithelial cell line as well as the opening of the tight junctions. It was expected that the formulations would not alter the tight junctions and transepithelial electrical resistance (TEER).

2. Materials and Methods

2.1 Chemicals and materials

Montelukast sodium was purchased from Sigma-Aldrich (Singapore). HPC (Klucel[®] HF pharm grade, MW 1,150 kDa) was obtained from Bronson and Jacobs International Co., Ltd. (Bangkok, Thailand). C940 (MW 1,044 kDa) was obtained from Sigma - Aldrich (Shanghai, China). Propylene glycol was purchased from S. Tong Chemicals Co., Ltd. (Bangkok, Thailand). Methanol and ethanol were from RCI Labscan Ltd., (Bangkok, Thailand). Disodium hydrogen orthophosphate dihydrate and triethanolamine (TEA) were obtained from Ajax Finechem Pty Ltd., (Bangkok, Thailand). Orthophosphoric acid was purchased from Merck KGaA (Darmstadt, Germany). Mucin from the porcine stomach type III, bound sialic acid 0.5 – 1.5% was

purchased from Sigma-Aldrich (St. Louis, USA). Eagle's minimum essential medium (EMEM), phosphate buffered saline (PBS) solution, penicillin/streptomycin, and trypsin - EDTA solution were purchased from ATCC[®] (Virginia, USA). Fetal bovine serum (FBS) was purchased from Gibco[®] Thermo Fisher Scientific Inc. (New York, USA). Dialysis membrane (Cellu-Sep[®] H1 molecular cut-off 1 kDa) was obtained from Membrane Filtration Products, Inc. (Texas, USA). Nupore nylon-66 membrane disc filter - type HNN pore size 0.45 μm was from Nupore Filtration Systems Pvt. Ltd. (Ghaziabad, India). Distilled water was immediately filtered through a 0.22 μm cellulose acetate membrane filter before use. All chemicals and reagents were of analytical grade.

2.2 Preparation of the mucoadhesive polymers dispersions

The polymer concentrations were chosen based on the formulations which obtain proper viscosity and droplet size for nasal spray (100-300 mPa.s and 10-100 μm). The increase of the viscosity impacts the droplet size distribution, resulting in altered deposition in the nasal cavity (Yu et al. 2014). Therefore, it is crucial to find and set the correct viscosity for the special application. The polymers were prepared in various concentrations by using one gram of HPC dissolved in 100 mL of distilled water and stirred continuously overnight at room temperature. Thereafter, the HPC solution was diluted to make different formulations (HPC: 0.005, 0.01, 0.05, 0.10, 0.30, and 0.50 %w/v). C940 solution was prepared by dispersing 0.5 g into 100 mL of distilled water to obtain a colloidal dispersion. The C940 colloidal solution was diluted to obtain various concentrations (C940: 0.005, 0.01, 0.05, 0.10, and 0.15 %w/v). After that, they were neutralized with 20 μL /mL of TEA to obtain pH values of 6.0 to 6.5.

2.3 Preparation of the MTS nasal sprays

The dose of MTS was calculated from the pharmacokinetic (Cheng et al. 1996, Zheng 2010) and human nasal anatomy (Wadell 2002, Harkema et al. 2006). The calculated dose to the nasal cavity is 240 ng/dose and 15 µg/dose of MTS to the lung. Due to the dose to the nasal being very small, the dose for lung delivery was chosen for this study. The maximum dose is dependent on the nasal cell toxicity. However the dose estimation may depict variability based on the performance of the device, anatomical and personal variations and the stage of disease.

MTS (3.75 mg) was dissolved in 5 mL of the mixed ethanol-propylene glycol (2:3 volume ratio) at 25°C and pH 7.2 to obtain a clear solution of MTS (Final concentration is 0.0375 %w/v). Afterwards, the MTS solution (5 mL) was slowly added to the mucoadhesive polymer solution (5 mL of either HPC or C940). The mixture was mixed and stirred continuously until a clear solution was obtained.

2.4 Evaluation of physicochemical properties

2.4.1 Clarity Test

The prepared formulations were visually examined for any foreign particles and were discarded if any foreign particles were present (Worthey 1985).

2.4.2 Determination of pH

The pH values of the MTS nasal spray formulations were measured using a pH meter (BP3001, Trans Instruments Ltd., Singapore).

2.4.3 Determination of hydrodynamic particle size and zeta potential

The hydrodynamic particle size and zeta potential of the formulations were measured using the Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK). The hydrodynamic particle size was measured based on the dynamic light scattering technique, while the zeta potential was calculated by determining the electrophoretic

mobility. The measurements were performed at 25°C. Due to knowledge obtained in a preliminary study, it was understood that the hydrodynamic sizes of the MTS, HPC and C940 were smaller than 200 nm. Therefore, filtering formulation with a 0.45µm nylon membrane filter would not impact the hydrodynamic size of the MTS and polymers. Before taking measurements of the hydrodynamic particle size and zeta potential of the samples, all solution samples were diluted 1:10 in volume with distilled water. The samples obtained were then filtered using a nylon membrane filter with a 0.45µm pore size.

2.4.4 Determination of viscosity

The rheological properties and the viscosity of the formulations were assessed using a Brookfield viscometer (Model DV-III, Brookfield Engineering Laboratories, Massachusetts, USA). The spindle sc4-31 was used and the sample volume was 10 ml. The torque should be in the range of 10 – 100% of 0.067 mN.m. Since the viscosity of the nasal sprays was very little, therefore, the rotational speed was adjusted to obtain the 50%torque. The viscosities of the formulations were examined at a rotational speed of 250 rpm at 25°C, while the assessment of the flow behavior was performed at 25°C with a shear rate of 17-85 s⁻¹.

2.4.5 Determination of surface tension and contact angle

The surface tension and contact angle were measured by drop shape analysis using a contact angle meter (OCA 15EC, DataPhysics Instruments GmbH, Filderstadt, Germany) to determine the spreadability of the formulations.

2.4.6 In vitro mucoadhesion testing of the nasal spray formulation

The method of Pardeshi et al. (2016) was modified to measure the mucoadhesion of the formulation systems (Pardeshi and Belgamwar 2016). The mucoadhesive property was measured using a texture analyzer (TA.XT plus Texture

Analyser, Stable Micro Systems, Surrey, England). The load cell was set up with a capacity equivalent to 5 kg. The cylindrical probe (P/0.5R) was utilized in this experiment. The dialysis membrane was previously immersed in 0.5%w/v mucin overnight. The sample solution was kept below the probe. The upper probe was then lowered at a speed of 0.5 mm/s to touch the surface of the solution. A force of 6.0 N was applied for 20 sec, and the probe was lifted upwards at a similar rate (0.5 mm/s). The dialysis membrane wrapped the 1.2 cm diameter probe. The surface area of the exposed dialysis membrane was 1.13 cm². The mucoadhesive force (detachment stress, N) required to detach each polymer compact from membrane was considered as an index of adhesiveness. The area under the curve was calculated by multiplying force and distance resulting in work of adhesion. In the work of adhesion, the greater value was the mucoadhesive property.

2.4.7 Droplet size and distribution analysis

To determine the potential deposition characteristics of the proposed nasal formulations, a multi-dose pump spray delivery system was used to characterize the formulation droplet size distributions using a laser diffraction technique. The Spraytec[®] (Malvern Instrument, Worcestershire, UK) system was used with a 300 mm lens (measuring range 0.1-900 µm). The droplet sizes of the MTS formulations were analyzed at room temperature. The nasal pump was vertically mounted 3 cm away from the laser path, and a vacuum source was mounted anterior to the pump system. Before measurement, 5 doses were discharged to waste. Then 5 puffs were sprayed at two seconds per puff. Data were reported as volume diameters at 10%, 50%, and 90% of the cumulative undersized volumes. Span was calculated using the equation: Span = [(Dv90-Dv10)/Dv50].

2.5 *In vitro* release studies of the MTS nasal sprays

The *in vitro* drug release profile of the formulations was carried out in the Franz diffusion cell (Variomag Telesystem, Thermo Fisher Scientific Inc., Waltham, MA, USA). Three puffs of the MTS formulation was sprayed onto a nylon membrane (1.77 cm², MW cut-off 250 kDa, pore size 0.45 µm) and the membrane was placed interfacing with the dissolution media (12 mL) in the lower chamber of the Franz diffusion cell. The donor compartments were placed on top of the receptor compartments and were clamped tightly. It is expected that the MTS will therefore release into the dissolution medium while the media is continuously stirred with magnetic bar (5 x 2 mm) at the speed of 400 rpm. The dissolution media was adapted from the US Pharmacopeia using 0.5% sodium dodecyl sulfate in water (U.S. Pharmacopeial Convention, 2016). Franz cells were fixed on a preheated water bath (PolyScience, Niles, IL, USA) at 37°C. Syringes were rinsed three times before sampling. One mL sample was withdrawn at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h from the receiver compartment and an equal volume of fresh medium at 37 °C was replaced after each sampling. The samples were analyzed by high-performance liquid chromatography (HPLC) (Thermo Fisher Scientific, San Jose, CA, USA).

2.6 *Stability Assessment*

All formulations were kept at room temperature for three months. The stability determinations were made once a month for three months. The formulations were excluded from further studies if the MTS cannot retain 90% content within the specified period (U.S. Department of Health and Human Services, 1990).

2.6.1 *Physical stability evaluation*

Physical stability of the MTS nasal spray formulations was observed for three months. Parameters included color change, drug and other excipient precipitation.

2.6.2 Chemical stability

2.6.2.1 High-performance liquid chromatography

The chemical stability of the formulations was analyzed using HPLC. The separation was done using an octadecylsilane column (C18). The mobile phase consisted of methanol: 10 mM disodium hydrogen orthophosphoric acid 85% (pH 6.5 adjusted with orthophosphoric acid) in 75:25 (v/v) with a flow rate of 1 mL/min at ambient temperature. The UV detector was operated at 285 nm (Ethiraj et al. 2011), and the injection volume was 20 μ L.

2.6.2.2 Mass spectrometry

The aim of this experiment was to investigate the parent drug and the metabolite. A sample of each formulation was dissolved in acetonitrile and 0.05 M aqueous ammonium acetate (adjusted pH to 3.7 with acetic acid) in the ratio of 75:25. The electrospray ionization and MS/MS studies were performed on an LC/MS/MS spectrometer (AB Sciex Instruments, Model API 3200, Ontario, Canada). The negative electrospray MS data were obtained at -4500 V. The MS-MS data were generated with the collision energy ramping from 30 to 60 V in a nitrogen atmosphere (Saravanan et al. 2008).

2.7 Evaluation of toxicity of nasal epithelium cells

2.7.1 Culture of human nasal epithelial cells

The human nasal epithelial cell line (RPMI 2650; ATCC, Virginia, USA) was used for toxicity evaluation. The cells were cultured in complete growth medium (EMEM: Eagle's Minimum Essential Medium) supplemented with 10% fetal bovine serum (FBS), 50 units/mL of penicillin, and 50 units/mL of streptomycin (100 U/mL penicillin/streptomycin). The RPMI 2650 was incubated at 37°C in a fully humidified atmosphere of 5% CO₂ in the air. The medium was changed every day, and the cells

were cultured for one week. When the cells reached 70-80% confluence, they were rinsed with phosphate buffered saline (PBS) solution pH 7.4. The PBS was aspirated, and the cells were detached from the plate by 0.25% trypsin - EDTA solution followed by the addition of fresh culture medium to create a new single cell suspension for further incubation. Detached cells were centrifuged, resuspended, and transferred into a new culture flask (Kreft et al. 2015, Pozzoli et al. 2016).

2.7.2 Determination of RPMI2650 cells viability after incubation with MTS and MTS formulation

Cell number and viability were determined by MTT assay. The cell suspension was diluted to 1×10^5 cells/well and added to a 96-well plate (Corning® Costar, Corning, New York, USA), 100 μ L per well or 10,000 cell per well. The medium was prepared by spraying 20 doses of the MTS solution into a blank well and waiting for 60 min until the solvent completely evaporated. The residue on the well was adjusted with 1 mL of the medium to obtain a final concentration of 300 μ g/mL. Thereafter, the MTS in the medium was diluted to a concentration of MTS 10 to 200 μ g/mL. Other samples were C940 and MTS formulation. After incubation of RPMI2605 cells overnight, 100 μ L of cell culture medium was aspirated from the well and replaced by 100 μ L of samples. Cells without a sample served as a negative control. After incubation for 24 h, methyl thiazol tetrazolium (MTT) assay was performed to evaluate cell viability. Briefly, the cells were treated with 100 μ L of fresh media along with 50 μ L of MTT solution and incubated at 37°C under 5% CO₂ for 4 h. Thereafter, media containing MTT was removed, and 100 μ L of dimethyl sulfoxide was added. The quantity of formazan (directly proportional to the number of viable cells) was measured by recording the changes in absorbance at 570 nm using a microplate reader (Biohit 830, Biohit Healthcare Ltd, Helsinki, Finland). Viable cells

with an active metabolism would convert MTT into a purple colored formazan product which has an absorbance maximum at 570 nm (Riss et al. 2016). The percentage of cell viability was calculated and compared to a negative control.

2.8 TEER measurement and permeability studies across nasal epithelial cells

2.8.1 TEER measurement

The RPMI 2650 were seeded into a 6 Transwell plate (Corning Costar, Corning, New York, USA) with polycarbonate translucent membrane. The membrane pore size was 0.4 μm , membrane thickness was 10 μm , cell growth area was 4.67 cm^2 and nominal pore density was 1×10^8 pores per cm^2 . The cells were seeded at a density of 1×10^6 cells/well and cultured in EMEM. The cells were incubated at 5% CO_2 at 37°C for 1 week with the culture medium changed daily. Transepithelial electrical resistance (TEER) of the cell monolayer was measured using the epithelial voltohmmeter (Merck KGaA, Darmstadt, Germany) every day for 1 week (Kreft et al. 2015). When the TEER value was constant for 2 days, the experiment was performed by adding 2.5 mL of the medium to the basolateral compartment. 2 mL of either medium with MTS solution, polymeric solution or suitable formulation to the apical compartment. The medium with those samples was similarly prepared by spraying 40 doses of the MTS solution, polymeric solution or suitable formulation onto the blank well and waited for 60 min until the solvent completely evaporated. The residue on the well was adjusted with 2 mL of the EMEM. The cells were then incubated, and the TEER measurement was performed every 3 h for 24 h in a laminar airflow hood. A sample solution (500 μL) from the basolateral compartment was taken and replaced with 500 μL of cell culture medium every 3 h followed by incubation. The sampling solutions were analyzed to determine the MTS by the HPLC.

2.8.2 Sample preparation for HPLC analysis

The sample solution from section 2.8.1 was subjected to liquid-liquid extraction. MTS was extracted from the sample (500 μ L) with 1 mL ethyl acetate and the solution was vortexed for 10 sec. After that the clear solution was pipetted. Nitrogen gas was used to evaporate the ethyl acetate and the final volume was adjusted to 2 mL with the mobile phase. The quantity of MTS was analyzed by HPLC

2.9. Statistical analysis

All experiments were performed in triplicate and the data were represented as mean $\pm$ SD. Statistical analysis was done using a Student's t-test with GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com) and a significance level (*p-value*) of <0.05 was considered statistically significant.

3. Results

3.1 Improvement of MTS solubility

MTS is an acidic drug and is categorized as BCS class II (Okumu et al. 2008). The dielectric constant of MTS is 13. To dissolve MTS clearly, ethanol and propylene glycol were used as cosolvents. Moreover, the advantage of propylene glycol is that it acts as a preservative in concentrations of 15-30% (Rowe et al. 2006). All formulations of the prepared nasal sprays were inspected visually with black and white backgrounds. The formulations were clear without any foreign particles.

3.2 pH of the MTS nasal sprays

The pH of the HPC and C940 formulations are shown in Table 2. The pH of all formulations were in the range of 6.5-7.5 since the suitable pH is 4.5-7.5 (Bitter et al. 2011). The pH of the C940 formulations were significantly lower than the HPC

formulations ($p < 0.05$) because the C940 formulations were neutralized using TEA to obtain a proper viscosity of the carbomer (Rowe et al. 2006). Nevertheless, the pH of the formulations with C940 could be controlled in a suitable range for the nasal spray.

3.3 Viscosity of the MTS nasal sprays

The flow behavior of the formulations with 0.05% HPC depicted a linear relation between shear stress (N/m^2) and shear rate (s^{-1}) and categorized as “Newtonian” flow (Figure 1). However, a formulation containing C940 behaved like a plastic flow where at initial there was a yield stress. At a particular temperature, the samples of both polymers had a constant viscosity for both shear rates.

The viscosities of the HPC and C940 formulations were in the range of 6 – 320 and 6 – 120 mP.s, respectively (Table 1). The viscosity of the formulation with HPC depended only on the concentration of the polymer. The acceptable limit is 3 – 6 mP.s (Menaka and Pandey 2014). Therefore, the formulations with acceptable viscosities were HPC 5, HPC 10, HPC 50, C940 5, and C940 10.

3.4 Particle hydrodynamic size and zeta potential of the MTS nasal sprays

The hydrodynamic size of the MTS without the polymer was 53 nm with a polydispersity index of 0.126 indicating a monodisperse system. The polymer formulations gave larger hydrodynamic sizes compared to the MTS particle. The HPC formulations had a slightly smaller particle size than the C940, but at the same concentrations, they were not significantly different (Table 1).

The zeta potential of the MTS without polymer was -28.5 mV. The zeta potentials of the HPC formulations were in the range of -25 to -3 mV. The more negative zeta potentials were found in the formulations which contained lower quantities of HPC. Moreover, the zeta potentials of the samples of HPC formulations were different from the C940 formulations at the same concentrations (Table 1). The

zeta potentials of the C940 formulations ranged from -45 to -30 mV and they were more negative than -30 mV in the higher C940 concentrations.

3.5 Surface tension and contact angle of the MTS nasal sprays

The spreadability of the polymer solutions was observed as it could increase the contact area in the nasal cavity. The contact angle of the solution without the polymers was 67°, whereas the solutions containing the 0.005-0.05% HPC or C940 had a contact angle in a range of 0-30° and they were categorized as having good spreadability (Yuan and Lee 2013). Other solutions containing concentrations of 0.15%, 0.30% and 0.50% polymers gave contact angles in the range of 31-90°. Therefore, adding polymer in the formulations gave a better spreadability than the solution without the polymers. The degree of contact angle was slightly different between the samples of HPC formulations and C940 formulations at the same concentrations (Table 1). The surface tension of the solution without the polymers was 35.48 mN·m⁻¹. Table 1 shows the surface tension of all formulations. The cohesive forces of the formulations were dependent on the polymer concentration. A greater surface tension resulted in a greater contact angle but with less spreadability.

3.6 Droplet size and distribution of the MTS nasal sprays

The droplet size of the nasal spray gradually increased with the increased polymer concentration. The droplet diameter obtained from the formulations with C940 were significantly smaller than the HPC formulations ($p < 0.05$) when compared with the same concentration. To ensure retention of the drug within the nasal passages, the cut-off points for the droplet sizes should be greater than 10 µm but less than 100 µm (Aurora 2002, Ghorri et al. 2015). The formulations with 0.005% and 0.01% w/v HPC and the formulations with 0.005%, 0.01%, 0.05% and 0.10% w/v C940 demonstrated sizes in the range of the criteria (Table 2). It was observed that the

size of the droplets relied on the contact angle of the formulation. The higher polymer concentrations provided greater cohesive forces which gave larger droplet sizes.

3.7 Texture analysis of the nasal spray formulation

Table 3 shows the adhesive forces between the formulations and mucin as a work of adhesion. Overall, the results showed that the work of adhesion of the formulations with mucoadhesive polymers gave dramatically higher adhesions than the solution without polymer. However, there were no significant differences between each formulation on the work of adhesion.

3.8 Stability of the MTS nasal sprays

Optimized formulations were prepared and kept in clear bottles. It was observed that HPC precipitated at temperatures above 40°C. Therefore, the stability test of all formulations was carried out at room temperature for three months. All formulations were stored at 25°C. After one month, the solutions precipitated as yellow crystals. The precipitation occurred in all samples of the solution containing HPC. Similar circumstances also occurred in the samples containing C940 and even the zeta potential was less than -30 mV. Surprisingly, no precipitation occurred in the formulation containing 0.01% w/v C940. Table 4 shows the % drug content in all of the formulations. The formulation containing 0.01% w/v C940 had a drug content of 95.50% on average. Therefore, a lower concentrated formulation could produce an appropriate zeta potential for the hydrodynamic size in the proper viscous media. In conclusion, only the formulation of 0.01% w/v C940 demonstrated good stability when stored at 25°C for three months. In addition, the yellow crystals were further analyzed by MS-MS spectrometry using 1 µg/mL of MTS as a standard. Figure 2 shows the MTS spectra. The molecular weight was about 584 Da and the highest peak was the yellow crystal peak which was 584 Da but some other peaks were degradation

products of MTS, such as the molecular weight of 600 Da which was montelukast sulfoxide (Al Omari et al. 2007, Saravanan et al. 2008). This degradation may occur from the oxidation of MTS in the solution. From overall physicochemical properties evaluation, the formulation with 0.01% w/v C940 had good physicochemical properties and was also stable. Therefore, this formulation was chosen for further investigation including MTS release profile and *in vitro* evaluation.

3.9 *In vitro* drug release of the MTS nasal sprays

Figure 3 shows the release profiles of the film from the solution without the polymers. The nasal spray formulations with 0.01% w/v HPC and 0.01% w/v C940 were stored at 37°C for 24 h in 0.5% sodium dodecyl sulfate (Okumu et al. 2008). The release rate of the film without the polymers and the solution with 0.01% w/v HPC gradually increased until it reached the maximum at 24 h, while the MTS formulation with 0.01% w/v C940 released continuously until the maximum concentration achieved at 3 h. The % cumulative drug release was then obtained as 44.6, 41.8 and 50.1, respectively. Indicating that the MTS with 0.01% w/v C940 would have provided rapid and highly efficient delivery of the MTS into the nasal cavity. The amount of MTS delivery is expected to be controllable by the application of metered dose nasal spray bottle.

3.10 *Effect of MTS nasal spray formulation on the nasal epithelial cells*

In this study, RPMI 2650 line was used as a model of the progressive formation of the cellular toxicity, the effect of formulation on the tight junction and drug permeation. Figure 4 shows the viability of the different concentrations of MTS on the nasal epithelial cells and the formulation with 0.01% w/v C940. The results showed that the cellular viability of the samples was comparable to the control (95-109%). Therefore, the MTS 15 µg/dose and the formulation with 0.01% w/v C940

were chosen to monitor the alteration of transepithelial electrical resistance (TEER) of the nasal epithelial cells. Before the experiment was performed, the TEER value was constant around $515 \Omega \cdot \text{cm}^2$ for two days. Therefore this TEER value was assumed as 100% TEER. Figure 5 provides the %TEER of the RPMI 2650 cell monolayers incubated with different samples including the control, the polymeric solution, MTS solution, and the formulation as a function of time. The %TEER value of all samples from 0 h to 24 h were significantly higher than the control ($p < 0.05$) which indicated no interference of the formulation on the tight junction. The permeation of the MTS was evaluated by using the apparent permeability coefficient (P_{app}). The P_{app} was calculated from the following equation (Blaser 2007).

$$P_{app} = \frac{V_R * d_{CR}}{d_t * A * C_{D0}}$$

Where P_{app} is the apparent permeability coefficient, V_R the volume of the receiver chamber, A is the area of the permeability barrier, C_{D0} the initial drug concentration in the donor chamber, and d_{CR}/dt the change in concentration of compound in receiver compartment over time.

Only a small amount of MTS permeation could be detected in the basolateral compartment. Figure 6. shows the apparent permeability (P_{app}) of the MTS solution which was calculated from the equation above (Blaser 2007) at 3 h was 3.73×10^{-6} cm/s but after 3 and 6 h later, the P_{app} decreased to 0.43×10^{-6} cm/s and 0.49×10^{-6} cm/s, respectively. After medium replacement at 12 h, P_{app} of the MTS solution was raised to 2.65×10^{-6} cm/s and P_{app} decreased to less than 2×10^{-6} cm/s until 24 h. For the formulation with 0.01% w/v C940, the P_{app} at 3 h was 1.79×10^{-6} cm/s and was decreased to 0.74×10^{-6} cm/s and 0.73×10^{-6} cm/s at 6 and 9 h, respectively. The P_{app} raised again to 1.79×10^{-6} cm/s at 12 h which was the time of medium replacement.

When the P_{app} was less than 2×10^{-6} cm/s, it was an indication of the low permeability but if the P_{app} was between 2×10^{-6} cm/s and 20×10^{-6} cm/s, it was an indication for medium permeability but if it was more than 20×10^{-6} cm/s, it indicated high permeability. However, the P_{app} of formulation with 0.01% w/v C940 from 3 to 12 h was less than 2×10^{-6} cm/s. Therefore, using the 0.01% w/v C940 in the formulation showed less drug permeation into the basolateral compartment compared to MTS without the C940. The addition of C940 in the formulation showed significantly slower drug permeation into the basolateral compartment compared to MTS without the C940 ($p < 0.05$). It was concluded that using the C940 would retain the drug at the nasal epithelial cell longer than without the C940.

4. Discussion

The factors of nasal absorption and the evaluation of nasal sprays consists of many formulation parameters. The factors that affect nasal absorption were carefully considered. The first factor is the anatomy and physiology of the nasal cavity which is related to membrane transport, deposition, enzymatic degradation, and the mucociliary clearance mechanism. The second factor involves the physicochemical properties of the drug including size, molecular weight, solubility, lipophilicity (log P), charge, pKa, polymorphism, chemical state, and physical state. The third factor includes the formulation parameters, for example, the suitable pH for nasal sprays is 4 – 7.5 because the pH range of the human nasal mucosa is 5.5 – 6.5 which is slightly acidic (England et al. 1999, Washington et al. 2000). The adjustment of the pH is to avoid nasal irritation and obtain drug absorption. Because drugs are absorbed well in unionized forms and to prevent the growth of pathogenic bacteria in the nasal cavity, the lysozymes found in nasal secretions are destroyed by certain bacteria at an acidic

pH, whereas in the alkaline conditions lysozymes are not activated, and the nasal tissue is susceptible to microbial infection (Behl et al. 1998, Patil et al. 2012). The osmolarity of the formulation should be 200 to 500 mOsm/kg. The drug concentration should not exceed 5 mg/dose. The viscosity should be in the range of 3-6 cP. Finally, the applied volume of the solution should be 25 – 200 μ L per nostril (Illum 2002, Jadhav et al. 2007, Bitter et al. 2011, Menaka and Pandey 2014). In this study, the physicochemical properties of the formulations were dependent on the polymer concentrations. The sizes of the particles in the formulation solutions were in the range of nanometers. Since the drug could dissolve in the co-solvent, the detected particles were colloids of polymer which covered the drug in the formulation. The zeta potential measurement was used to predict the dispersion stability. The particles showed stable dispersion and a stable system with less chance of aggregation or precipitation when the particles carried more negative charges (-30 mV) or positive charges (+30 mV) (Salopek et al. 1992). MTS is an acidic drug, which has a negative charge after dissolution. Moreover, ethanol and propylene glycol also show negative charges as well. The zeta potentials of the HPC formulations tended to be higher as the concentrations of the polymer increased. This indicated that since HPC is a nonionic polymer, the drug molecules were covered by the polymer leading to a decrease in the zeta potential compared with the solution without polymer (Figure 7a) (Rowe et al. 2006). On the other hand, the C940 is an anionic polymer. The polymer had covered the MTS and promoted the negative charge of the system. Its zeta potential was lower than -30 mV when the concentration of C940 was higher (Figure 7b) (Rowe et al. 2006). Therefore, the formulations with C940 had more impulsive interaction between each particle than the HPC formulations. In brief, the solution containing C940 was found to be more stable with a lower chance of precipitation

than the HPC formulations according to their zeta potential. The contact angles of formulations HPC 5 – 50 and C940 5 – 50 were between 20 and 30 degrees on a cellulose membrane. This range indicates potentially effective wettability. The cellulose membrane was used to mimic human skin in contact angles measurement. The surface tensions of the formulations were low, suggesting good spreadability when applied to the nasal epithelial cells (Zheng 2010). Moreover, easy spraying and providing fine droplets could be obtained in the formulation that had less surface tension and cohesive force (Zheng 2010). The formulation which had a low polymer concentration obtained good spreadability and increased surface area for drug absorption on the nasal epithelial cell. The viscosity of the formulations was raised when the polymer concentrations increased. The advantages from the viscosity of the polymer include a prolonged residence time of the drug in the nasal cavity for sustained release of the drug. A greater viscosity impacts the droplet size distribution which results in an altered deposition in the nasal cavity. However, the viscosity of the MTS formulations prepared was still in the range that could easily be sprayed. Mucoadhesion is defined as a state where one biological material adheres to another material to prolong the contact time. The work of adhesion rose gradually as the concentration of polymers in solution increased. Since the HPC and C940 have hydroxyl and carboxyl groups, respectively, these chemical structures adhered to the tissue by using the net result of the forces including hydrogen bonding, hydrophobic bonding and van der Waal's forces between two surfaces. Mucoadhesion may be affected by a number of factors, including hydrophilicity, molecular weight, cross-linking, swelling, pH, and the concentration of the polymer (Rahamatullah et al. 2011). MTS is sensitive to light and the pH of the solution. The major photodegradation product of MTS is the formation of its cis-isomer, whereas the

major degradation product from an acidic solution is montelukast sulfoxide (Al Omari et al. 2007). Therefore, MTS formulations should be kept in closed containers and protected from light. The TEER can be used as an indicator of the alternation of tight junctions. The TEER steadily increased with the density of the cells until day 7 when the TEER value was $122 \times 4.2 \text{ cm}^2$ or $515 \Omega \cdot \text{cm}^2$ (1×10^6 cells/well seeding). This trend is similar to previously published data (Pozzoli et al. 2016). The formulation with 0.01% w/v C940 was chosen in this study because it passed the stability exam with the suitable physicochemical properties required for the nasal spray. When the TEER decreases it means the tight junction opened (Kreft et al. 2015), but if the TEER value is the same as or higher than the control group, it indicates the tight junction did not open. However, some MTS could pass from the apical to the basolateral chamber of nasal epithelial cells (Srinivasan et al. 2015).

5. Conclusion

In general, the route of localized nasal drug delivery can reduce the amount of drugs entering the human body and also avoids systemic adverse effects of the drug. According to this work, the formulation with 0.01% w/v C940 was found to be an optimal MTS nasal spray comprised of MTS 0.0375% w/v as an active ingredient, 0.01% w/v C940 as a mucoadhesive polymer, propylene glycol as preservative and co-solvent, ethanol as a cosolvent. In addition, the selected formulation demonstrated suitable physicochemical properties as well as proper adhesiveness and an appropriate drug release profile. In other words, it was safe to nasal epithelial cells and did not alter the opening of the tight junction. It can be concluded that this MTS spray can be an effective candidate for nasal administration to obtain local drug delivery.

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Conflict of interest

The authors report no declarations of interest.

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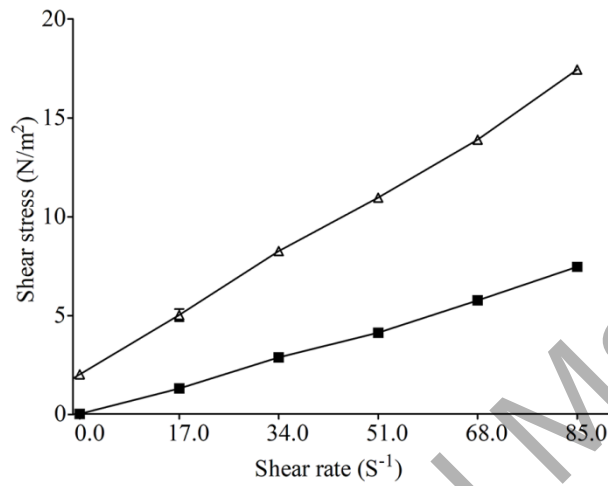
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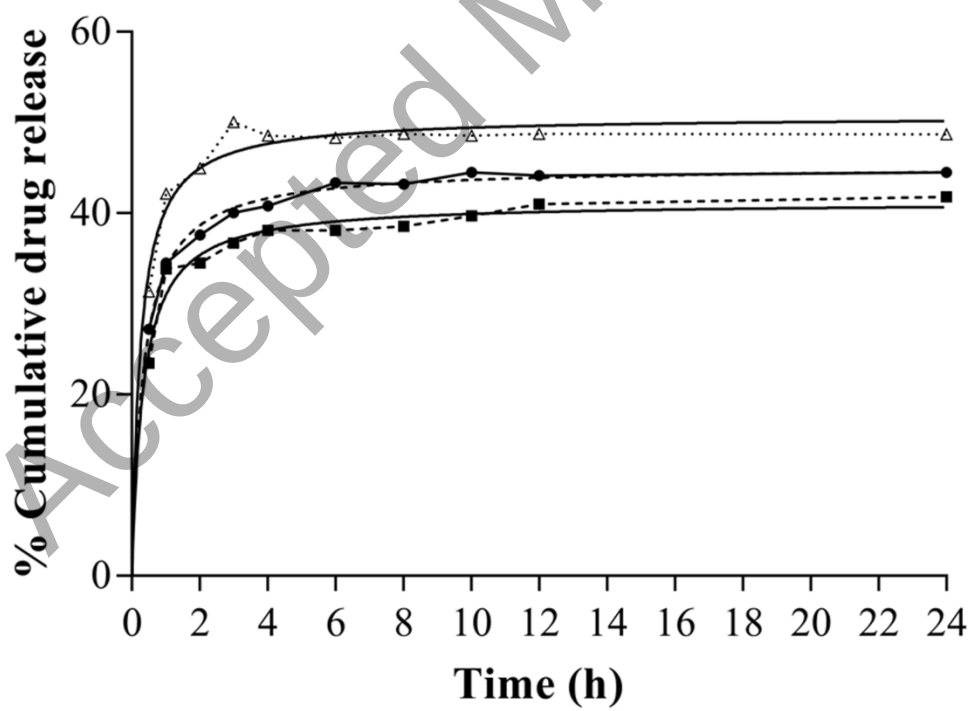
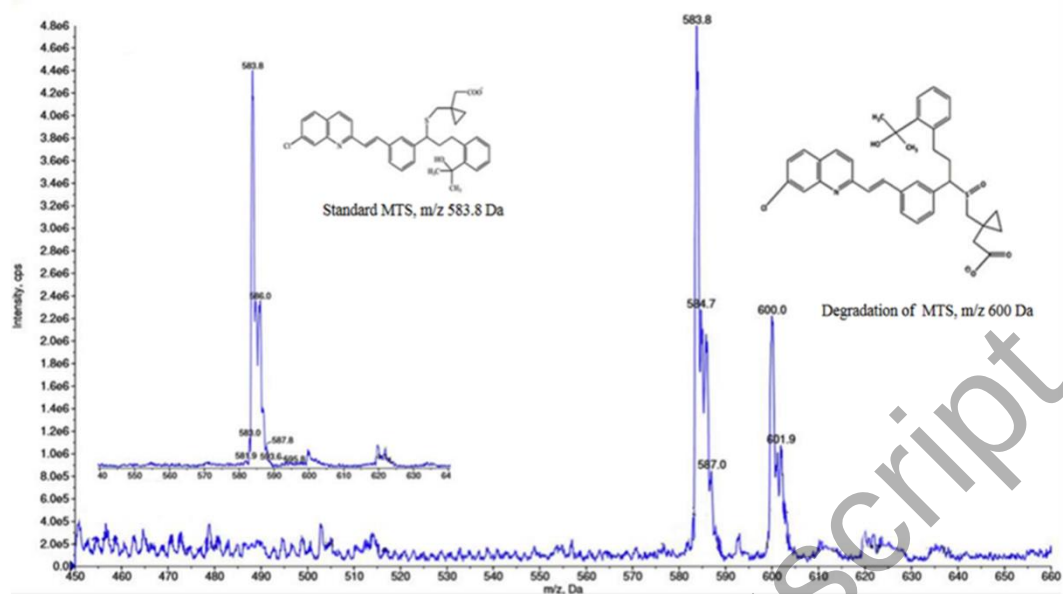
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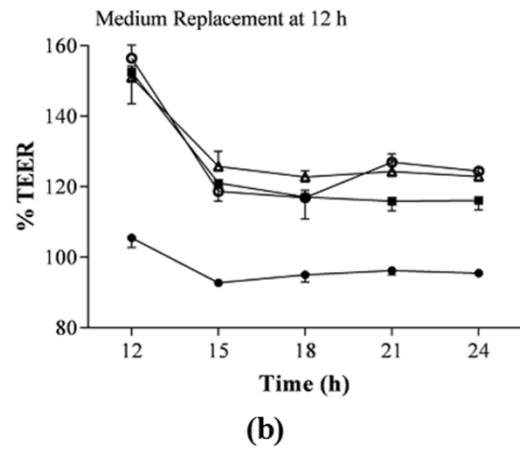
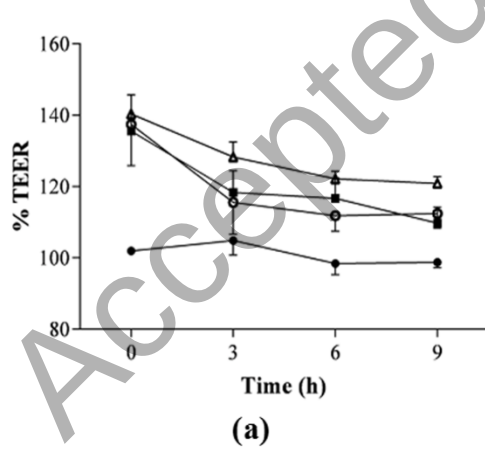
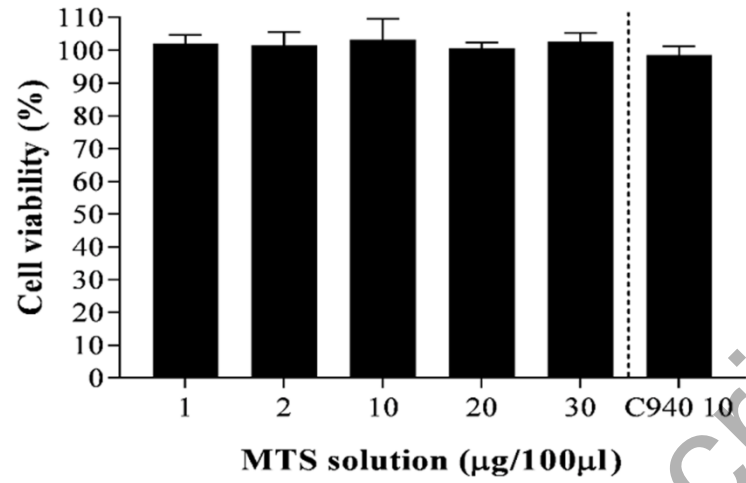
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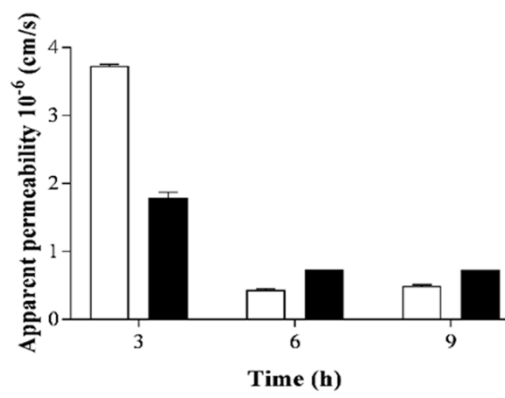
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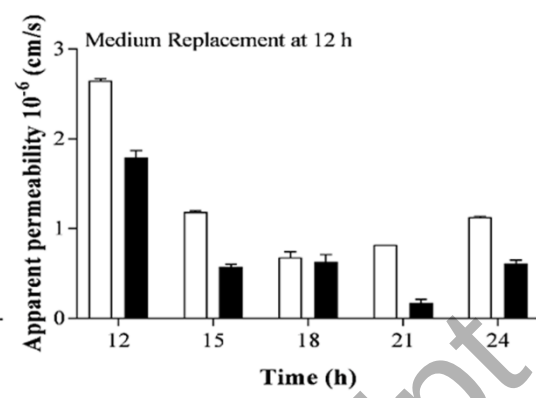




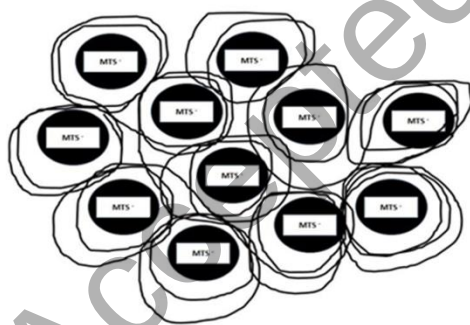




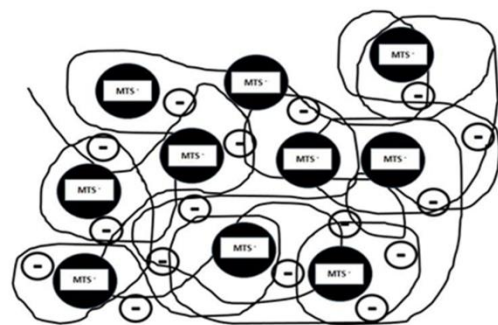
(a)



(b)



(a)



(b)

Table 1: Physical and chemical characterizations of the MTS nasal sprays(mean $\pm$ SD, n=3).

Formulation	pH	Particle size (nm)		Zeta potential (mV)*	Viscosity (cps)	Contact angle (degree)	Surface tension (mN/m)
		Z-average	PDI				
No polymer	7.2 $\pm$ 0.1	53.0	0.1	-28.5	5.7 $\pm$ 0.2	66.9 $\pm$ 1.3	0.5 $\pm$ 0.1
HPC 5	7.5 $\pm$ 0.1	70.6	0.1	-24.6	6.2 $\pm$ 0.0	23.7 $\pm$ 3.7	31.3 $\pm$ 0.7
HPC 10	7.5 $\pm$ 0.0	78.5	0.2	-21.4	6.7 $\pm$ 0.0	24.3 $\pm$ 2.3	32.6 $\pm$ 0.3
HPC 50	7.4 $\pm$ 0.0	80.5	0.4	-9.7	8.0 $\pm$ 0.0	29.0 $\pm$ 4.4	33.1 $\pm$ 0.1
HPC 100	7.4 $\pm$ 0.1	93.2	0.3	-6.3	11.4 $\pm$ 0.1	36.8 $\pm$ 3.1	34.5 $\pm$ 0.1
HPC 300	7.4 $\pm$ 0.0	96.8	0.2	-5.1	46.8 $\pm$ 0.1	42.8 $\pm$ 3.5	35.3 $\pm$ 0.2
HPC 500	7.3 $\pm$ 0.0	100.1	0.4	-3.7	318.7 $\pm$ 1.3	43.5 $\pm$ 4.6	35.4 $\pm$ 0.6
C940 5	6.8 $\pm$ 0.0	84.3	0.2	-32.4	6.8 $\pm$ 0.0	17.6 $\pm$ 1.8	33.2 $\pm$ 0.5
C940 10	6.7 $\pm$ 0.0	94.0	0.2	-37.8	7.3 $\pm$ 0.0	20.9 $\pm$ 1.4	34.0 $\pm$ 0.5
C940 50	6.6 $\pm$ 0.1	100.4	0.1	-44.3	18.2 $\pm$ 0.1	27.0 $\pm$ 2.9	34.4 $\pm$ 0.3
C940 100	6.6 $\pm$ 0.0	103.1	0.3	-43.9	115.1 $\pm$ 0.9	31.8 $\pm$ 0.9	35.5 $\pm$ 0.1
C940 150	6.5 $\pm$ 0.0	267.2	0.6	-43.3	NM	34.5 $\pm$ 0.3	37.8 $\pm$ 0.8

* Indicates significant difference between HPC and C940 formulations $p < 0.05$, NM is not measurable.

Table 2. Laser diffraction particle size analysis of montelukast nasal sprays(mean $\pm$ SD, n=3).

Polymer concentration (%w/v)	Diameter (μm)				
	Dv 10	Dv 50	Dv 90	VMD	Span
0	43.5 $\pm$ 4.4	87.7 $\pm$ 4.8	135.3 $\pm$ 0.3	88.8 $\pm$ 3.2	1.1 $\pm$ 0.1
HPC 5	35.4 $\pm$ 1.7	61.7 $\pm$ 0.7	205.7 $\pm$ 0.6	100.9 $\pm$ 1.0	1.2 $\pm$ 0.2
HPC 10	42.1 $\pm$ 1.1	94.7 $\pm$ 1.9	213.2 $\pm$ 0.3	116.5 $\pm$ 0.9	1.7 $\pm$ 0.1
HPC 50	133.7 $\pm$ 2.9	279.8 $\pm$ 0.2	557.2 $\pm$ 2.1	323.5 $\pm$ 1.7	1.2 $\pm$ 0.3
HPC 100	307.7 $\pm$ 2.4	557.5 $\pm$ 1.1	762.6 $\pm$ 1.6	542.6 $\pm$ 2.1	0.8 $\pm$ 0.1
HPC 300	324.8 $\pm$ 0.6	582.3 $\pm$ 1.1	827.3 $\pm$ 1.6	578.1 $\pm$ 1.1	0.5 $\pm$ 0.4
C 940 5	35.0 $\pm$ 0.1	71.8 $\pm$ 0.3	115.4 $\pm$ 0.1	74.1 $\pm$ 0.2	1.1 $\pm$ 0.1
C 940 10	38.8 $\pm$ 2.0	87.8 $\pm$ 0.5	143.0 $\pm$ 0.5	89.8 $\pm$ 1.0	0.9 $\pm$ 0.2
C 940 50	48.9 $\pm$ 2.7	92.2 $\pm$ 2.6	153.0 $\pm$ 0.6	98.0 $\pm$ 2.0	1.1 $\pm$ 0.2
C 940 100	65.8 $\pm$ 2.9	98.6 $\pm$ 1.1	155.8 $\pm$ 1.8	106.7 $\pm$ 1.9	1.1 $\pm$ 0.0
C 940 150	81.8 $\pm$ 0.9	108.7 $\pm$ 0.6	172.5 $\pm$ 1.1	121.0 $\pm$ 0.8	0.9 $\pm$ 0.1

Dv10 is the 10% of the cumulative undersized (volume); Dv50 is the 50% of the cumulative undersized; Dv90 is the 90% of the cumulative undersized; VMD is the volume mean diameter; and Span refers to relative span (Dv90-Dv10)/Dv50.

Table 3. Texture profiles of MTS nasal sprays (mean $\pm$ SD, n=3).

Polymer concentration (%w/v)	Work of adhesion (μ J)	
	Hydroxypropyl cellulose	Carbomer940
0	0.5 ± 0.1	0.5 ± 0.1
0.005	$0.9 \pm 0.1^{\dagger}$	0.2 ± 0.1
0.01	$0.9 \pm 0.1^{\dagger}$	$1.0 \pm 0.2^{\dagger}$
0.05	$1.1 \pm 0.1^{\dagger}$	$3.6 \pm 0.4^{\dagger}$
0.10	$1.3 \pm 0.4^{\dagger}$	$8.1 \pm 0.9^{\dagger}$
0.15	NA	$27.1 \pm 0.8^{\dagger}$
0.30	$1.8 \pm 0.6^{\dagger}$	NA
0.50	$3.9 \pm 0.7^{\dagger}$	NA

[†]Indicates significant difference between the solution without polymers and the formulation with HPC or C940 $p < 0.05$. NA is not applicable.

Table 4. %Drug content stability (mean $\pm$ SD, n=3).

Formulation Month	0	1	2	3
HPC 5	92.7 $\pm$ 1.5	70.4 $\pm$ 0.6	67.3 $\pm$ 0.8	60.9 $\pm$ 0.8
HPC 10	91.9 $\pm$ 0.4	86.39 $\pm$ 0.9	81.4 $\pm$ 0.4	75.5 $\pm$ 0.6
HPC 50	90.3 $\pm$ 0.5	75.7 $\pm$ 0.6	72.3 $\pm$ 2.9	70.6 $\pm$ 1.6
HPC 100	91.7 $\pm$ 0.9	85.9 $\pm$ 0.8	82.9 $\pm$ 3.3	78.5 $\pm$ 2.5
HPC 300	91.1 $\pm$ 1.4	87.7 $\pm$ 1.4	80.0 $\pm$ 2.3	75.1 $\pm$ 1.7
HPC 500	91.8 $\pm$ 0.5	60.6 $\pm$ 0.5	50.7 $\pm$ 0.5	48.6 $\pm$ 0.7
C940 5	97.2 $\pm$ 3.7	93.3 $\pm$ 1.5	91.9 $\pm$ 0.4	89.8 $\pm$ 0.6
C940 10	97.9 $\pm$ 2.9	96.9 $\pm$ 0.8	95.2 $\pm$ 2.9	94.8 $\pm$ 1.8
C940 50	92.7 $\pm$ 2.6	91.8 $\pm$ 1.9	90.6 $\pm$ 2.5	80.7 $\pm$ 1.4
C940 100	86.7 $\pm$ 4.2	80.7 $\pm$ 1.5	74.7 $\pm$ 1.0	69.8 $\pm$ 1.2
C940 150	84.5 $\pm$ 0.1	78.5 $\pm$ 0.8	71.37 $\pm$ 0.18	60.8 $\pm$ 0.4