Original Article

DOI: 10.5582/ddt.2018.01017

D-cycloserine nasal formulation development for anxiety disorders by using polymeric gels

Yeonoh Shin[1,2,§], Rutika Kokate[1,§], Vilas Desai[1], Alok Bhushan[1], Gagan Kaushal[1,*]

[1] *Department of Pharmaceutical Sciences, College of Pharmacy, Thomas Jefferson University, Philadelphia, PA, USA;*
[2] *Pensylvania State University, Old Main, State College, PA, USA.*

Summary **D-cycloserine (DCS), a partial agonist at N-methyl-D-aspartate (NMDA) receptors, is used as an enhancer of exposure therapy for anxiety disorders. The purpose of the present study was to investigate the feasibility of using polymeric gels to increase the viscosity of the formulation and thereby increase the nasal residence time and sustained release of DCS *in vitro*. Hydroxypropyl methylcellulose (HPMC), hydroxypropyl cellulose (HPC), and methyl cellulose (MC) were prepared at concentrations of 0.5 to 5% w/v. Pluronic F-127 (PF-127) was prepared at concentrations of 15 to 35% w/v. pH, viscosity and *in vitro* DCS release behavior of the formulated gels were analyzed. All four gels that were tested, demonstrated sustained DCS release behavior over a 24-hour period, but with different rates. Based on the results of this study, HPMC, HPC, MC, and PF-127 are capable of increasing the viscosity of nasal gel formulations and of releasing DCS in sustained manner. Therefore, these polymeric gels can be suitable carriers for DCS nasal gel formulation.**

Keywords: D-cycloserine, polymeric gels, hydroxypropyl methyl cellulose, hydroxypropyl cellulose, methylcellulose, Pluronic F-127

1. Introduction

Anxiety disorders are the most common mental disorders in America, affecting 15.7 million people each year and more than 30 million people at some point in the course of their lifetimes (*1,2*). Anxiety disorders have a serious impact on the society and health care system. According to data from the National Comorbidity Study (*3*), approximately $42.3 billion per year were spent on anxiety disorders in 1990 in the United States. Additionally, anxiety disorders may cause reduced productivity at work, which can be a burden on the society (*3*).

D-cycloserine (DCS), an antibiotic for tuberculosis, has been tried as an enhancer of exposure therapy for anxiety disorders (*2*) as it was discovered to act as a partial agonist at the glycine modulatory site of the N-methyl-D-aspartate (NMDA) receptor with high affinity for this receptor (*4,5*). DCS has also been used to improve the negative symptoms in schizophrenia (*6,7*), and to facilitate improvements in functional impairments among children with autism (*8*). Moreover, DCS has been used in the treatment of other psychiatric disorders such as acrophobia (*9*), social-phobia (*10*) and obsessive-compulsive disorder (*11,12*).

The availability of the DCS at the NMDA receptor site depends on its dosage and on the levels of the blood/cerebrospinal fluid (CSF). DCS has excellent central bioavailability (*13,14*) and is excreted primarily by the kidneys with a half-life of 9 hours (*15*). For treatment of tuberculosis, 250 mg tablets of DCS are typically used at 500-750 mg daily in chronic dosing (*16*). In contrast, utilization of DCS to enhance exposure treatment in humans has required only 50-500 mg in isolated dosing rather than in chronic dosing (*10*). While the pharmacokinetics of DCS used for treatment of tuberculosis is known, the relationship between pharmacokinetics of DCS and the effect on behavior is not firmly established.

The optimal delivery of drugs to the brain in conditions such as Alzheimer's disease and anxiety disorders is crucial. A drug can be more effective for the treatment of anxiety disorders if it is given direct access

[§]These authors contributed equally to this work.
**Address correspondence to:*
Dr. Gagan Kaushal, Department of Pharmaceutical Sciences, College of Pharmacy, Thomas Jefferson University, Philadelphia PA 19107, USA.
E-mail: gagan.kaushal@jefferson.edu

to the brain through the nasal passage than if it is given in the current capsule-based oral formulation. Nasal delivery could significantly improve onset of action for DCS and reduce the required dose (*17*). For these reasons, the nasal administration method has received lot of attention lately (*18-20*), and it has become clear that a new formulation and delivery system for DCS will have important treatment implications. Because the olfactory receptor cells are in direct contact with both the environment and the central nervous system (CNS), delivering the drug *via* nasal passage allows drugs to bypass the blood brain barrier (BBB) and to be delivered to the CNS directly (*21,22*).

Many researchers have tried to use a nasal delivery system to bypass the BBB since many drugs are not able to cross the BBB (*18-20*). Particularly, interest is shown in nasal delivery of drugs for neurodegenerative diseases such as Alzheimer's disease (*23*) and Parkinson's disease (*24*). Nasal delivery has many advantages besides circumventing the BBB. The nasal epithelium is composed of monolayer of cells with a relatively high permeability (*25*). The nose has a large surface area with numerous microvilli that enhance drug absorption rate, which may provide a rapid onset of action. Additionally, the sub-epithelial layer is highly vascularized and venous blood from the nose does not pass the liver, therefore, it circumvents first-pass metabolism in the liver. Nasal delivery also avoids acid and gastrointestinal enzyme degradation. Consequently, it will require lower doses, have more rapid onset of pharmacological actions, and have fewer side effects (*26,27*). By eliminating the need for systemic delivery, unwanted systemic side effects are reduced (*21,22*). Nasal delivery could be used for targeted drug delivery that releases the drug at or near the intended physiologic site of action. Nasal delivery also could be a strategy for an extended-release or sustained-release drug delivery if the dosage frequency allows at least a two-fold reduction as compared to conventional dosage form (*28*).

One of the limitations of nasal drug delivery is its inadequate nasal drug absorption due to nasal mucociliary clearance. The clearance function of the nose is a protective system against foreign materials such as bacteria and viruses from reaching the lungs and is very important in order to prevent respiratory tract infections (*29*). The mucocilliary clearance function moves noxious substances towards the nasopharynx and the substances are eventually transported into the gastrointestinal tract (*30*). Increasing formulation viscosity with polymeric gels may provide a means of increasing the residence time in the nasal cavity by decreasing the mucociliary clearance. Increasing the residence time in the nasal cavity may prolong the absorption and facilitate the uptake of the drug, therefore providing longer therapeutic effects of nasal preparations (*31*).

In the present study, different concentrations of four polymeric gels; Hydroxypropyl methylcellulose (HPMC), methylcellulose (MC), hydroxypropyl cellulose (HPC), and Pluronic® F-127 (PF-127); that are known to increase viscosities of solutions in concentration dependent manner were prepared and analyzed. We hypothesized that if a polymer (HPMC, HPC, MC, or PF-127) is used as a drug carrier in DCS nasal gel formulation, then the polymer will increase the viscosity of the formulation and facilitate sustained DCS release. The aim of this preliminary study was to screen DCS gel formulations prepared using different polymers for optimal pH, viscosity and *in vitro* release behavior. Overall, the long-term goal of this study was to help evaluate the feasibility of using these polymers for the development of DCS nasal gel formulations in the future.

2. Materials and Methods

2.1. *Materials*

DCS was obtained from Acros Organics™ (NJ, USA). HPMC, MC, and HPC were also purchased from Acros Organics™ (NJ, USA). Pluronic F-127 gel was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). A ServoDyne™ Digital Mixer (Cole-Parmer®, Vernon Hills, IL, USA) was used to mix the powdered polymers into distilled water. Distek dissolution apparatus (Distek, North Brunswick, NJ, USA) and high-performance liquid chromatography (HPLC) (Waters, Milford, MA, USA) were used for the *in vitro* DCS release study. The solvents used were HPLC grade distilled water and phosphate buffer (Fisher Scientific, Hampton, NH, USA). RPMI 2650 cells (ATCC® CCL-30™), Eagle's minimal essential medium (EMEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, and trypsin EDTA solution (1X ATCC® 30-2101™) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Microplate reader used was Epoch 2 Microplate Spectrophotometer, BioTek® (Winooski, VT, USA).

2.2. *Preparation of gels with HPMC, MC, and HPC*

Different concentrations of HPMC, MC, and HPC were prepared. "Hot/Cold technique" was used as these gels are non-Newtonian pseudo-plastics that must be heated first for an even suspension in distilled water (*32*). Then, the gels were dissolved in the distilled water by cooling in an ice bath. Briefly, 400 mL of distilled water was heated on a hot plate until it reached approximately 100°C. The beaker was removed from the hot plate and placed in a rubber ice bucket under a mixing apparatus so that the propeller was submerged but not touching the bottom of the beaker. When the stirring process began, weighed amounts (for 0.5, 1, 2, 3, 4, 5%, w/v) of HPMC, MC, and HPC powders were slowly added and mixed into the distilled water. After 2.5 minutes, ice

was added around the sides of the beaker in the rubber ice bucket. Stirring was continued at 190 rpm until the 20-minute interval had come to completion.

2.3. *Preparation of gels with PF-127*

Pluronic gels were prepared by cold method (*33*) in different concentrations (15, 20, 25, 30, 35%, w/v) and screened to compare effect of different concentration. Briefly, a weighed amount of Pluronic F-127 was slowly added to around 60-70 mL of water (at 10°C) in a beaker with continuous magnetic stirring. Aqueous PF-127 mixture was kept overnight at 4°C, and the final volume was adjusted to 100 mL with deionized water.

2.4. *pH and viscosity*

The pH of polymeric gels was analyzed for 3 weeks using Mettler Toledo pH meter (Columbus, OH, USA). The viscosities of prepared solutions were analyzed with a Brookfield DV-III Ultra Rheometer (Brookfield Engineering Laboratories, Middleboro, MA, USA) using a cone and cup attachment system. The prepared gels were placed in the cup and the spindle used was lowered perpendicular into the sample. The spindle was rotated at constant rpm. The temperature was set at 32°C as the physiological temperature of the nasal mucosa ranges between 32-34°C (*34*). Each concentration (0.5 mL) of HPMC, MC and HPC was analyzed in Brookfield's RheoCalc computer program using the Bingham equation. The unit commonly used is centipoise (cP), where 1 cP = 10^{-2} P = 10^{-3} Pa·s = 1 mPa·s. The viscosity of PF-127 was not measured because it was beyond the limit of the instrument and the spindle available. Few literatures (*35,36*) were selected for the relationship between the concentration of PF-127 and the viscosity.

2.5. *In vitro DCS release behavior study*

In vitro release of DCS from the gels was performed using a Distek dissolution apparatus (Distek, North Brunswick, NJ, USA). The procedure was modified so that it is suitable for the analysis of the gels. Briefly, 15 mL of each gel and 300 mg DCS was added to a 50 mL centrifuge tube with plug seal cap (Corning®, Corning, NY, USA). The tube was wrapped in aluminum foil and left on a Wrist Action Shaker (Burrell, Pittsburgh, PA, USA) overnight in order to ensure uniform mixing. A blue membrane clip shut (Spectrum Laboratories, Rancho Dominguez, CA, USA) was used to clip one side of an 8 cm piece of molecular-porous membrane tubing (Spectra/Por®, Rancho Dominguez, CA, USA) that was soaked in a beaker of phosphate buffered saline (PBS) buffer for 20 minutes. DCS gel (2 mL) was added using a positive displacement pipet (Eppendorf, Westbury, NY, USA) into the membrane and the top of the bag was closed with a piece of string. Then, the attached string was used to tie the bag onto the bottom of the drive assembly of the Distek system. Once the vessels filled with 500 mL of PBS each had equilibrated to water temperature, the assembly drives were lowered into the vessels and locked into place to submerge the membranous sack in 500 mL PBS buffer. The Distek system was turned on and rotated at 50 rpm to mimic flow of body fluids. Samples (2 mL) were collected at predetermined time intervals for up to 24 hours (every 10 minutes for the first half hour, then every 15 minutes for the following half hour, and then every hour after that for a total of 6 hours as well as one 24-hour sample the next day) and analyzed by HPLC (Waters, Milford, MA, USA). Whenever each sample was removed, an equivalent amount of buffer was added. The samples were run on HPLC for 3.5 minutes at 220 nm wavelength, 30°C, 10 µL injection volume and flow rate of 0.5 mL/min. The mobile phase was a 90:10 mixture of a 10 mM sodium phosphate buffer (pH 7.4): acetonitrile. The peak areas were converted to the amount of DCS in mg using the previously calibrated standard curve of DCS. The amount of DCS release from each gel in phosphate buffer was plotted against time to know the release pattern. Based on the data, drug release behavior of polymeric gels was observed.

2.6. *Drug transport assay in Calu-3 cells*

For the drug transport assay Transwells were obtained from Corning Incorporated (Corning, NY). T-75 flasks were obtained from Thermo scientific (Rochester, NY, USA). Dimethyl Sulfoxide (DMSO) was obtained from Sigma (St. Louis, MO, USA). PBS 1X, sterile was used and was obtained from Mediatech, Inc (Manassas, VA, USA). Trypan Blue was obtained from MP Biomedicals, LLC (Solon, OH, USA). Serum-free cell freezing media, Trypsin EDTA, Fetal Bovine Serum, EMEM cell culture medium, and Calu-3 cell line (HTB 55) were all obtained from ATCC (Manassas, VA, USA). DCS was obtained from Research Products International Corp. (Mt. Prospect, Illinois, USA) and was used without any further purification. All chemicals, buffer reagents, and solvents used were of analytical grade and were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). HPLC grade water and acetonitrile were also purchased from Fisher Chemicals (Fair Lawn, NJ, USA) and used throughout this study.

2.7. *In vitro cytotoxicity test in RPMI 2650 cells*

The cell line was grown in the T75 tissue culture flasks at 37°C in 95% air-5% CO_2. Culture medium used was EMEM with 10% FBS (v/v). When cell line was 70-80% confluent the cells were detached with Trypsin-EDTA solution and 100 µL of cells with media were

seeded onto 96-well plate at a final density of 10,000 cells/100 µL per well. After overnight incubation, various concentrations of DCS dissolved in autoclaved distilled water were added to each well to determine the cytotoxicity. The concentrations of DCS used were in the range of 1.25-100 µM. The cells were incubated for 72 hours at 37°C in 5% CO_2. After incubation, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well. The cells were incubated at 37°C for 4 hours. The formazan crystals formed were dissolved in a solubilizing buffer containing 20% SDS and 0.1 N HCl. The plate was left overnight, and absorbance values of the samples were read at wavelength of 570 nm with microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek®, VT, USA). The relative cell viability was calculated from the absorbance values as a percent of untreated cells (control).

2.8. *Cell culture for drug transport assay*

The Calu-3 cell line (HTB-55) purchased from the American Type Cell Culture Collection (ATCC, Rockville, IN) were grown in 75 cm^2 flasks in complete Eagles's minimal essential medium (EMEM) containing 10% (v/v) fetal bovine serum solution and maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Cells were propagated and subcultured according to ATCC recommendations. To establish the air-liquid interface model, cells were seeded onto Transwell polyester inserts at a density of 5×10^5 cells/cm^2 in 1.5 mL apical and 2.6 mL basolateral medium. The apical medium was removed 24 hours after seeding and cells were fed every alternate day with fresh basolateral medium only. The monolayers were allowed to differentiate under air interface feeding conditions over 10-15 days.

2.9. *Transepithelial electrical resistance (TEER) of cell layers*

The transepithelial electrical resistance (TEER) of Calu-3 monolayers was measured over time using a Millicell ERS-2 Epithelial Volt-ohm meter (EMD Millipore Corporation, Billerica, MA) with STX-01 chopstick electrodes. Pre-warmed sterile PBS 1× was added to the apical and basolateral sides of the Calu-3 monolayer. The monolayer was equilibrated for 30 minutes in a humidified atmosphere of 95% air/5% CO_2 at 37°C prior to resistance measurements. TEER was calculated by subtracting the resistance of a blank insert. The resistance of the cell monolayers in each well was measured 7 times between days 2 and 15 of culture.

2.10. *Transport experiments*

Transport experiments were conducted on days 10-15 in culture. Before each experiment, the cells were washed three times with sterile PBS 1×. The apical and basolateral layer were washed with pre-warmed sterile PBS and allowed to equilibrate for 30 minutes at 37°C. After the equilibration, the TEER of the monolayer was checked, and then the entire medium in both compartments was discarded. Fresh pre-warmed sterile PBS was acquired, and 2.6 mL of this were placed in the basolateral compartment and 1.5 mL of a solution of DCS was placed in the apical compartment. A sample of 1 mL was collected from each basolateral compartment at specific time intervals within a 3-hour period: 0.25, 0.5, 1, 2, and 3 hours. After each sampling period, the PBS in the basolateral compartment was discarded and replaced with a fresh 2.6 mL of pre-warmed sterile PBS to each basolateral compartment, and then placed in a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of the entire sampling period, the remaining solution in the apical compartments was also collected for HPLC analysis. The samples were purified by filtration through 0.45 µm membrane filter and transferred to a HPLC vial for analysis. After the collection of the last samples, the TEER of the monolayers was again monitored.

2.11. *HPLC method for DCS analysis*

For the analysis of DCS in unknown samples, a previously published stability-indicating HPLC method developed in our lab for the separation and the detection of DCS was used (*17*). All the chromatographic studies were performed on a Waters Alliance e2696 separations module/2489 UV/Vis detector. The separations were performed on Atlantis T3 5 µm Column (250 × 4.6 mm, Waters, Milford MA, USA). Column effluents were monitored at the wavelength of 220 nm for a run time of 8 minutes at the temperature of 30°C. For the mobile phase, 90% of 10 mM sodium phosphate buffer (pH = 7.5) and 10% acetonitrile was used. The mobile phase was filtered and degassed before use. The flow rate was 0.5 mL/min with the injection volume of 10 µL.

2.12. *Statistical analysis*

Experiments were performed at three different times and means were compared by analysis of variance (ANOVA). Data analysis was performed with Microsoft Excel (Microsoft Corp, Redmond, WA, USA) and a p-value of < 0.05 was considered statistically significant.

3. Results

3.1. *pH*

The pH of gels was found to be in the range of 6.66 (5% HPC) to 7.57 (35% PF-127) (Figure 1). Table 1 shows the pH value of each gel for three weeks. All

polymeric gels showed general trend of stability (The pH difference of ≤ 0.1 from week 1 to week 3) except 5% MC (0.14), 2% HPMC (0.19) and 30% PF-127 (0.11).

3.2. *Viscosity*

Table 2 shows the viscosity profile of HPMC, MC, and HPC polymeric gels at different concentrations. The viscosity of the preparation used in this study ranged between 1.4 cP to 43,000 cP. 4% HPMC showed the highest viscosity (43,000 cP) and 0.5% HPC showed the lowest viscosity (1.4 cP). Viscosity of the 5% HPMC could not be measured because it was beyond the limit of the instrument and spindle available but the viscosity of 5% HPMC was higher than 4% HPMC by visual inspection. Likewise, the viscosity of PF-127 was not measured because of instrument limitations. For the relative comparison to cellulose derivative gels, the data from previously published literature (*35,36*) was used.

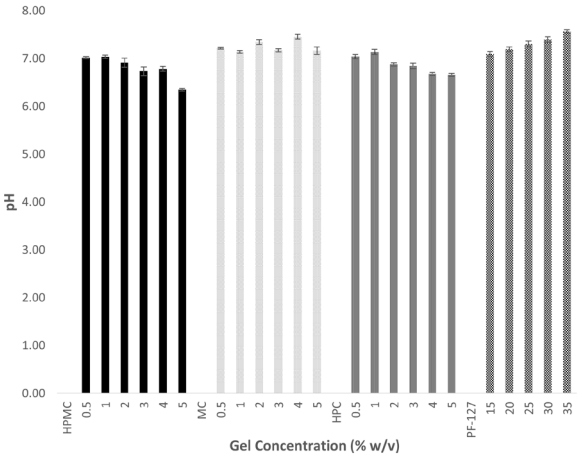

Figure 1. Average pH of Polymeric Gels. The pH of polymeric gels was analyzed using Mettler Toledo pH meter. Data are expressed as mean ± standard deviation (n = 3).

The data and visual inspection clearly indicate that as the concentration of polymer increased, there was an increased viscosity. HPMC, HPC, and MC showed trend of exponential increase in viscosities with an increase in concentration (analyzed by trend line equations). These polymeric gels showed strong correlation between the concentration of gel and the viscosity (R^2 > 0.97-0.99) (Figures 2A-2C). Compared to MC and HPC, the viscosities of HPMC and PF-127 increased relatively much higher as concentration increased. The viscosity of HPMC was in the range of 14.4 cP to 42,296.0 cP for 4% HPMC and it is expected that 5% HPMC would be much higher. The viscosity of MC was in the range of 1.69 cP to 137.5 and HPC was in the range of 1.4 cP to 54.3 cP.

3.3. *Comparison of the effect of different polymeric gels on DCS release*

The drug-release behavior from four different polymeric gels was performed *in vitro* using Distek dissolution apparatus discussed in methods section. All four gels

Table 1. Comparison of pH of gels over 3 weeks

HPMC concentration	Week1	Week2	Week3	AVG pH	ST DEV	CV
0.50%	7.04	7.01	7.00	7.02	0.02	0.00
1.00%	7.01	7.08	7.02	7.04	0.04	0.00
2.00%	7.01	6.91	6.82	6.91	0.10	0.01
3.00%	6.74	6.82	6.64	6.73	0.09	0.01
4.00%	6.83	6.79	6.73	6.78	0.05	0.01
5.00%	6.33	6.38	6.34	6.35	0.03	0.00
MC concentration	Week1	Week2	Week3	AVG pH	ST DEV	CV
0.50%	7.20	7.20	7.23	7.21	0.02	0.00
1.00%	7.11	7.14	7.16	7.14	0.03	0.00
2.00%	7.40	7.32	7.31	7.34	0.05	0.01
3.00%	7.17	7.20	7.14	7.17	0.03	0.00
4.00%	7.52	7.42	7.43	7.46	0.06	0.01
5.00%	7.25	7.12	7.11	7.16	0.08	0.01
HPC concentration	Week1	Week2	Week3	AVG pH	ST DEV	CV
0.50%	7.09	7.01	7.02	7.04	0.04	0.01
1.00%	7.20	7.11	7.10	7.14	0.06	0.01
2.00%	6.91	6.87	6.85	6.88	0.03	0.00
3.00%	6.91	6.82	6.81	6.85	0.06	0.01
4.00%	6.71	6.65	6.67	6.68	0.03	0.00
5.00%	6.69	6.64	6.64	6.66	0.03	0.00
PF - 127 concentration	Week1	Week2	Week3	AVG pH	ST DEV	CV
15.00%	7.15	7.06	7.07	7.09	0.05	0.01
20.00%	7.25	7.17	7.16	7.19	0.05	0.01
25.00%	7.35	7.23	7.33	7.30	0.06	0.01
30.00%	7.45	7.40	7.34	7.40	0.06	0.01
35.00%	7.60	7.56	7.54	7.57	0.03	0.00

Table 2. Comparison of viscosities of polymer gels

Polymeric Gel Type	Concentration (% W/V)	Viscosity (Cp)	Trend line equation	R-squared value
HPMC	0.5	14.4	$y = 8.7768e^{223.24x}$	0.97
	1	94.4		
	2	1,728.3		
	3	7,930.7		
	4	42,296.0		
	5	Not Done		
MC	0.5	1.69	$y = 1.4428e^{94.927x}$	0.98
	1	4.26		
	2	11.53		
	3	29.17		
	4	66.53		
	5	137.5		
HPC	0.5	1.4	$y = 1.108e^{81.172x}$	0.99
	1	2.5		
	2	6.6		
	3	14.8		
	4	29.4		
	5	54.3		

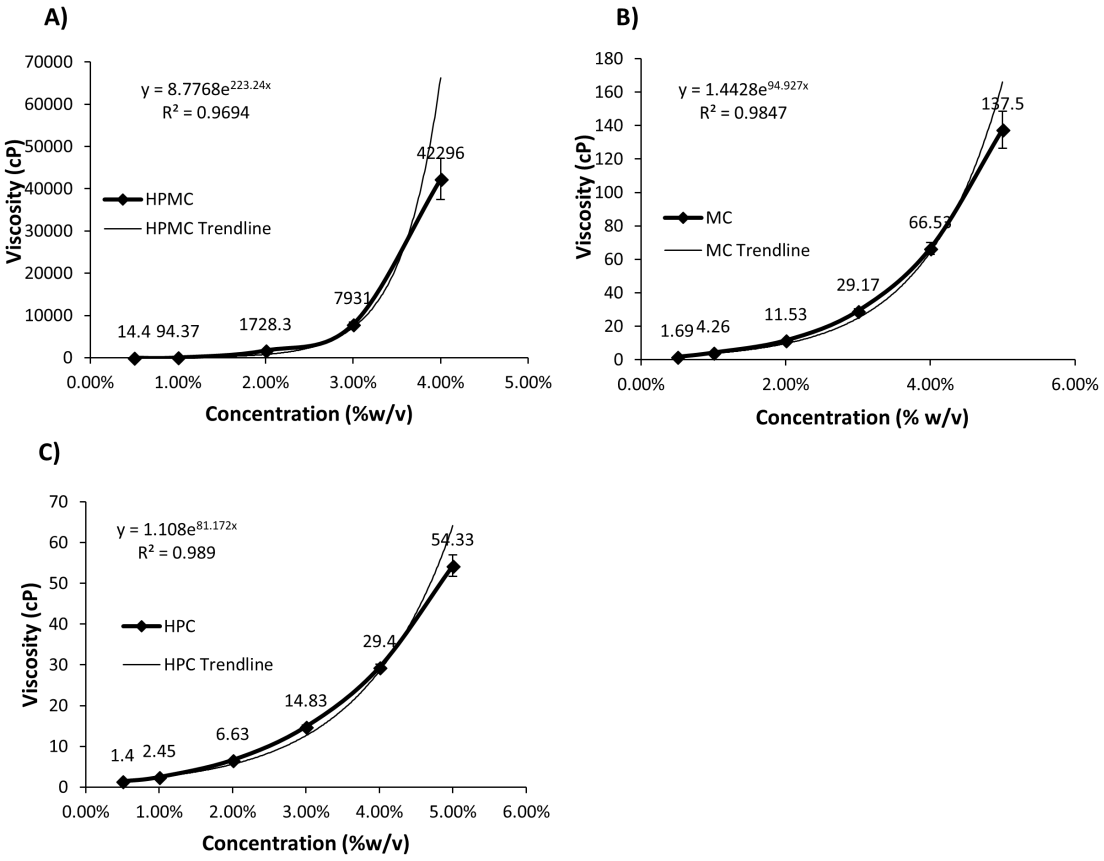

Figure 2. Relationship between the concentration of polymeric gels and the viscosities. The viscosity of polymeric gels was analyzed using the Brookfield DV-III Ultra Rheometer. The data is expressed as a function of concentration of polymeric gels (% w/v) versus the viscosity (cP). Data are expressed as mean ± standard deviation ($n = 3$).

tested showed sustained DCS release over a 24-hour period, but with different rates. All cellulose derivative polymers (HPMC, MC, and HPC) showed the general trend of burst release resulting in greater than 50% of DCS release in the first hour except 5% HPMC, 4% HPC, and 5% HPC. On the other hand, PF-127 could not release 50% of DCS in 24 hours (Figure 3). One percent and 0.5% of HPMC could release > 90% of DCS in two hours. In 24 hours, 99% and 96% of DCS were released from 0.5% and 1% HPMC. Two, 3, and

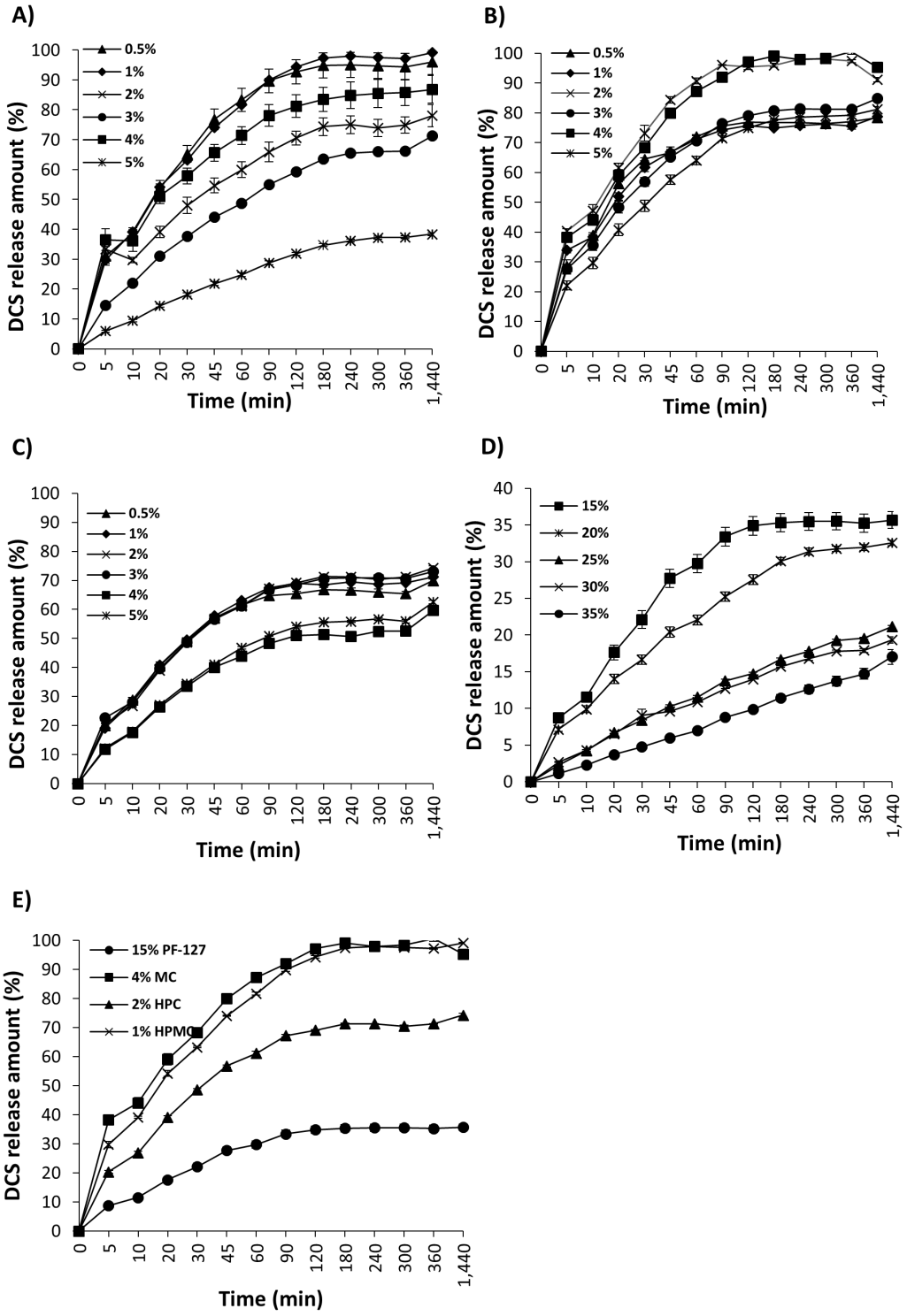

Figure 3. Dissolution of DCS 40 mg/2 mL from polymeric gels. *In vitro* DCS release behavior from **(A)** HPMC, **(B)** MC, **(C)** HPC and **(D)** PF-127 gel was analyzed with the Distek dissolution apparatus and HPLC. (E) Comparison of *in vitro* DCS release behavior of four polymeric gels (HPMC, MC, HPC and PF-127). The data are expressed as a function of time (min) versus cumulative DCS release amount (%). Data are expressed as mean ± standard deviation (n = 3).

4% HPMC were able to release 71, 59, 81% of DCS in two hours and 78, 71, and 87% in 24 hours. Therefore, there was a lack of correlation between the viscosity of HPMC and the drug release behavior. Five percent HPMC significantly retarded drug release to only 32% at 2 hours and 38% at 24 hours. The result may suggest that 5% HPMC is not suitable for the maximum release rate (Figure 3A).

All concentrations of MC gels could release > 50% of DCS at 1 hour and > 75% of DCS at 2 hours. 2 and 4% MC released > 90%, 3 and 5% released > 80%, and 0.5 and 1% > released 78% at 24 hours. Therefore, there was a lack of correlation between the viscosity and drug release behavior for MC as well (Figure 3B).

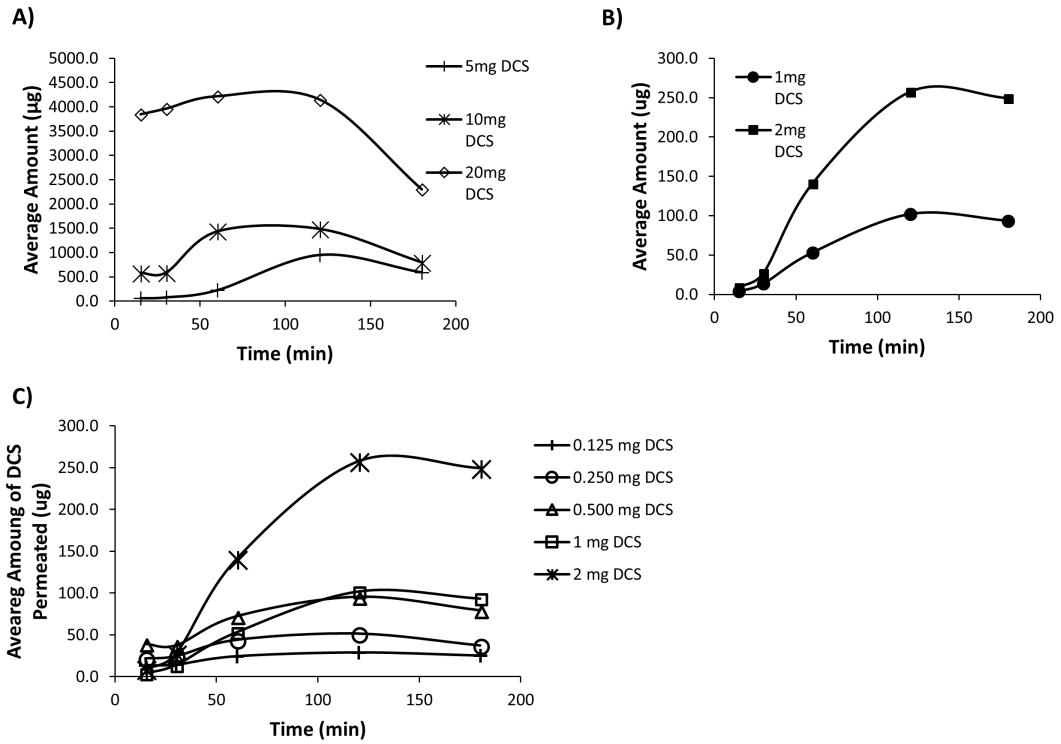

Figure 4. Drug Transport Assay on Calu-3 cells. (A) The analysis of the transport experiment using 5 mg, 10 mg, and 20 mg of DCS. **(B)** The analysis of the transport experiment using 1 mg and 2 mg of DCS. **(C)** The analysis of the transport experiment using 0.125 mg, 0.250 mg, 0.500 mg, 1 mg, and 2 mg of DCS.

The lowest concentration (0.5%), 1%, 2%, 3% of HPC gels could release 50% of DCS at 1 hour. 4% and 5% of HPC gels could release 50% of DCS at 2 hours. However, no HPC gels could release greater than 75% at 24 hours. Among 0.5-5% concentrations of HPC, 2% released the highest concentration (74%) while 4% released the lowest concentration (60%). Again, there was lack of correlation between the viscosity and drug release behavior for HPC gels (Figure 3C).

Unlike other cellulose derivative gels (HPMC, MC, HPC), there was inverse relationship between the concentration of PF-127 gels and DCS release kinetics. As concentration of PF-127 increased, the viscosity increased, and drug release amounts decreased. Moreover, 15% PF-127 was only able to release 36% from initial DCS amount (the highest in PF-127), and 35% PF-127 could release 17% from initial amount of DCS (the lowest in PF-127) (Figure 3D). Figure 3E shows the relative comparison of four polymeric gels with concentrations that showed the highest amount of DCS release. The rank order of the highest to the lowest DCS release profile among them was 1% HPMC > 4% MC > 2% HPC > 15% PF-127. Overall, the rank of polymeric gels that released the highest amount of DCS to the lowest was HPMC > MC > HPC > PF-127.

3.4. *Drug transport assay*

For the drug transport assay, Calu-3 cells which were continuously propagated, grew rapidly and consistently, and were subcultured about once a week. Calu-3 monolayers generally consisted of cuboidal and polygonal cells. Confluent monolayers were generally formed after about 3 days post-seeding when plated at 1×10^5 cells/cm^2 in 6-well cluster plates. Histological staining of cross sections (using a hemocytometer, Bright-Line (Horsham, PA) revealed that Calu-3 cells, when plated at 5×10^5 cells/cm^2, retained a predominant monolayer condition in Transwells.

The chromatogram of DCS standards showed a peak at retention time of 5.6 minutes. A blank sample was also injected to the HPLC system and no peak was observed from this sample. A good linearity was exhibited in the concentration range (1-1,000 µg/mL) by using the presently developed HPLC method. The average coefficient of determination of 0.99 was observed for the standard curve. The slopes of the curves illustrated an excellent agreement with coefficient of variability.

To determine the concentration of DCS that will be used in the study, several concentrations were evaluated in transport experiments: 0.125 mg, 0.250 mg, 0.500 mg, 1.00 mg, 2.00 mg, 5.00 mg, 10.0 mg, and 20.0 mg of DCS were mixed in a 1.5 mL solution. From the Figure 4A, one can see that there is not much of a correlation with the amount of DCS that passed through the monolayer over time. The amount of DCS that passed through the monolayer does however seem to go down after 2 hours of experiment. As a result, lower concentrations of DCS (< 5 mg) were also evaluated.

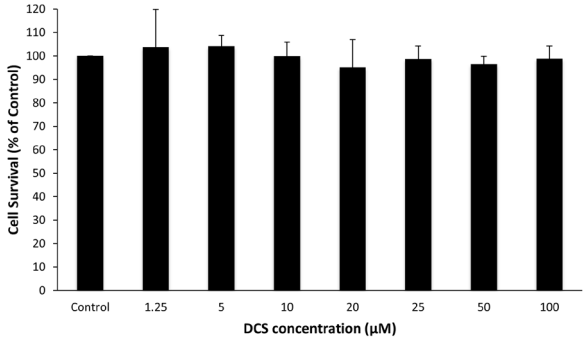

Figure 5. Cytotoxicity of DCS on RPMI 2650 cells. RPMI 2650 cells were treated with 1.25-100 µM concentrations of DCS and the cell viability was determined using the MTT colorimetric assay. Data are expressed as mean ± standard deviation (n = 3).

Figure 4B demonstrates that there is a closer correlation between the amounts of DCS that passed through the monolayer with time. Over the first 2 hours, there is an exponential increase in the amounts of DCS that penetrated through the monolayer, but after two hours, the graph plateaus. However, we further investigated DCS penetration at still lower concentrations (< 1mg). Figure 4C indicates that the lower the concentration the better the correlation between the passage of DCS over time. However, below 1mg of DCS, the correlation starts to deteriorate making 1mg a better choice over other concentrations.

3.5. *In vitro cytotoxicity test in RPMI 2650 cells*

The cytotoxicity of DCS was tested on RPMI 2650 nasal squamous cell carcinoma cells *in vitro*. The cell viability was determined using the MTT colorimetric assay after RPMI 2650 cells were treated with 1.25-100 µM concentrations of DCS. Since four polymeric gels: HPMC, HPC, MC and PF-127 are FDA approved non-toxic polymers, DCS dissolved in distilled water was used alone for the cell viability assay. Figure 5 shows the *in vitro* viability of RPMI 2650 cells after 72-hour treatment with 1.25-100 µM of DCS. Data were compared to the control (cells not treated with DCS) for the relative cell viability. There was no direct relationship between the dose and cell survival. p value of 0.51 indicates no statistically significant result.

4. Discussion

Nasal delivery of DCS, a partial agonist at NMDA receptors, *via* nasal passage could have important applications in many psychiatric disorders such as anxiety disorders (*2,4,5*). However, because DCS is only available in tablets for tuberculosis treatment, which requires higher dosage for longer period than anxiety disorders, the utilization of DCS to enhance exposure treatment in humans, is not firmly established. Besides, the hydrophilic nature of DCS and BBB limits the bioavailability of DCS at NMDA receptors. It could have important implication in anxiety disorders if DCS could be delivered directly into the brain. Nasal delivery system could be an attractive strategy as a means of delivering a drug into the brain due to direct contact of olfactory receptors to both the environment and the CNS. Additionally, the nature of highly vascularized nasal mucosa could provide rapid systemic effect and avoid hepatic first pass metabolism. Delivering DCS through nasal passage with polymeric gels, which bypass crossing BBB, may have advantages in terms of dosage requirements, bioavailability and onset time of action. A major barrier of nasal drug delivery is mucocilliary clearance which leads to low absorption of drugs. Using polymeric gels to prepare DCS nasal formulation could increase viscosity of the formulation, which would increase residence time in the nose thereby facilitating sustained release of DCS (*31*). Prolonging nasal residence time may lead to longer absorption time for the drug to be permeated through nasal mucosa and it could lead to better therapeutic effects. Some polymeric gels are suitable carriers to be used in nasal gel formulation because of their thermoreversible property and biocompatibility. Also, the nasal gel does not require a specialized administration device. In the present study, four polymeric gels with thermoreversible property were selected and screened for the feasibility of using these gels for future development of DCS nasal gel. HPMC, MC, HPC, and PF-127 are polymeric gels that are frequently used as a delivery vehicle for drugs due to their unique characteristics, including thermos-reversibility and safety (*37-41*). In this study, HPMC, MC, and HPC at 0.5-5% w/v and PF-127 at 15-35% w/v were prepared and screened for pH, viscosity and DCS release behavior *in vitro*. Additionally, the permeability and *in vitro* cytotoxicity of DCS in the nasal cells was also evaluated.

pH is a physical parameter that indicates the stability of the products as the change in pH can alter the property of solutions (*42*). "Average baseline human nasal pH is approximately 6.3". It is recommended to keep the final formulation at a pH of 4.5 to 6.5 in order to have the best efficacy (drugs are absorbed in the un-ionized form). The pH of a nasal formulation is also important to avoid irritation of nasal mucosa, prevent growth of pathogenic bacteria, and maintain functionality of excipients such as preservatives (*43*). The pH value ranged from 6.35 to 7.57 and could be easily adjusted with buffer (NaOH and HCl) to maintain proper pH. Furthermore, the prepared gels showed stability over three weeks (Table 1 and Figure 1).

The viscosity of a formulation is directly proportional to the nasal residence time. Ibrahim *et al*. have shown previously, with increasing pluronic concentration, the viscosity increases (*36*). In another study done by El-Kamel, the viscosity of PF-127 gel also increased as the

concentration increased (*35*). All four polymers tested in this study could enhance nasal residence time owing to increased viscosity in a concentration-dependent manner. The exponential increase in viscosity of all gels was observed with the increase in concentration with strong correlation between the concentration of gel and the viscosity (R^2 > 0.97-0.99). Viscosities of the gels ranged between 1.4 cP to 43,000 cP. A relatively large change in viscosity of gels was observed with HPMC and PF-127 compared to HPC and MC (Table 2 and Figure 2). HPMC, HPC, and MC showed exponential increase in viscosities with an increase in concentration and strong correlation between the concentration of gel and the viscosity could be established. Although viscosity of PF-127 could not be measured with the instrument available, other researchers reported that there is a direct relationship between PF-127 concentration and viscosity as well (*35,36*). Viscosities of HPMC and PF-127 were found to be in wider range compared to HPC and MC. This means if we need relatively higher viscosity formulation, we could manipulate HPMC and PF-127 concentrations for future development of DCS nasal gel as long as DCS release behavior is appropriate for anxiety disorders.

Furthermore, these polymeric gels showed that they release DCS in a sustained manner. The release of DCS was almost complete from 1% HPMC, 0.5% HPMC, and 4% MC (> 95%) within 24 hours, while 5% HPMC and PF-127 gels significantly retarded DCS release (< 40%) at 24 hours (Figure 3). In theory, DCS release rate should be decreased as polymeric gel concentrations increase. Overall, our results show that the rate of drug release decreased with increasing PF-127 viscosity (Figure 3D). However, there was a lack of correlation between the rate of drug release and the viscosity of cellulose derivative gels used in this study (Figures 3A-3C). Therefore, complex drug-polymeric gels-water interactions need to be investigated in the future. Currently, there is no defined or optimal viscosity that should be used in nasal formulation. It will depend on the patient's physiological condition. Therefore, the viscosity of the gel may be manipulated for different formulations as desired with the amount of drug that would want to be released to human nasal cells.

The permeability of drug is an important factor for BBB permeation to achieve desired and optimal therapeutic effects (*44*). D-Cycloserine is an ideal drug for intranasal administration as the drug's low molecular weight may provide good absorption of the drug regardless of its hydrophilicity and ionization state (*45*). Also, DCS should be absorbed well through the nasal cavity as DCS shows very good water solubility (*17*). In this project, along with the DCS release behavior of polymeric gels we also conducted *in vitro* permeation studies on Calu-3 cells. A close correlation with an exponential increase (for the first two hours) was observed between the amounts of DCS that passed through the monolayer over time. From Figure 4 we observed that the correlation deteriorates below 1 mg of DCS. However, the space between 2 mg and 1 mg curve was found to be relatively large compared to the space between the 1 mg curve and the lower DCS concentration curves, making the 1 mg concentration a better choice for future studies.

When the nasal formulation is applied to a patient, it should not cause any cell death in nasal cells. The potential toxicity of DCS was tested in RPMI 2650 nasal cells to partially improve the concept of the drug delivery *via* nasal passage. RPMI 2650 cells are derived from squamous cell carcinoma of the human nasal septum, and the cells resemble normal human nasal epithelium (*46*). RPMI 2650 cells are a valid model for an *in vitro* study of nasal drug absorption (*47,48*). No significant cytotoxicity occurred at any concentration between 1.25 µM - 100 µM of DCS in distilled water (p = 0.51). No dose dependent cell death occurred either (Figure 5). Therefore, in conclusion polymeric gels of DCS can be applied safely in nasal cells.

5. Conclusion

The optimal viscosity of the gel that should be used in nasal passage is not defined. Patient's physiological condition, the amount of drug needed, and the implication will define the type and the concentration of polymeric gels. This study was used as an initial screening of the feasibility of using polymeric gels for DCS delivery *via* nasal passage. Based on the overall results of this study and considering the data available in the literature, we firmly believe that HPMC, MC, HPC, and PF-127 could be used in a nasal gel formulation to increase the viscosity and release DCS from formulations in sustained manner at different rates. In the current study, we only looked at DCS release behavior of polymeric gels. In future, we also plan to alter and test the formulation after adding excipients such as buffer (NaOH and HCl) to maintain pH, sodium chloride to adjust osmolarity, and preservatives such as Parabens to prevent microbial growth. Additionally, we also plan to monitor the gelation temperature of the final formulation since it needs to be in the range of 25°C to 37°C to function as a thermoreversible gel. Finally, formulation with minimum concentration of polymeric gel showing acceptable gelation temperature will be selected for the animal studies.

References

1. Lépine JP. The epidemiology of anxiety disorders: Prevalence and societal costs. J Clin Psychiatry. 2002; 63:4-8.
2. Rodrigues H, Figueira I, Lopes A, Gonçalves R, Mendlowicz MV, Coutinho ESF, Ventura P. Does D-cycloserine enhance exposure therapy for anxiety disorders in humans? A meta-analysis. PloS One. 2014;

9:e93519.
3. Greenberg PE, Sisitsky T, Kessler RC, Finkelstein SN, Berndt ER, Davidson JR, Ballenger JC, Fyer AJ. The economic burden of anxiety disorders in the 1990s. J Clin Psychiatry. 1999; 60:427-435.
4. Henderson G, Johnson J, Ascher P. Competitive antagonists and partial agonists at the glycine modulatory site of the mouse N-methyl-D-aspartate receptor. J Physiol. 1990; 430:189-212.
5. Tomek SE, LaCrosse AL, Nemirovsky NE, Olive MF. NMDA receptor modulators in the treatment of drug addiction. Pharmaceuticals. 2013; 6:251-268.
6. Goff DC, Coyle JT. The emerging role of glutamate in the pathophysiology and treatment of schizophrenia. Am J Psychiatry. 2001; 158:1367-1377.
7. Goff DC, Tsai G, Levitt J, Amico E, Manoach D, Schoenfeld DA, Hayden DL, McCarley R, Coyle JT. A placebo-controlled trial of D-cycloserine added to conventional neuroleptics in patients with schizophrenia. Arch Gen Psychiatry. 1999; 56:21-27.
8. Posey DJ, Kem DL, Swiezy NB, Sweeten TL, Wiegand RE, McDougle CJ. A pilot study of D-cycloserine in subjects with autistic disorder. Am J Psychiatry. 2004; 161:2115-2117.
9. Ressler KJ, Rothbaum BO, Tannenbaum L, Anderson P, Graap K, Zimand E, Hodges L, Davis M. Cognitive enhancers as adjuncts to psychotherapy: Use of D-cycloserine in phobic individuals to facilitate extinction of fear. Arch Gen Psychiatry. 2004; 61:1136-1144.
10. Hofmann SG, Meuret AE, Smits JA, Simon NM, Pollack MH, Eisenmenger K, Shiekh M, Otto MW. Augmentation of exposure therapy with D-cycloserine for social anxiety disorder. Arch Gen Psychiatry. 2006; 63:298-304.
11. Chasson GS, Buhlmann U, Tolin DF, Rao SR, Reese HE, Rowley T, Welsh KS, Wilhelm S. Need for speed: Evaluating slopes of OCD recovery in behavior therapy enhanced with D-cycloserine. Behav Res Ther. 2010; 48:675-679.
12. Kushner MG, Kim SW, Donahue C, Thuras P, Adson D, Kotlyar M, McCabe J, Peterson J, Foa EB. D-cycloserine augmented exposure therapy for obsessive-compulsive disorder. Biol Psychiat. 2007; 62:835-838.
13. Conzelman GM Jr. The physiologic disposition of cycloserine in the human subject. Am Rev Tuberc. 1956; 74:739-746.
14. Nair C, Ray A, Singh J. Studies on Nuri strain of P. knowlesi. XI. Comparative studies on quinine and chloroquine administered intravenously. Indian J Malariol. 1956; 10:85-93.
15. Baron H, Epstein IG, Mulinos MG, Nair KG. Absorption, distribution, and excretion of cycloserine in man. Antibiot Annu. 1955; 3:136-140.
16. Crofton J, Chaulet P, Maher D, *et al*. Guidelines for the management of drug-resistant tuberculosis. Geneva: World Health Organization, 1997 (Publication no. WHO/GTB/96.210.).
17. Kaushal G, Ramirez R, Alambo D, Taupradist W, Choksi K, Sirbu C. Initial characterization of D-cycloserine for future formulation development for anxiety disorders. Drug Discov Ther. 2011; 5:253-260.
18. Dahlin M, Bergman U, Jansson B, Björk E, Brittebo E. Transfer of dopamine in the olfactory pathway following nasal administration in mice. Pharm Res. 2000; 17:737-742.
19. Dahlin M, Björk E. Nasal absorption of (S)-UH-301 and its transport into the cerebrospinal fluid of rats. Int J Pharm. 2000; 195:197-205.
20. Jogani VV, Shah PJ, Misra AR, Mishra P, Mishra AK. Nose-to-brain delivery of tacrine. J Pharm Pharmacol. 2007; 59:1199-1205.
21. van Berckel BN, Lipsch C, Timp S, Gispen-de Wied C, Wynne H, Van Ree J, Kahn R. Behavioral and neuroendocrine effects of the partial NMDA agonist D-cycloserine in healthy subjects. Neuropsychopharmacology. 1997; 16:317-324.
22. Wen MM. Olfactory targeting through intranasal delivery of biopharmaceutical drugs to the brain: Current development. Discov Med. 2011; 11:497-503.
23. Qian S, Wong YC, Zuo Z. Development, characterization and application of *in situ* gel systems for intranasal delivery of tacrine. Int J Pharm. 2014; 468:272-282.
24. Md S, Haque S, Fazil M, Kumar M, Baboota S, Sahni JK, Ali J. Optimised nanoformulation of bromocriptine for direct nose-to-brain delivery: Biodistribution, pharmacokinetic and dopamine estimation by ultra-HPLC/mass spectrometry method. Expert Opin Drug Deliv. 2014; 11:827-842.
25. Chaturvedi M, Kumar M, Pathak K. A review on mucoadhesive polymer used in nasal drug delivery system. J Adv Pharm Technol Res. 2011; 2:215-222.
26. Kissel T, Werner U. Nasal delivery of peptides: An *in vitro* cell culture model for the investigation of transport and metabolism in human nasal epithelium. J Control Release. 1998; 53:195-203.
27. Shaikh R, Raj Singh TR, Garland MJ, Woolfson AD, Donnelly RF. Mucoadhesive drug delivery systems. J Pharm Bioallied Sci. 2011; 3:89.
28. Narasimharao R, Anusha Reddy M, Swetha Reddy N, Divyasagar P, Keerthana K. Design and evaluation of metformin hydrochloride extended release tablets by direct compression. Int J Res Pharm Biomed Sci. 2011; 2:1118-1133.
29. Brime B, Ballesteros M, Frutos P. Preparation and *in vitro* characterization of gelatin microspheres containing Levodopa for nasal administration. J Microencapsul. 2000; 17:777-784.
30. Ugwoke MI, Agu RU, Verbeke N, Kinget R. Nasal mucoadhesive drug delivery: Background, applications, trends and future perspectives. Adv Drug Deliv Rev. 2005; 57:1640-1665.
31. Fini A, Bergamante V, Ceschel GC. Mucoadhesive gels designed for the controlled release of chlorhexidine in the oral cavity. Pharmaceutics. 2011; 3:665-679.
32. Williams RO, Sykora MA, Mahaguna V. Method to recover a lipophilic drug from hydroxypropyl methylcellulose matrix tablets. AAPS PharmSciTech. 2001; 2:29-37.
33. Schmolka IR. Artificial skin I. Preparation and properties of pluronic F-127 gels for treatment of burns. J Biomed Mater Res. 1972; 6:571-582.
34. Sharwaree H, Priyanka B, Ashok B, Swapnil M. Development of mucoadhesive nasal *in situ* gel to sustain the release of amitriptyline hydrochloride. Int J Biol Pharm Res. 2012; 3:980-989.
35. El-Kamel A. *In vitro* and *in vivo* evaluation of Pluronic F127-based ocular delivery system for timolol maleate. Int J Pharm. 2002; 241:47-55.
36. Ibrahim el-SA, Ismail S, Fetih G, Shaaban O, Hassanein

K, Abdellah NH. Development and characterization of thermosensitive pluronic-based metronidazole *in situ* gelling formulations for vaginal application. Acta Pharm. 2012; 62:59-70.
37. Carotenuto C, Grizzuti N. Thermoreversible gelation of hydroxypropylcellulose aqueous solutions. Rheologica Acta. 2006; 45:468-473.
38. Chen E, Chen J, Cao SL, Zhang QZ, Jiang XG. Preparation of nasal temperature-sensitive *in situ* gel of Radix Bupleuri and evaluation of the febrile response mechanism. Drug Dev Ind Pharm. 2010; 36:490-496.
39. Joshi SC. Sol-gel behavior of hydroxypropyl methylcellulose (HPMC) in ionic media including drug release. Materials (Basel). 2011; 4:1861-1905.
40. Majithiya RJ, Ghosh PK, Umrethia ML, Murthy RS. Thermoreversible-mucoadhesive gel for nasal delivery of sumatriptan. AAPS PharmSciTech. 2006; 7:67.
41. Nasatto PL, Pignon F, Silveira JL, Duarte MER, Noseda MD, Rinaudo M. Methylcellulose, a cellulose derivative with original physical properties and extended applications. Polymers. 2015; 7:777-803.
42. Kaushal G, Sayre BE, Prettyman T. Stability-indicating HPLC method for the determination of the stability of oxytocin parenteral solutions prepared in polyolefin bags. Drug Discov Ther. 2012; 6:49-54.
43. Washington N, Steele R, Jackson S, Bush D, Mason J, Gill D, Pitt K, Rawlins D. Determination of baseline human nasal pH and the effect of intranasally administered buffers. Int J Pharm. 2000; 198:139-146.
44. Misra A, Ganesh S, Shahiwala A, Shah SP. Drug delivery to the central nervous system: A review. J Pharm Pharm Sci. 2003; 6:252-273.
45. Hussain AA. Intranasal drug delivery. Adv Drug Deliv Rev. 1998; 29:39-49.
46. Salib R, Lau L, Howarth P. The novel use of the human nasal epithelial cell line RPMI 2650 as an *in vitro* model to study the influence of allergens and cytokines on transforming growth factor-β gene expression and protein release. Clin Exp Allergy. 2005; 35:811-819.
47. Reichl S, Becker K. Cultivation of RPMI 2650 cells as an *in-vitro* model for human transmucosal nasal drug absorption studies: Optimization of selected culture conditions. J Pharm Pharmacol. 2012; 64:1621-1630.
48. Wengst A, Reichl S. RPMI 2650 epithelial model and three-dimensional reconstructed human nasal mucosa as *in vitro* models for nasal permeation studies. Eur J Pharm Biopharm. 2010; 74:290-297.

(Received April 10, 2018, Revised June 21, 2018, Accepted June 23, 2018)