Ischemic Stroke is the third leading cause of death and the main cause of long-term disability in western societies. It has been estimated that worldwide approximately 5.7 million people die from acute ischemic stroke per annum. Stroke is defined as sudden onset of a neurologic deficit that is attributable to a focal vascular cause. Reduction in blood flow to the brain for more than several seconds will cause cerebral ischemia. Changes in neurologic symptoms are observed within seconds as neurons lack glycogen leading to energy failure. If blood flow to the ischemic zone is quickly restored, apoptotic brain tissue can be recovered fully. This situation is called as transient ischemic attack (TIA). Hemorrhagic stroke, intracranial aneurysms and arteriovenous malformations (AVMs) are some other examples of cerebrovascular diseases.

Pathophysiology of the cerebral stroke involves complex cascade events which lead to cellular death via necrotic pathway and apoptotic pathway. In first mechanism, due to the energy failure or loss, rapidly breakdown of cytoskeletal occurs. While in apoptotic pathway, programmed death of cells occurs. Depletion of glucose leads to failure of mitochondria to produce ATP. As a consequence of lack of ATP production, membrane function stops and neurons becomes depolarise and intracellular level of calcium increases. Depolarization of cells further cause the release of excitatory neurotransmitter glutamate in the synaptic terminals causing excitotoxicity of the cells. Free radical species generated by dysfunctioning of mitochondria and lipid degradation of membrane causes catalytic degradation of membranes and damage other vital cellular functions. Oxygen radicals serve as important signaling molecules that trigger inflammation and apoptosis.

Currently only approved available treatment therapy for cerebral stroke is rt-PA (alteplase) which is licensed in most countries for clinical use and given within 3 h of onset. Other treatment therapies include thrombolitics, antiplatelets, anticoagulation and neuroprotective agents. One of the most promising therapies is, thrombolitics, which restore cerebral blood flow in some patients with acute ischemic stroke and may lead to improvement or resolution of neurologic deficits. Thrombolitics administered via intra-arterial route is growing interest to minimised systemic bleeding complications and to rise the drug concentration at clot. Even though thrombolitics are still not used regularly, due to their narrow therapeutic time window
and hemorrhage risk. On the other hand, anticoagulants, due to risk of hemorrhagic events rather than reduction in recurrence of stroke, have shown to be ineffective and even more detrimental than placebo. Now a days, use of neuroprotectives, has emerged as a approach to potentially protect the brain in different cerebral conditions like ischemic stroke without significant side effects.

Agents that can produce neuroprotection by primarily targeting the cerebral vasculature includes agents like anti-thrombotics and antiplatelets that can prevent clot formation and agents like thrombolytics that can break down existing clots considered as indirect neuroprotectants which are mentioned above. While agents that directly act upon the neuron itself are considered direct neuroprotectants. Neuronal death occurs by the cerebral stroke cascade events. Within these cascade events, many molecular targets can be modulated pharmacologically to produce neuroprotection. Neuroprotectants can target cascade events like free radical production, intracellular \( \text{Ca}^{2+} \) influx, glutamate release, excitotoxicity, nitric oxide production, inflammation and apoptosis. Even though there are many numbers of clinical trials, neuroprotective agents yet have not been proven to beneficial in humans due to lack of suitable delivery approach to improve bioavailability and target selectivity.

Nicergoline is an ergoline derivative which is used to treat cognitive deficits, dizziness and its having positive effects on psychomotor performance, concentration and neurophysiological parameters. Nicergoline increases the cerebral blood flow and mono amine turnover and affects cholinergic function in the aged rat brain. It was reported that nicergoline has a protective effect against ischemic brain damage in ischemic brain models. One study demonstrated that nicergoline suppressed the production of pro-inflammatory cytokines and superoxide anion generation by activated microglia and astrocytes. Through the effect on glutamate transporters nicergoline reduces the extracellular glutamate and plays a role as a neuroprotective. Further, nicergoline by inducing TGF-\( \beta \) and glial-derived neurotrophic factor protects cultured neurons against \( \beta \)-amyloid toxicity.

Recently, there are many types of drug delivery carriers are being developed or in developing stage with an objective to improve drug bioavailability, to reduce toxicity and side effects, to prevent degradation or loss upon administration. These drug delivery carriers include
liposomes, nanoparticles, microcapsules, microemulsions, micelles etc. Micelles as drug carriers have gained more attention in recent years, among those delivery systems. Bioavailability of poorly soluble drugs can be improved by incorporating drug into core-shell structure of micelles. Due to smaller particle size of micelle carriers, they can accumulate spontaneously in damaged vasculature via Enhanced Permeability and Retention (EPR) effect.

Micelles spontaneously form when the concentration of the amphiphile unimers is higher than a critical concentration (CMC). Below the CMC amphiphilic molecules absorbed at the air water interface. At the CMC the amphiphile becomes saturated at this interface and in the bulk solvent, and it becomes entropically favorable for micelles to form to minimize the free energy of the system.

Mixed micelles system increases the solubility of poorly soluble drugs, having physicochemical properties for tumor targeting by passive targeting via EPR effect. Micelle drug carriers can be targeted at particular specific site by ligand coupling. Various ligands include monoclonal antibodies-mAbs (Immunomicelles), folate residues, peptides, luteinizing hormone–releasing hormone, epidermal growth factor (EGF), α2-glycoprotein, transferrin, sugars etc. Functionalization of polymeric micelles can modify the physicochemical and biological properties of micelles systems for receptor mediated Drug delivery.

Nicergoline is poorly soluble slightly yellow colored powder insoluble in water, soluble in acetic acid or acetone and slightly soluble in the ether in chloroform or benzene. Presently in the market nicergoline is present in form of tablet, slowly-releasing granule, capsule, powder-injection and injection. Oral bioavailability of nicergoline is low. The mucosal administration i.e nasal administration of nicergoline will improve its bioavailability and reduces dose and thereby its associated side effects. In comparison to injectable dosage administration mucosal administration is simple and convenient. As nicergoline acts directly with the brain receptors its intranasal dosage form will carry the nicergoline easily to brain. The nasal cavity is used as the method of administration equivalent to systemic therapy from last few decades. Because there is unique contact between the olfactory lobe and the brain, it’s an effective way for brain targeting. The nasal administration of drug will become an effective method to replace long-term injection.
Hence, Nicergoline was formulated into suitable intranasal micelle drug delivery. The drug was entrapped into TPGS/PF 127 surfactant micelle and the surface of the micelle was conjugated with stroke homing peptide and characterized for particle size, zeta potential, entrapment efficiency, surface morphology and in vitro drug release. It was hypothesized that incorporation of the drugs in the micelle will improve solubility and reduce dose of drug and thereby improve its pharmacological activity and targeting efficiency to ischemic tissue, due to attached stroke homing peptide, following intranasal administration. The efforts were made to elucidate the brain targeting efficiencies of the two different formulations developed viz. stroke homing peptide conjugated micelle and non-conjugated micelle.

Calibration curve of nicergoline was developed by HPLC. The linearity of method was found between concentrations 2 – 100 ppm. In the standard addition method, the mean % recovery analyte were in the range of 99.33 to 102.13. The % RSD for intermediate precision, with intra-day variation was not more than 2.94 % and inter-day variation not more than 2.06% . In the MeOH:ACN:KHP 0.01M (pH 7) (50:30:20) DL and QL were found to be 1.55 μg/ml and 4.71 μg/ml, respectively. The % RSD of the area responses are less than 2%. The method was found to be robust as variation of flow rate, pH, mobile phase composition did not affect % recovery significantly. Similarly variation in the analytical instrument did not affect % recovery significantly, so the proposed method was found to be rugged as well. The results of solution stability showed that prepared samples were stable since % RSD was less that 5 % with respect to initial sample. Therefore the samples can be analyzed after storing for 48 hr. In conclusion developed analytical method was found to be accurate with respect to all the validation parameters and therefore was considered suitable for the analysis of the NG during the course of formulation development of micelle nano-concepts to determine drug loading, assay, entrapment efficiency of Nicergoline.

Use of non-ionic surfactant such as Poloxamer F 127 and TPGS alone helped in entrapment of nicergoline to some extent, but it was mainly concentration dependent. When micelles were prepared using TPGS and PF 127 in different w/w ratio, at lower concentration of polymers precipitation of drug was observed after dilution in water. But at higher concentration i.e Poloxamer F-127, and nicergoline a stable micelle dispersion (50% entrapment) was formed and got precipitated after 2 hrs of dilution and in case of TPGS:Nicergoline with 10:0.1 ratio a stable
solution (98% entrapment) was obtained, but it also got precipitated after 24 hrs. In terms of solubility of nicergolene forming micelle it was increased from 5 ppm to 50 ppm in combination with PF 127 and 80 ppm in combination with TPGS. However combination of Pluronic F127 with TPGS, prohibited the precipitation of drug after dilution and remain stable for approximately 7 days.

After applying optimization the major factors which influenced formulation were found. Concentration of Pluronic F127 and TPGS had a quadratic effect on entrapment. Initially increase in TPGS concentration showed linear increase with that being constant at later stage. However, in case of Pluronic F127, increase in concentration showed decline in drug entrapment with slight initial increase. This result might be related to the stable interaction among the aromatic ring in TPGS and hydrophobic part i.e Polyoxyl groups in PF 127. Increase in concentration of drug caused a linear decline in the drug entrapment. This can be explained by the fact that at higher drug concentrations, surfactants were unable to entrap the whole amount of drug. TPGS increases the size as well as hydrophobicity of the core because of the organic ring moiety of Vitamin E. Hence, as concentration of TPGS increases entrapment increases. But the TPGS moiety alone is unable to hold the drug in the core as after a period of 2-3 days of storage of formulation of TPGS alone showed precipitation of drug from core. But in combination with Pluronic F 127 stability was achieved. The solubility of Nicergolin in obtained micelle formulation is 3mg/ml, while in water is very poorly soluble. We believe that combined interaction between the drug and PF-127/TPGS mixture stabilize the drug moiety inside the micelle core. Also the blocklength of the hydrophobic polypropylene oxide (PPO) segment and hydrophobic nature of tocopherol succinate moiety helps in drug stabilisation.

The size of micelle depends upon the composition of block copolymer, ratio at which two polymers are used and the drug loading. Once the micelle is formed, the micelle size is decided by the hydrophobic interaction between the hydrophobic fractions of polymer and drug. The size of micelle is one of the factors that influence the in vivo distribution of micelle and drug delivery. The in vivo extracellular space in rat brain is estimated to be about 38-64 nm at least two folds greater than estimate from fixed tissue. Other nanocarriers such as liposome have been used in size range from 50-150 nm. Size of some of the nanocarriers was greater than
extracellular space width. Because of shrinkage of extracellular space relatively smaller sized nanocarriers may facilitate interstitial diffusion in ischemic tissue.

Micelle nanocarriers of Nicergoline were developed to exploit their advantages such as low particle size, enhanced permeability across nasal mucosa and ability to incorporate varying ingredients, which would allow targeting of the solubilized Nicergoline, longevity and higher retention effects in the ischemic area, in turn providing an enhanced neuroprotection. From the optimization of formulation we obtained 20 nm micelle with an acceptable polydispersity index of 0.3.

Nicergoline-loaded Poloxamer 407/TPGS mixed micelles were negatively charged with zeta potential of about -13.57 mV. In the structure of Poloxamer 407 both the polypropylene oxide and polyethylene oxide segments were non-ionic, so change of the surface charge of the micelles must have resulted from addition of Nicergoline and/or TPGS.

The size of Nicergoline + PF 127 + TPGS (20.52 nm) was smaller than that of Nicergoline + PF 127 (25.04 nm). This may be due to the impact of polymer composition and the compact structure of combination as well as smaller structure of TPGS compared to PF 127. Hence TPGS easily replaced PF 127 form the inner core of micelle with smaller diameter, which might result in smaller size. Concentration of PF 127 had a quadratic effect on entrapment with a bell shaped curve. Initially increase in PF 127 concentration led to increase in particle size up to around middle level of factor which then led to reduction in particle size. Because initially at lower concentration PF 127 tries to accommodate with the micelle structure with TPGS. But after middle level of concentration they are seggregated and form their own micelles of smaller size. In case of TPGS and drug, both led to linear rise in the particle size with increase in their concentraion. In case of TPGS and drug, increase in concentration leads to increase in entrapment and increase in core size because it increases the core size.

Stable and small particle size ( <200 nm) could reduce the uptake of reticuloendothelial system and provide efficient passive targeting ability via the enhanced permeability and retention effect. Therefore the size of prepared micelle was suitable for ischemia specific accumulation with help of targeting moiety.
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The low CMC of the micelles infers to high stability and ability to maintain integrity of the micelles even upon dilution in the blood circulation. The addition of TPGS to micelle formulation did not result in notable variation in the CMC. In previous results it is shown that the CMC of micelle prepared from individual polymer i.e Pluronic alone and TPGS alone is higher than the micelle prepared from combination of Pluronic and TPGS. Therefore, we prepared mixed micelles which were more stable than pure polymeric micelles. The lower CMC and higher stability of micelle could be explained by the fact that TPGS polymers increase the hydrophobic interactions between the hydrophobic part of Pluronic and the drug in the micellar core and stabilize the structure.

Additionally, synergistic interaction between poly(propylene oxide) moieties of Pluronic and the hydrophobic part of TPGS improves the stability of the mixed micelles and lead to increased core of micelle. Both courses of action had significant impact on the drug solubilization and entrapment efficiency of mixed micelles. But the stability of combination is limited to some extent. As the concentration of TPGS was increased, it increased hydrophilic segments i.e succinate-PEG which raise the chances of interaction between hydrophilic and hydrophobic segments and a reduced hydrophobicity of the core subsequently leading to an increased CMC and instability.

Entrapment of nicergoline inside the micelle was confirmed by DSC and FTIR. The thermogram of Nicergoline exhibited sharp endothermic peak at 134.60°C indicated melting point which was reported in literature. Characteristic peak of Nicergoline was well recognized in the physical mixture. The thermogram of Nicergoline loaded Poloxamer 407/TPGS micelles shows all endothermic peaks of polymers as shown in the blank micelles thermogram, but the characteristic peaks (at 135°C) of Nicergoline was absent, which revealed that nicergoline was molecularly dispersed inside the micelle.

IR spectrum of nicergoline exhibited the characteristic bands corresponding to the functional groups of the drug at 3423.99 cm\(^{-1}\) characteristic for (\(-\) NH stretch) from (3500--3300), at 1720.19 cm\(^{-1}\) from (1760--1665 cm\(^{-1}\)) characteristic for (C = 0 stretch), at 1081.87 cm\(^{-1}\) characteristic for (C--O) and at 1463.71 and 1427.07 cm\(^{-1}\) for (C = C) stretching of aromatic rings. The spectra of the Nicergoline encapsulated mixed micelles showed the absence of
characteristic peaks for Nicergoline, which suggested that the drug was localized and entrapped within the hydrophobic core of micelle.

Nicergoline release occurred in 2 phases: a phase of burst release followed by a phase of slow and gradual release. Only 45% of Nicergoline was released from Nicergoline –PF 127-TPGS in pH 7.4 and 15% at pH 5.5 within the first 4 h, while almost all Nicergoline was released from the tartaric acid drug solution during the same time period. There are two probable reasons for the initial burst release. Quick disruption of the micelle system due to cohesion, higher concentration gradient, and sink conditions in the system. A small quantity of nicergoline located at the interface of the micelle hydrophobic core and hydrophilic corona, or even within the micelle corona compartment, releases either by hydration of the interfacial nicergoline molecules and passive diffusion. The nicergoline incorporated into the hydrophobic core remains inside the micelles maintaining a slow release. This initial burst release would help in achieving the required therapeutic concentration and the sustained release would maintain the concentration. The lower drug release at pH 5.5 could be beneficial because target for drug action is brain not the nasal mucosa and also it avoids the drug associated side effects. Burst release in pH 7.4 is required as it is physiological pH in brain. However, Nicergoline-loaded mixed micelles could have improved therapeutic efficiency because of their amplified mean residence time in ischemic zone through EPR effects and enhanced drug release in the ischemic tissue.

The micelle carrier not only solubilized the poorly soluble nicergoline, but also sustained the nicergoline release for more than 24 hours. Drug diffusion, polymer erosion or swelling might be possible mechanisms for release of nicergoline from micelles. After 24 h, 10-12% of the initially incorporated drug still existed in the micelles at pH 7.4. The result indicated that the micelles showed a sustained-release property for the incorporated Nicergoline.

Generally drug release from micelle occurs by diffusion through the polymer matrix, release by polymer degradation and solubilisation, or diffusion through micro channels that exist in the polymer matrix. Nicergoline is actually captured in the hydrophobic core of the Poloxamer and TPGS, which controlled its release. Nicergoline from the hydrophobic core is released mainly through diffusion. As a result, the in vitro release profile of nicergoline from the polymeric micelle system is largely dependent on the interaction with hydrophobic core
properties. The strong hydrophobic interaction of Nicergoline with PPO segments of PF 127 and aromatic ring of TPGS may result in a longer diffusion time, leading to a sustained release property.

Optical microscopic images of sheep nasal mucosa treated with IPA, Phosphate buffer (pH 6.4), drug solution, drug loaded micelle formulation and drug loaded peptide conjugated micelle formulations. The prepared formulations were subjected to nasal toxicity to evaluate the safety of the ingredients used in the formulation. The nasal mucosa treated with PBS pH 6.4 showed intact epithelial layer without any damage while mucosa treated with isopropyl alcohol (mucociliary toxic agent) showed complete destruction of epithelial layer and even deeper tissues

Mucosa treated with Blank micelles and drug loaded micelle were found with intact epithelial layer and there were no alterations in basal membrane and superficial part of sub mucosa even after 2 hr of treatment as compared with phosphate buffer (pH 6.4) treated mucosa. This may be due to the lower concentration of Pluronic and TPGS in formulation. Thus, the developed blank micelle and drug loaded micelle seem to be safe with respect to nasal administration.

The presence of maleimide group in the functional phospholipids, DSPE-mPEG2000-Maleimide was tested using Fourier transform infrared spectrophotometer. FTIR spectrum confirms the presence of maleimide group in lipid. The spectrum depicts (3441 cm\(^{-1}\): N-H bending; 2917 cm\(^{-1}\): C-H stretching; 1739 cm\(^{-1}\):C=O stretching ester; 1709 cm\(^{-1}\):C=O stretching imide; 1657 cm\(^{-1}\): C=O stretching amide; 1103 cm\(^{-1}\):C=O stretching imide; 836 cm\(^{-1}\):C=O imide.

In our study the functionalized micelle were first prepared as per the early reported methods with slight modifications and then we used these micelle to prepare immune-micelle. Solution of 1 mol% of DSPE-mPEG\(_{2000}\)-Maleimide was then inserted into the preformed Nicergoline loaded functionalized micelle as described in method. The micelles were slightly diluted after separation but no change in total drug content and mean particle size were observed. The insertion of functional phospholipids, DSPE-mPEG\(_{2000}\)-Maleimide, on micelle was confirmed by Elman’s sulphhydryl group estimation assay (thermo scientific protocol with slight modifications) and as early the reported method with slight modifications. The amount of maleimide is calculated as
the difference between the initial amount of thiol (cysteine) and the amount of unreacted thiol (cysteine) after complete reaction of all maleimide groups. Prepared a set of test tubes (Blank, positive control and sample tubes) (yellow product) analyzed using UV-visible spectrophotometer at 412 nm. The absorbance positive control tubes and sample tubes were subtracted from blank sample absorbance. Using the absorbance values we have calculated the amount thiol (cysteine) remained unreacted in samples as below mentioned.

Moles of sulfhydryls present in test sample were calculated in comparison to positive control solution. The amount of sulfhydryls reacted with functionalized micelle were calculated by subtracting test sample values from positive control sample values. The obtained value of sulfhydryls reacted with functionalized micelle indicated as the availability of sufficient maleimide for conjugation of ligand.

The peptide conjugated micelle were prepared by incubating CLEVSRKNC peptide with functionalised PEGylated micelles overnight in cold room followed by incubation with excess amount of cysteine and purification of peptide conjugated micelle from unconjugated CLEVSRKNC peptide fragments and cysteine by using ultracel-50 membranes (50kDa MWCO). CLEVSRKNC peptide ~20µg (~0.4µM) was incubated with functionalized micelles of 0.018mM of total polymer/mL (20 mM of functionalized polymer/mL). The weight ratio of peptide to functionalized lipid is 1:50, w/w (or 1:100, µM/µM). The peptide conjugated micelle were identified by SDS-PAGE gel permeation chromatography. 10% and 8%, SDS-PAGE gel was used for identification of CLEVSRKNC peptide. The permeation chromatography was performed under non reducing condition (loading buffer containing no reducing agent) because right end of peptide has –SH group susceptible for reduction when sample was heated in the presence of reducing agent in loading buffer during sample preparation. Finally, silver staining method was used as it is very sensitive method and we can easily detect small amount of peptide. The molecular weight of separated proteins are determined using software Alpha ease (Alpha Innotech). The gel was incubated in the developing solution as long as possible to obtain all possible bands with minimum background color.

The conjugation of CLEVSRKNC peptide over micelles can be confirmed by both the disappearance of intact CLEVSRKNC peptide band and appearance of new band corresponding
to peptide conjugated micelle in SDS PAGE. The peptide conjugated micelle (composed of many TPGS and PF 127 molecules and CLEVSRKNC peptide) are of high molecular weight, as compared to other proteins used in the study, diffuse very slowly in the gel (8%) and show band near the well. Instead, a new band at ~1.3 kda corresponding to peptide conjugated micelle was observed in our study.

The prepared immunomicelle were evaluated for the mean particle size, zeta potential, drug content, and total peptide content. The concentration of peptide attached over micelles was determined by Bradford assay and it was found to be ~14.42±0.12µg/mL (0.0255mM of total lipid). Therefore, the peptide conjugated over micelles was found to be 90.13%. The prepared peptide conjugated micelle have zeta potential of -20 ± 3.1mV and they gain still more negative potential during sample preparation for loading. No change in the zeta potential was observed between functionalized micelle and immunomicelle. The % drug content of immunomicelle was slightly decreased and is might be due to dilution that occur during the preparation and purification of immunomicelle.

In in vitro neuroprotective efficacy study, H$_2$O$_2$ treated SH-SY5Y cells were incubated with 0.5mM MTT at 37°C for 30 min, followed by measurement of absorbance at 450 nm. As can be seen in exposure to 40 µM H$_2$O$_2$ decreased cell viability to 50.03 ± 3.72. In H$_2$O$_2$ challenge study it can be seen that pre-treatment with 0.6 and 0.8 µM nicergoline loaded micelle and peptide conjugated drug loaded micelle significantly increased cell viability compared to drug suspension. The results of the current study demonstrated that Nicergoline solution alone had no significant effect on cell survival but, Nicergoline at the concentration of 0.4, 0.6 and 0.8 µM in the form of micelle formulation were able to counteract the deleterious effect of 40µM H$_2$O$_2$. The positive results demonstrating neuroprotection in the above mentioned formulations may be attributed to resultant improvement of micelle permeability through SH-SY5Y cell membrane. Hence, one can conclude that the permeability of Nicergoline can be increased by incorporating it in micelle formulation.

To illustrate the safety of micelle formulations and its effect on the cell viability In-vitro cytotoxicity study was performed using MTT assay. A substantial decrease in the cell viability was observed after incubation with Triton X 100 compared to HBSS-HEPES, blank micelle,
drug solution, drug loaded micelle and peptide conjugated micelle (p <0.001). *In-vitro* cytotoxicity study demonstrated that the higher concentration of blank micelle has less viable cell in RPMI 2650 cells and SH SY5Y in comparison to drug solution and HBSS-HEPES. The drug loaded micelle had higher cell viability than the blank micelle suggesting neuroprotective effect of drug and membrane sealing activity of Pluronic protecting cells. This proved safety of formulation for the nose to brain delivery.

Although the formulations are devoid of any microbial growth supporting ingredients the high water activity values for aqueous formulations (> 0.9) warrant the chances of contamination from the both bacteria and fungus. Therefore, the prepared formulation was also evaluated for antimicrobial efficacy to select suitable preservative concentration. For this microbial strains in the form of lyophilized kwick-sticksto culture plates were successfully sub-cultured. All the revived cultures were confirmed for purity from microscopic examinations, staining and macroscopic appearance of colonies formed. Further, all the strain meets the requirements of minimum number of passage required for the preservative efficacy testing as per US pharmacopoeia.

The method of serial dilutions was used to prepare the inoculum and for characterizing the standard microbial solutions required for inoculating the formulations to be tested. The 10 ml of pooled samples of each formulation was inoculated for microbial challenge to a concentration of $10^5$ - $10^6$/mL of formulation. The growth of microorganisms in zero day count confirms that the challenge level of the test indicating the contamination level of $10^5$-$10^6$ organisms/ml of formulation. NSs 1, 2, 3 and 4 contained different concentrations of Benzalkonium chloride (BKC) 0.025%, 0.05% and 0.1% respectively. The control and NS2 formulation failed in microbial challenge test while NS3 and NS4 passed the test based on the acceptance criteria given by USP. A significant microbial count was observed on the day 7 and 14, however; there was reduction in microbial count in preservative containing formulation. The day 14 and 28 showed very low microbial counts in all the cases except the control formulations, which proves the susceptibility of the preservative free formulations to support growth of microorganisms, which will be further increased when used in contact with the external body cavities like nostrils. Since, it was proposed to choose the minimum preservative concentration effective in
preservative efficacy test, the NS3 formulation containing 0.1 % of BKC can be considered to be acceptable.

The developed nasal spray was finally evaluated on SympaTEC for globule size distribution and effect of different factors on globule size distribution. Changes in D90 as a result of rotating the actuator (30°, 60° and 90° angles) suggest that the spray formation from a nasal device is unbalanced. It is evident from the images that there exists localized greater concentration of droplets (so-called ‘‘hotspots’’) within a nasal-spray pattern, which appear asymmetrically and vary according to pump design and orientation. Such kind of different spray leads to difference in droplet size distribution (DSD). The profiles show a DSD pattern of an emitted spray from the initial nasal spray formation, middle stable phase and last dissipation phase. After looking into the data, one can conclude that extreme changes in all spray parameters (D10, D50, and D90) can occur from formation to dissipation phase. It is obvious from data that D10 and D50 levels were fairly stable throughout the spray period, whereas D90 fluctuated in the dissipation phase.

It can be seen that increase in distance from the laser beam, force applied at the bottom of spray and angle of spray bottle increases D50 with highest effect from distance and force. Response surface plots indicate that lowest D50 was observed at lowest levels of force, distance and angle.

FDA recommends study of DSD at two distances ranging from 2 to 7 cm from the nozzle tip, where distance between the two should be 3 cm or more. To compare the effect of distance on DSD the data to should be collected only during the fully developed phase and should comprise the droplet size expressed in terms of D10, D50 and D90 and the span value defined as (D90 − D10)/D50 as an indicator for the width of the distribution and, for NDAs, additionally, the fraction of droplets smaller than 10 μm. Distance between the spray nozzle and the laser beam influence the DSD measurement because of diverse settling velocities of the droplets, the plume dynamics and the diverse demonstration of the true DSD in the measurement zone.

Many scientists have explained the impact of distance between nasal spray tip and the laser beam on droplet size distribution data. But contradictory results were obtained on the effects of actuation distance on DSD. None of them have explored the effect of distance on DSD
from different distances. In our study we examined our developed nasal product and obtained impact of distance of on DSD. We varied the nasal spray tip to measuring zone distance from 2 to 7 cm and observed decrease in $D_{50}$ and $D_{90}$ value but $D_{10}$ remain unaffected. The Spraytec laser beam has a sampling zone width of 30 cm and diameter of 1 cm. As the distance from the laser beam increases the area of the resultant actuated spray also increases which results in larger percentage of droplets missing the beam. Thus, this is not the actual DSD as fewer droplets represent the DSD of the spray. As a result, actuation of spray from short distance from the laser beam may provide better information of the DSD from nasal sprays. But, analysis at a short distance may lead to multiple scattering, due to the high density of droplets in the measuring zone, which can result in misinterpretation of data of droplet size and, thus, a distance has to be chosen based on the obscuration levels that reduce multiple scattering events. FDA draft guidance states that DSD must be checked from two distances. Guidance has specified a maximum distance of 7 cm from the beam and the second distance should be at least 3 cm from the first. So, a short distance should be choosen or vacuum must be applied at the top after the beam of analysis to characterize the droplet distribution. Applied vacuum will reduce the loss of droplets from the measurement zone during flight. Hence, distance selection must be done carefully or experiments must be carried out at fixed force and vacuum but at different distances from laser beam. From the data one must choose two distances which have significantly different DSD. For our device 30 N force was not sufficient for actuation of device. Hence, we were unable to collect data below 35N actuation force. We finalized 45N as reliable for our device.

It was observed that increase in distance, force and angle show quadratic effects with significant being that of distance and angle. Increase in distance and angle, initially reduces shot weight up to around zero level and then increases shot weight. When the force applied at the bottom of bottle increases, shot weight increases because more is the force, more will be gap covered between nozzle and bottle. The force affects the shot weight mainly by determining the weight of formulation to be pulled into the dip tube and sprayed. A force higher than the actuation limit of pump may affect the functionality of nasal spray valve. Hence, proper force selection should be done taking care of valve. In case of angle vs shot weight, the shot weight is more at an angle where the dip tube in the nasal spray bottle is completely dipped. In case of conventional bottles which were used in our studies were flat based, but the newer modified
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bottles have conical base which will help in increasing number of dosing and uniformity of dosing.

As per FDA acceptance criteria include the weight of the individual sprays to within 85% to 115% of the target weight and mean weight to be within 10% of the target weight. Guo et al. have studied the impact of surfactant such as Na-CMC and Polysorbate 80 and their concentration on shot weight and found out that the concentration of polymer and surfactant have a very slight effect. In our study we found that delivered volume by the selected spray falls within the specified limit.

In case of effect of resting on priming it was observed that after initial priming, if the spray is used up to 24 hrs there is no need of repriming. But, if the spray bottle is kept unused for 36, 48, 60 or 72 hrs re-priming up to 2 sprays is required in order to achieve the required spray weight. Hence, the spray bottle should be labeled as to be primed if bottle is left unused for 24 hrs or more.

The results for stroke length on shot weight were in accord with prior results i.e., the shot weight increases with increasing stroke length and required shot weight of 100 mg is achieved at the optimal stroke length of 4–5 mm for used nasal spray pump. RSD for stroke length settings of 1, 2, 3 and 6 mm was on higher depicting the variability in results and for stroke length of 4 and 5 mm was less than 5% depicting the reproducibility of results. No shot weight is outside the limit of ±15% of the average value at 4 -5 mm stroke length. At stroke length of 6 mm, low shot weigh and variable results were obtained. It may be due to over actuation spray pump, which affected the normal working of the spray pump valve.

Span value represent the width of the DSD from the median droplet range (D_{50}) and, hence, a smaller the span value narrow is the DSD. Till date, there is no literature support on how span value affects nasal deposition and thereby nasal bioavailability. But, span values can be used to evaluate the quality of the spray. If the span value is large, it indicates that liquid dispersion has high surface tension, cohesiveness, and/or a relatively low amount of energy representing low dispersion efficiency of the nasal device. The span value is dependent upon on pump design and type of formulations. It can be seen that increase in distance and force showed quadratic effect while angle showed linear effect on span value. Increase in distance led to initial
increase followed by prominent decrease in span. Increase in angle linearly and significantly caused rise in span. Highest span value has been predicted at lowest level of force 30 N i.e and highest level of angle 90. Span is a consolidated measure of broadness of the DSD. The span values are computed from the measured $D_{10}$, $D_{50}$ and $D_{90}$ values.

Till date no one has studied the impact of force on stages of spray development. It was observed that increase in distance and force showed linear effect on stable phase. Rise in these factors led to decline in stable phase. Angle and force time had no main effects on stable phase but were involved in significant two way interactions with distance and force respectively. Maximum stable phase was predicted from highest levels of angle (90°) and distance (7 cm) as well as at highest levels of force (55 N) and force time (3 sec).

Impact of force on formation phase was found to be negligible as it remained constant throughout different force. A gradual decrease in stable phase and gradual increase in dissipation phase was observed. If force applied at the bottom of the nasal spray pump increased, time of dissipation phase increases leading to formation of large droplets. As force applied at the bottom of the nasal spray pump increases the stable phase time decreases i.e. total percentage or concentration of globule size decreases up to 50N force. The reduction in the duration of the stable phase as the force i.e velocity with which force is applied is increased, indicating a faster delivery of the dose.

The findings suggest that along with drug product, experimental test method and the methodology, along with device actuation parameters can vary output. Spray pattern was analyzed means of automated machine and plume geometry was analyzed manually. Plume geometry as carried out manually it is subjected to operator bias.

To conclude Nicergoline Micelle formulation was successfully prepared and optimized by using optimization designs. Micelle formulation was successfully conjugated with stroke homing peptide. Result of this study clearly showed that targeting efficacy of developed peptide conjugated micelle. From the result of this study we can conclude that the peptide conjugated micelles are promising tool for the specific delivery of anti-ischemic drugs. Further the developed formulations were found to be safe from patient compliance perspective. Therefore the combined result of improved efficacy and safety should result in enhancement of current therapeutics regimen for the patients suffering from cerebral ischemia.