



NANOSTRUCTURED LIPOSOMES FOR NOSE TO BRAIN DELIVERY OF CARMUSTINE: *IN VIVO* EVALUATION

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Nano sized particles are promising mediators for the administration of active compounds in the nasal and cerebral systems. However, the ability to achieve relevant therapeutic concentrations of exogenous molecules in the body is dependent mainly on the capability of nanoparticles to break down biological obstructions. In this work, nanoscale formulations conveying the minimally soluble model drug Carmustine (CS) were discussed. Box Behnken design (QbD tool) was applied to predict the interactions between the independent variable (drug-lipid ratio (X1), cholesterol (X2) and Critical Process Parameters (sonication time, X3) on the dependent variable (Entrapment efficiency (Y1), Particle size (Y2) and in vitro drug release (Y3)). The nano scale liposomal optimized Carmustine formulation (NSL-OCS) was investigated for their effect on the biopharmaceutical facets decisive for nose-to-brain delivery such as permeation across the nasal mucosa. An in vivo pharmacokinetic study was performed in Wistar albino rats via intravenous routes. Histopathologic studies support the absence of evidence of toxicity. In vitro drug dissemination studies show rapid drug release followed by extended release of CS for up to 24 hrs. The Pharmacokinetic parameters of NSLs in brain were higher in intranasal route compared to NSLs administered by intravenous route. The findings showed that the intranasal pathway can be an effective approach to administering the drug straight to the brain and improve the drug's efficacy in the brain to treat brain tumors and become a good substitute to oral medication.

Keywords: Box Behnken design; Carmustine; intranasal; Liposomes

INTRODUCTION

Carmustine (CS) has been proven to be effective chemotherapy for treating brain tumors¹. It is a nitrosourea compound that is very lipophilic. It hydrolyses in vivo to generate reactive metabolites that cause alkylation and cross linking of DNA and RNA. It is known to inhibit repair and produce elevated molecular complexes by the Denovo purine synthesis². Targeted administration of CS in the brain was challenged due to bioavailability issues. The systemic drug route has a short elimination half-life, which frequently leads to side effects such as liver toxicity and pulmonary fibrosis³.

Nasal administration gets one of the most appealing routes for systemic administration of drugs and a possible choice to most traditional oral and enteral administration. It allows a simple and non-invasive administration, avoids the hepatic metabolism,⁴ and possibly provides direct access to the central nervous system circumvention of the blood-brain barrier (BBB). Specifically, administration intranasally is a great opportunity. to transport the active ingredient into the brain, Benefit from the innervations of the nasal cavity, that is, the olfactory nerve, linking the olfactory bulb to the olfactory region of the nasal cavity, and the trigeminal nerve⁸. In fact, the quantity of drugs transported from the nose to the general

circulation varies widely., from nearly 100% to less than 1% of the dose assumed to be given^{9&10}. For molecules whose bioavailability is less after nasal administration., it has been shown that the administration of therapeutic-relevant quantities of drugs has been shown to be highly dependent on the availability of effective formulations and supports¹¹. In recent years, numerous research groups have demonstrated that nanoparticles considerably improve the convey of therapeutic products through the mucosa of nose^{12&13}.

This research assumes that CS-sized liposomes will be an effective treatment strategy to target glioma or brain tumors. Liposomes loaded with CS will be target-specific, increase therapeutic efficiency, bioavailability and stability. As a result, this article discusses liposomes loaded with CS and their efficacy in treating brain tumors in vivo.

MATERIALS AND METHODS

Carmustine (CS) was acquired as a gift sample of SP Accure Labs Private Ltd., Telangana, India. Cholesterol, Soya-L- α -lecithin, was procured from Merck (Mumbai, India). Chloroform was purchased from Hi Media Laboratories Pvt. Ltd (Mumbai, India). U87MG human glioma cells were procured from National Center for Cell Science (Pune, India). HPLC-quality solvents were used in the analysis of HPLC and all other chemicals and reagents were of analytical quality.

Preparation and optimization of Nano sized Liposomes

CS-loaded nanoscale liposomes (NSL) were developed with the help of conventional thin-film hydration method^{4, 15}. To evaluate the interaction effects of the drug-lipid ratio (X1), cholesterol (X2) and sonication time (X3) in formulations, the 3-level and 3-factor Box

Behnken method was used. A total of 17 tests have been done by Design Expert Version 11. The independent variables were drug to lipid ratio(X1), Cholesterol (X2) and Sonication time (X3) while Entrapment efficiency (Y1), Particle size (Y2) and *in vitro* drug release (Y3) were the dependent variables which are given in Table 1.

Nasal Mucosal Permeation / Retention Study

Mucous permeation surveys were evaluated by the Franz diffusion cell. The rabbit's nasal mucosa was obtained and plunged into a phosphate buffer of pH 6.8 for 15 min. The nasal mucosa was fixed between the donor compartment and the acceptor compartment so that the mucous surface was oriented towards the donor compartment and the mucosal surface facing the recipient compartment. Nasal mucosa of thickness 0.3mm was chosen for this study. A dissemination study was conducted on NSL-OCS containing an equivalent quantity of 10mg of medication in the donor compartment. 20 ml of phosphate buffer saline (pH 6.8) was filled into the receiver compartment and continuously stirred using magnetic bead at 300 revolutions per minute. While shaking, the phosphate buffer solution should be in contact with the serosal surface of the nasal mucosa. A temperature of 37 ± 1 °C was maintained throughout the study and the tissues were aerated to maintain the living conditions of nasal mucosa. The diffusional surface area of nasal mucosa was 0.885 cm², sample volume of 1ml was sucked at a time intervals of 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 20 and 24 hrs. Immediately replace same volume with phosphate buffer saline solution (PBS). Filter samples using a 0.45 μ membrane filter (Millipore Merck, Mumbai India) and the quantity of drug released was assessed using HPLC. Percentage of drug diffused was plotted against time (hr), and slope of the line represents flux ($\mu\text{g}/\text{cm}^2/\text{h}$) of formulated OCS-NSLs^{16&17}.

Table 1: Independent and dependent variables used in Box–Behnken design for the development and optimization

Factor	Level used, actual coded		
Independent variables	Low (-1)	Medium (-1)	High (+1)
X1=Drug to lipid ratio	1:3	1:4	1:5
X2=Cholesterol (%)	2	4	6
X3=Sonication time (min)	5	10	15
Dependent variables	Goal		
% EE(Y1)	Maximize		
Particle size (nm) (Y2)	Maximize		
<i>In vitro</i> release (%) (Y3)	Maximize		

Nasal Histopathology

A tissue assessment was performed on the nasal mucous membrane of the goat to envisage pathological changes following application of OCS-NSL. Three membranes of 0.3 mm thickness of nasal was selected. For 1 hr, the first piece was treated as a positive control, while the second and third pieces which were treated with a pH of PBS of 6.8 as a negative control and OCS-NSLs respectively. Following treatment, the nasal septum was set to 10% HCHO for 24 hrs, decalcified and washed with tap water, dried with ethanol. The paraffin blocks were cut with the 5 µm thick blade to drown the nasal mucous membrane. Tissue sections have been dewaxed and coloured with hematoxylin - Eosin. Treated and untreated tissue slides were observed using an optical microscope to assess mucosal lesions¹⁸.

Assessment of *in vitro* Cytotoxicity

The cytotoxic effect of the OCS-NSLs was assessed on U87MG human glioblastoma cell line and the response was compared against the equivalent free CS suspension for the MTT test. The cell line which is tested was cultured inside a CO₂ incubator at 37 °C in a medium called Dulbecco's modified eagle's medium contained 10% fetal bovine in a 96 well culture plate. The cells treated with diverging concentrations of OCS-NSLs, free drug suspension and blank NSLs after reaching the necessitate density of cells in the plates (~5000 cells per well). Among them, some wells were treated as a negative control treated with culture medium. The media from each well was discarded and approximately 100 µl of MTT solution (1 mg/ml) was added to each well after 48 hrs and incubated in incubator for other 4 hrs. Discard MTT solution from the each well and add dimethyl sulfoxide (100 µl). The addition of dimethyl sulfide resulted in the solubilization

of formazan crystals to produce a purple color. The color intensity depends on the number of viable cells after processing in the well. The optical density was measured at 560 nm by micro plate reader (Bio-Red model 680)¹⁹. The percent viability of the tested formulation and the free suspension of the drug were assessed using the following formula.

$$\% \text{ Cell viability} = \frac{\text{Optical density of the sample at 560nm of treated cells}}{\text{Optical density of the sample at 560nm of treated cells}} \times 100$$

Animals

Male Wistar rats from 200 to 250 g were used in the pharmacokinetic studies. They were bought from NCLAS, Hyderabad. The animals were held in polypropylene cages at standard ambient temperature, in a relative humid environment of 55% with a normal day and night cycle. The study was approved by the Institutional Animal Ethical Committee, IAEC No: CBRLC/IAEC/01/01-2021. Prior to the experiments, animals have been fed with required quantity of drinking water. They were kept in this environment for 21 days prior to the study²⁰.

In Vivo Pharmacokinetic Study

All animals were accommodated at 20 °C - 24 °C and fasting overnight. Total Animals have been divided in 3 groups: Group 1 - Control, Group 2 - drug suspension (CS) Intranasal (I.N), Group 3 - I.N OCS-NSLs treated group. Drug suspension was fixed, and OCS-NSLs suspensions were administered, 20 µL in each nostril which was set up to be containing CS equivalent to 0.81 mg/kg. Blood samples were collected from a retro-orbital plexus of rat containing anticoagulants and centrifuged. The important PK parameters, i.e. C_{max} (ng/ml), T_{max} (h), AUC₀₋₂₄ (ng.h/L), AUC_{0-infi} (ng.h/L), Kel

(h⁻¹), t ½ (h) were determined by using ThermoKinetica (ver. 5.0; Thermo Fischer scientific)²¹.

Statistical analysis

Data for various endpoints were statistically evaluated using variance analysis (ANOVA) followed by Tukey tests, and average values were considered for the respective endpoints. All the values were expressed as mean ± SEM (n= 6)

Stability Study

To investigate physical stability, OCS-NSLs were store up for 3 months at ambient temperature and conditions at 25 ± 2°C/60% RH and 40 ± 2°C /75% RH. Samples were collected at specified time intervals of 0, 1 and 3 months and analyzed to determine the change in percentage of EE, drug content, physical properties and particle size^{22&23}.

RESULTS AND DISCUSSION

As part of this study, various formulations given in design containing CS were prepared. The optimum formulation of nano sized liposomes (OCS-NSL) was selected based on the criteria of attaining the required value of % EE, particle size and *In vitro* drug release by applying numerical point prediction method. The formulation composition with drug to lipid ratio (1:4), Cholesterol (25 mg), and Sonication

time (10 min) has been set up to comply with the demands. The OCS-NSLs presented the practical values of %EE of CS is 94.27 ± 0.25%, Particle size of 235.65 ± 12.87 %and *in vitro* drug release of CS 97.089 ± 1.76%. These practical values of %EE of CS, Particle size and *in vitro* drug release yielded by the OCS-NSLs formulation were found in conformity with the predicted value of %EE of CS is 91.27 ± 0.25%, Particle size of 242.35 ± 10.87 and *in vitro* drug release of CS 96.099 ± 1.66% developed by expert design software, suggesting that the optimized formulation was efficient and reliable. This formulation was successful in permitting the permeation of CS through nasal route.

Nasal Mucosal Permeation / Retention Study

Ex vivo permeation studies of the dug solution and OCS-NSLs have been tested in the nasal mucous membrane and the corresponding drug permeation through the mucous membrane is shown in Figure 1. Flux for OCS-NSL was found to be 240.43 ± 1.76 µg/cm²/h. This flow is caused by the barrier properties of the nasal mucosa and lipid matrix (membrane limiting the rate of permeation). Subsequently, the nasal mucosa has no lipid digestive enzymes so OCS-NSL will not be destroyed and remains to protect the drug from degradation. By adjusting the data in kinetic models, r² values were compared and a good correlation was conflated in the zero-order kinetics which represents that rate of drug release was independent on concentration^{24& 25}.

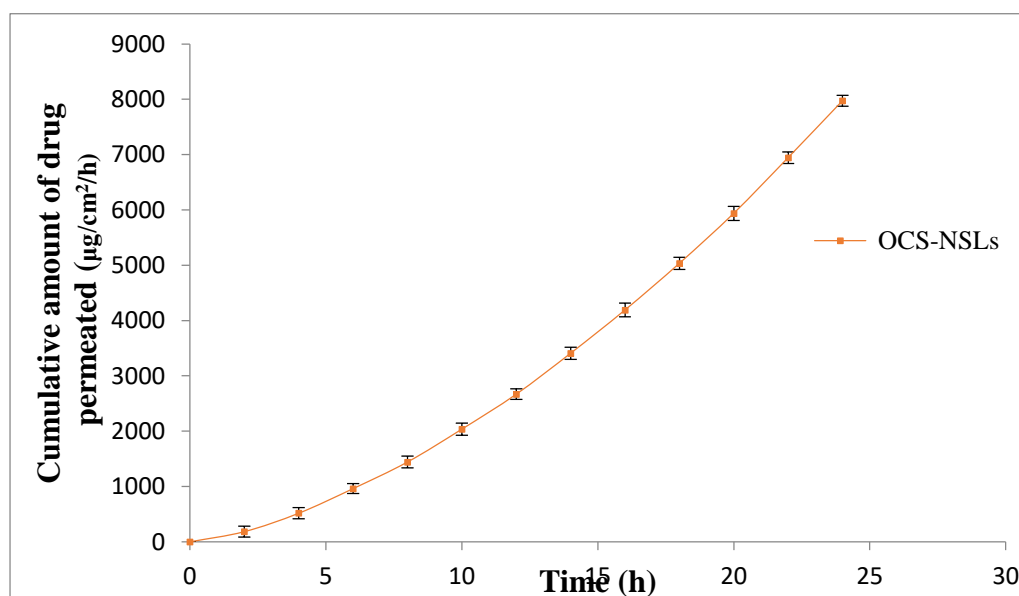


Fig. 1: *Ex-vivo* drug Diffusion Release Studies of OCS-NSL in Phosphate buffer Saline (pH 6.8) Values were expressed as mean ± SD, n=3

Nasal Histopathology

The anterior cross-section of the nasal chamber after instillation of OCS-NSL was used for the examination. The test results are reproduced in the Figure 2 and the results shows that after IN administration of OCS-NSL for 15 consecutive days, there are no sign of histopathological lesions was observed and compared the nasal mucosa with control group for clinical investigation shown in Figure 2. Formulated loaded OCS-NSL does not exhibit any toxicity indicators like fibrosis and inflammation. Hence, OCS-NSL is safer for nasal route administration^{26& 27}.

Assessment of *in vitro* Cytotoxicity

The percent cell viability versus dose (nM) graph (Figure 3) shows that as the concentration of CS formulation increased, the rate of cell

mortality increased. Cell death mediated by OCS-NSL was observed to be greater than cells treated with free solution and Control, respectively. The value of the maximum half-inhibitor concentration (IC₅₀) of OCS-NSL decreased considerably compared to the others. IC₅₀ values of CS from OCS-NSLs was found at 105.26 ± 2.8 nM, which is significantly very less in comparison to free-CS (IC₅₀ value, 233.64 ± 2.1 nM) and control (IC₅₀ value, 757.575 ± 1.3 nM) (Figure 3). It was clear that at equivalent concentration, there is high death rate for cells treated with OCS-NSLs as that of free suspension. The data showed a clear improvement in the antitumour effectiveness of the tested formulation compared to the free drug. A higher cytotoxicity of the optimized U87MG cell formulation is very important as it would promote future *in vivo* applications.

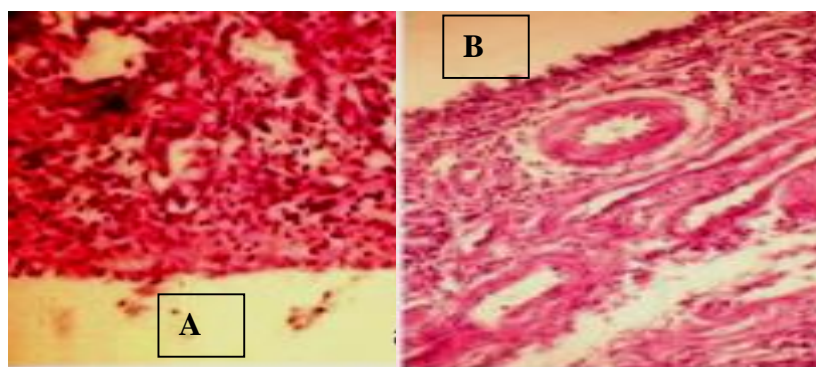


Fig. 2: *In vivo* nasal toxicity Studies - Histopathological section: (A) Normal nasal mucosa; (B) OCS-NSL (Images were recorded at a magnification of 100X)

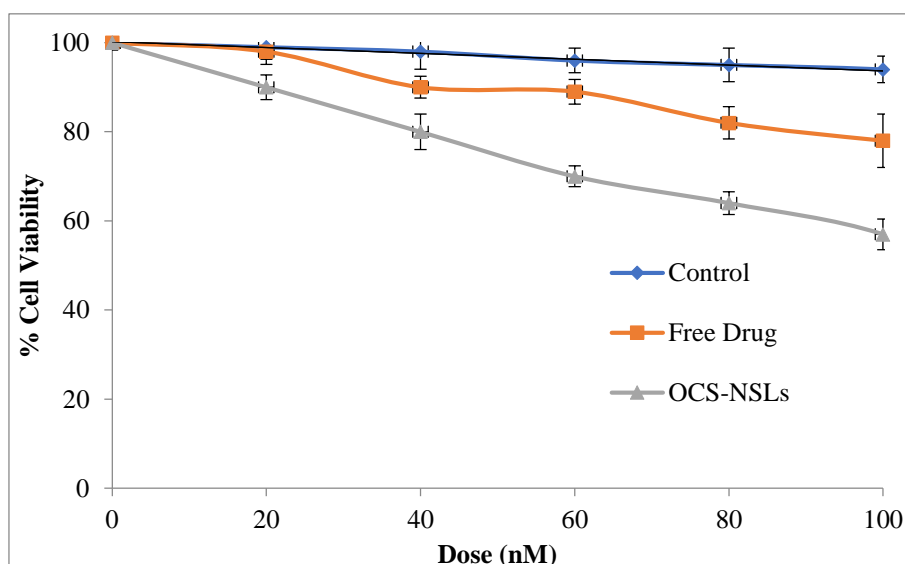


Fig. 3: Comparison of U87MG cell viability (%) upon treatment with optimized formulation (OCS-NSLs), free drug, and blank NSLs.

In Vivo Pharmacokinetic Study

The parameters were calculated using the ThermoKinetica (ver. 5.0; Thermo Fischer scientific). Various computed pharmacokinetics were presented in Table 2. Here we compared the distribution of CS intranasally in the brain for OCS-NSLs with respect to free CS suspension and oral CS suspension during administration to rats. Figure 4 shows the level of CS with the time profile following administration of OCS-NSL and CS suspension. T_{1/2} (half-life) of the OCS-NSLs 13.60 ± 0.23hr was found to be superior to CS I.N solution 6.34 ± 0.07hr. It has been demonstrated that NSLs are more effective at improving the bioavailability of CS.²⁸ As shown in Figure 4, concentration of drug that reaches after intranasal administration was much higher than

that of oral administration. and the plasma drug concentration was much lower which demonstrates less drug distribution to other organs compared to oral administration, hence the peripheral effects of CS are minimized.

Stability Study

The stability studies of OCS-NSLs at room temperature and at accelerated conditions are presented in Table 3 which was carried as per ICH (Q1A) guidelines. The OCS-NSL was evaluated for EE percentage, drug content, physical properties and particle size. The evaluated OCS-NSLs showed no change in the EE%, drug content, physical properties, particle size and stability at 25 °C and at 40 °C (accelerated conditions). OCS-NSLs were found to be stable at 25 °C and 40 °C for 3months²⁹.

Table 2: Pharmacokinetic Parameters for Formulation and Drug Solution in Brain (n = 12)

S.No.	Pharmacokinetic Parameter	Control	Drug Solution (I.N)	OCS-NSLs (I.N)
1	C _{max} (ng/ml)	206.62±13.15	198.18±11.52	143.62±11.06
2	T _{max} (h)	6±0.23	4±0.53	6±0.06
3	AUC ₀₋₂₄ (ng.h/L)	1424.79±3.15	1962.94±2.41	2616.94±1.41
4	AUC _{0-infi} (ng.h/L)	1616.126±2.42	2165.10±1.74	3784.63±1.15
5	K _{el} (h ⁻¹)	0.088±0.03	0.10±0.02	0.059±0.03
6	t _{1/2} (h)	6.06±0.01	6.34±0.07	13.60±0.23

^aData show mean ± SD (n=6). ^{*}Data were significantly different (p<0.05) where free CS drug solution and OCS-NSLs were compared.

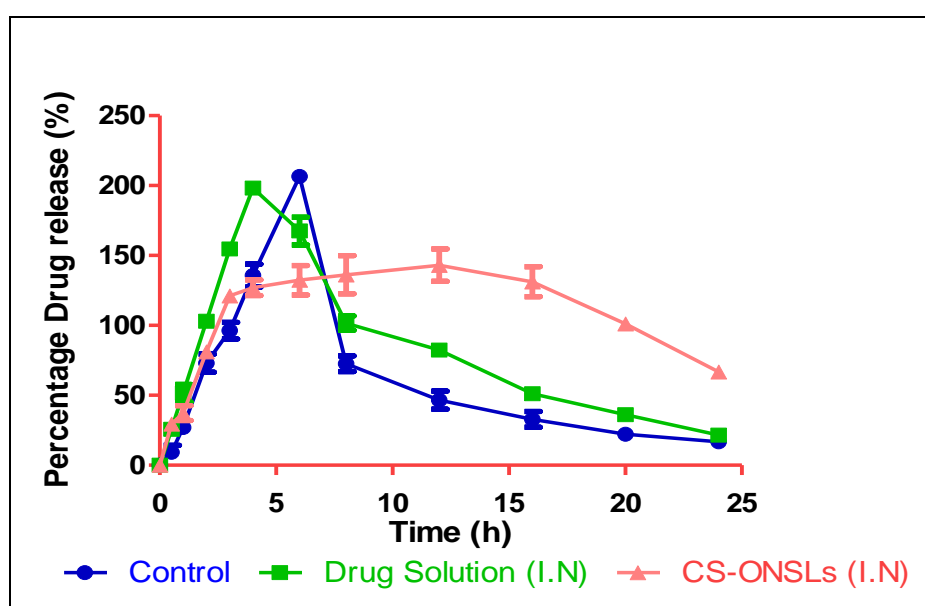


Fig. 4: Concentration-time curve of CS oral solution, Drug Solution (I.N) and OCS-NSLs (I.N) after delivery from various routes

Table 3: Stability data for various parameters after 3 months

S.No.	Parameters	Stability Data after 3 months	
		25 ± 2 °C/60% RH	40 ± 2 °C /75% RH
1	EE %	95.13 ± 1.39	94.07 ± 1.24
2	Drug Content	98.65 ± 2.32	97.98 ± 1.98
3	Particle size	236 ± 26.54	235 ± 18.35
4	Physical properties	No Change	No Change

Conclusion

In this study, we successfully designed a lipid based nanotransporter for the nasal administration of CS. The QbD approach allows us to recognize the critical attributes of materials and processes, such as drug to lipid ratio (X1), Cholesterol (X2) and Sonication time (X3) in the formulations using 3-factor, 3-level Box Behnken design. The optimized (OCS-NSL) was selected by point prediction method with in design space which shown a maximum entrapment efficiency (Y1), minimal Particle size (Y2) and maximum *in vitro* drug release (Y3). Nasal permeation studies showed a greater permeation of the drug NSLs through the nasal mucosa because of its minimal particle size and improvement of the diffusion surface. Histo-pathological studies demonstrate that NSL is safe for IN administration. *In vivo* pharmacokinetic studies have shown the IN pathway to be a potential non-invasive method of targeting the brain, of a hydrophilic drug with respect to the IV pathway and the loading of the drug (CS) into the NLC meets the challenges of drug permeation across BBB. In short, current research is opening up new possibilities for the administration of drugs to the brain via trans-nasal flow.

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Declarations

The authors declared that they have no conflicts of interest.

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نشرة العلوم الصيدلانية جامعة أسيوط



الليبوزومات النانوية المهيكلة لتوصيل كارموستين من الأنف إلى الدماغ: التقييم داخل الجسم الحي

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الجسيمات النانوية هي وسيط واعد لتناول المركبات النشطة خلال أنظمة الأنف والدماغ. ومع ذلك، فإن القدرة على تحقيق التركيزات العلاجية ذات الصلة للجزيئات الخارجية في الجسم تعتمد بشكل أساسي على قدرة الجسيمات النانوية على تحطيم العوائق البيولوجية. في هذا العمل، تمت مناقشة الصيغ النانوية التي تنقل العقار النموذج قليل الذوبان كارموستين (CS). تم تطبيق تصميم Box Behnken أداة (QbD) للتنبؤ بالتفاعلات بين المتغير المستقل نسبة الدهون إلى الدواء (X1) والكوليسترول (X2) ومعلمات العملية الحرجة وقت الصوتنة، (X3) على المتغير التابع (كفاءة احتجاز العقار (Y1)، حجم الجسيمات (Y2) نطلاق العقار في المختبر (Y3) تم فحص تركيبة كارموستين المحسنة على نطاق النانو (NSL-OCS) لتأثيرها على الجوانب الصيدلانية الحيوية الحاسمة للتوصيل من الأنف إلى الدماغ مثل التخلل عبر الغشاء المخاطي للأنف. تم إجراء دراسة الحرائك الدوائية في فئران ويستار البيضاء عبر الطرق الوريدية. و أظهرت الدراسات النسيجية المرضية عدم وجود دليل على السمية. تظهر دراسات انطلاق العقار في المختبر إنطلاقاً سريعاً للدواء متبوعاً بإنطلاق ممتد لمدة تصل إلى 24 ساعة. كانت معلمات الحرائك الدوائية لـ NSLs في الدماغ أعلى في تناول عن طريق الأنف مقارنة بـ NSLs في تناول عن طريق الوريد. وأظهرت النتائج أن المسار الأنفي يمكن أن يكون نهجاً فعالاً لإيصال العقار مباشرة إلى الدماغ وتحسين فعالية الدواء في الدماغ لعلاج أورام الدماغ ويصبح بديلاً جيداً للأدوية التي تؤخذ عن طريق الفم.