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Development and *Ex vivo* evaluation of Rasagiline Mesylate mucoadhesive microemulsion for intranasal delivery using Box-Behnken design

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Abstract: Rasagiline mesylate (RM), an irreversible, selective inhibitor of MAO-B enzyme, is used in the treatment of Parkinson's disease as oral tablets. It has low oral bioavailability (36%) due to hepatic first pass metabolism. Oral route of administration is associated with nausea and vomiting. Hence present research work was aimed to develop intranasal RM- loaded mucoadhesive microemulsions for brain targeting via olfactory pathway. The microemulsions were developed using Box Behnken design and evaluated for globule size, PDI, Zeta potential, pH, viscosity and *ex vivo* permeation on excised porcine nasal mucosa. Based on drug solubility, Capmul MCM, Tween 20 and Transcutol P were selected as oil, surfactant and cosurfactant respectively. Microemulsions were prepared by water titration method. Pseudoternary phase diagrams were constructed and the levels of surfactants, oil were selected. The influence of independent variables such as oil, Smix and water on responses size, zeta potential and flux were studied with the help of polynomial equations, contour plots and 3D response surface plots generated by design expert software. Optimized microemulsion formulation (ME18) was composed of oil (Capmul MCM), Smix (Tween 20: Transcutol P; 1:1), water and drug in the ratio 5:42:65:5. The globule size, zeta potential and flux of the optimized microemulsion was 150 nm, -29.6 mV and 291.7 µg/cm²/h respectively. Mucoadhesive agent (Chitosan) was added at 0.5% concentration to optimized microemulsion formulation (MME18). The size, zeta potential and flux of the MME18 was 176.4 nm, 12.1 mV and 323.1 µg/cm²/h respectively. The flux of ME18 and MME 18 was significantly higher than drug solution. The enhancement ratio of MME 18 was 4.2 times to that of drug solution, indicating potential advantage of microemulsion formulation.

Key words: Rasagiline mesylate; microemulsion; Box Behnken; Intranasal delivery; *ex vivo* permeation.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder. Globally 6.2 million people affected by the disease and approximately 11 lakh people died due to parkinsonism in 2015 [1, 2]. PD symptoms include abnormal movements, such as tremor, rigidity, bradykinesia and postural instability. PD is characterized by progressive loss of dopaminergic neurons in substantia nigra of the mid brain region which result in imbalance between dopaminergic and cholinergic system. Dopamine functions as a neurotransmitter, coordinates movement and controls muscle tone [3, 4].

Monoamine oxidase enzyme is responsible for oxidation of dopamine, generates hydroxyl free radical which results in damage of DNA, lipid membranes of neurons [5]. Rasagiline mesylate (RM) is a second-generation irreversible inhibitor of MAO-B enzyme with dopamine agonist activity [6]. Rasagiline mesylate was prescribed as monotherapy to treat early PD symptoms and in advanced cases as adjuvant along with levodopa [7]. Rasagiline is available in market as oral tablet (Azilect, 1mg) once daily. The oral route of administration is associated with GIT adverse effects such as headache, nausea, vomiting and dizziness. The bioavailability of orally administered drug is 36% as it undergoes hepatic first pass effect [8]. Therefore, there is a need to develop safe and effective formulation with improved bioavailability and reduced side effects. Nasal delivery of rasagiline could be a promising approach to improve the bioavailability [9]. Hence, mucoadhesive microemulsions of rasagiline mesylate for intranasal delivery were developed and optimized by Box Behnken experimental design.

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Materials and Methods

Rasagiline mesylate was procured as a gift sample from Apotex Research Pvt. Ltd., (Bangalore, India). Capmul MCM was from Abitec Corporation Ltd. (Mumbai, India). Labrasol was from Gattefosse Pvt. Ltd. (Mumbai, India). Chitosan (low molecular weight), Transcutol P were from Sigma-Aldrich (Bangalore, India). Isopropyl myristate were purchased from Qualikems Fine Chem Pvt. Ltd (Vadodara, India). Propylene glycol, Polyethylene glycol 400, Tween 80 and Tween 20 were purchased from S.D Fine Chemicals (Mumbai, India).

Spectrophotometric determination, UV method

Rasagiline mesylate was dissolved in methanol and stock solution (1 mg/mL) was prepared. Different concentrations (20 to 200 µg/mL) of RM were prepared from stock solution by diluting with PBS pH 6.4. The absorbance of the samples was measured at 265 nm and calibration curve of rasagiline mesylate was plotted. The standard graph in methanol was also plotted [10].

Solubility studies

The solubility of rasagiline mesylate in different oils, surfactants and cosurfactants was determined by equilibrium solubility method at room temperature, by adding excess amount of rasagiline mesylate into screw caped glass vial containing solvent. The mixture was agitated in water bath shaker at room temperature for 48 hours. The supernatant was filtered through 0.22 µm membrane filter and the filtrate was diluted suitably with methanol and the content of drug was determined by UV spectroscopy [11].

Pseudoternary phase diagrams

Determination of microemulsion region and concentration range of ingredients was selected from pseudoternary phase diagrams. The surfactant and cosurfactant were studied at different ratios 1:1, 2:1, 3:1 and 1:2. To the oil and surfactant mixture at varying ratios of 1:9 to 9:1 water was added drop wise under stirring until homogeneous mixture turns to turbid. The amount of water added was noted and pseudoternary phase diagrams were constructed using Chemix software [12].

Experimental design

A three level, three factor Box Behnken experimental design (Design expert software version 11, State Ease, Inc., MN) was used for formulation optimization [13]. Box Behnken design is categorized under response surface designs, with three levels, coded as -1, 0 and +1. The 3 major factors affecting the formulation, oil (A), Smix (B) and Water (C) were selected as independent variables and the dependent variables selected were globule size (Y1), zeta potential (Y2) and flux (Y3). Design matrix was comprised of 17 experimental runs. The polynomial equation generated for nonlinear quadratic model was as follows.

$$Y_i = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2.$$

Where Y_i is the measured response of each factor level combination; b_0 is intercept; b_1, b_2, b_3, b_{12}, b_{13}, b_{23}, b_{11}, b_{22} and b_{33} are regression coefficients calculated from the measured experimental response Y. A, B and C are the coded levels of independent variables. The terms A^2 and AB represent the quadratic and interaction terms respectively.

Preparation method of microemulsion (ME) and mucoadhesive microemulsion (MME)

Microemulsions were prepared by water titration method. Accurately weighed amount of rasagiline mesylate was dissolved in oil (Capmul MCM) and Smix mixture by vortexing for 15 minutes. Water was added under stirring to make up the weight to form a clear and transparent microemulsion [14]. Chitosan dissolved in 1% acetic acid at 0.5% concentration was added to the optimized microemulsion and stirred continuously obtain clear formulation [15].

Characterization of microemulsions

Globule size, zeta potential and Polydispersity index (PDI)

The Globule size, PDI and Zeta potential were determined using Zeta sizer (Nano-ZS 90, Malvern Instruments Ltd.UK) on 100 times dilute the sample with water, at room temperature 90^{o}angle.

Measurement of viscosity, pH and drug content

The viscosity of the formulation was measured using Brookfield viscometer at room temperature, the pH of formulation was determined by using digital pH meter.

To determine the drug content formulations were suitably diluted in methanol and analyzed using UV spectrophotometer at 265 nm.

***Ex vivo* permeation studies**

Ex vivo permeation studies were conducted using vertical diffusion cell apparatus mounted with excised porcine nasal mucosa. The porcine nose was collected from the local slaughter house and kept in Krebs bicarbonate ringers' solution and then nasal mucosa was carefully isolated using scalpel blade and blunt forceps. The isolated mucosa was rinsed with the PBS pH 6.4. The receptor compartment was filled with fresh buffer and the nasal mucosa was mounted between receptor and donor compartment allowed 30 min for equilibrate. The formulation was placed in donor compartment. Two ml of samples were withdrawn at specified time intervals up to 8 h and the samples were analyzed by UV method at 265 nm. The cumulative amount of rasagiline mesylate permeated at different time points was calculated using following formula

$$Q = [C_n V + \sum_{i=1}^{n-1} C_i S]$$

Where, Q= Cumulative amount of drug permeated

C_n= Concentration of drug (µg/mL) in n^{th} sample interval

V= Volume of Franz diffusion cell,

$\sum_{i=1}^{n-1} C_i S$ = Sum of drug concentration of sample (1 to n-1) multiplied with sample volume (S)

Analysis of permeation data

A graph was plotted between cumulative amount of rasagiline mesylate permeated through the nasal mucosa (µg) and time (h) for each formulation. Flux of formulation (µg/cm²/h) at steady state (J_{ss}) was calculated by dividing slope of linear portion of curve with effective mucosal area. Permeability coefficient (K_p) was calculated by dividing the steady state flux with the initial concentration of the rasagiline mesylate in the formulation. The ratio between steady state flux of formulation and drug solution was calculated to obtain enhancement ratio.

Check point analysis and optimization

To determine the significance of the main and interactive effects of factors on responses, analysis of variance (ANOVA) was performed. The independent variables were used at low, medium and high levels. The constraints chosen were minimum size, within the range of zeta potential and maximum flux.

Among the design matrix six formulations were selected by grid search for check point analysis. The formulations were prepared and evaluated for the response properties. The experimental values were compared with predicted values and the percentage prediction error was calculated. The optimized formulation was selected based on desirability near to 1.

Stability studies

The physical stability of optimized formulations ME18 and MME18 were studied at refrigerated temperature and at room temperature for three months. The samples were analyzed for size, zeta potential and drug content after 3 months. Physical stability was evaluated by centrifugation of formulation at 10,000 rpm for 30 minutes [16].

Results and Discussion

Calibration curve of rasagiline mesylate

The calibration curve of rasagiline mesylate in PBS pH 6.4 and methanol showed good linearity with correlation coefficient value of 0.999.

Solubility studies

Solubility of rasagiline mesylate in different oils, surfactants and cosurfactants was shown in Fig.1. Rasagiline mesylate was having highest solubility in Capmul MCM (90.3 ±1.7 mg/mL) among the oils. Tween 20 and Transcutol P were selected as surfactant and cosurfactant based on their solubilizing capacity. Nonionic surfactant mixture in a suitable proportion is crucial for the stability and globule size of microemulsion formulation. Transcutol P imparts flexibility to the interfacial film [17].

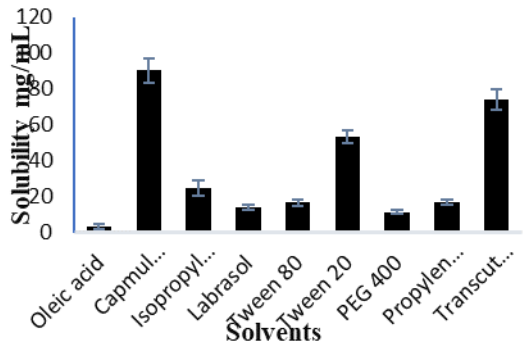

Fig.1. Solubility studies of Rasagiline mesylate

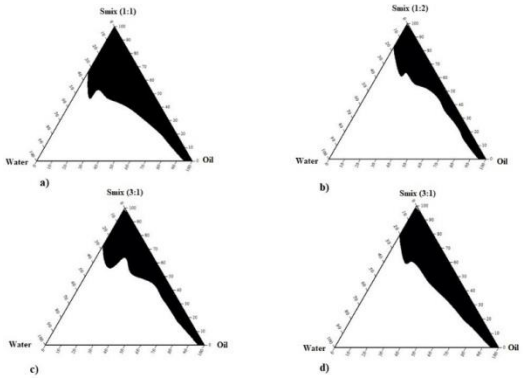

Fig.2. Pseudoternary phase diagrams composed of oil (Campul MCM), Smix (Tween 20, Transcutol P) and water

Pseudoternary phase diagrams

Capmul MCM (oil phase) Tween 20, Transcutol P (Smix) at varying ratios (Smix 1:1, 2:1, 3:1, and 1:2) were shown in Fig 2. Shaded area in the ternary phase diagram indicates the microemulsion region. Maximum isotropic region was observed at 1:1 ratio of Tween 20 and Transcutol P. Hence 1:1 ratio of Smix (Tween 20 and Transcutol P) was selected for the development of microemulsion formulations.

Optimization of formulation by Box Behnken design

All the independent variables (factors) and the measured responses of 17 experimental runs were shown in Table 1. The high-medium-low levels of oil were 5-10-15; Smix were 30-45-60, water were 25-45-65. The contour and 3D response surface plots were shown in Fig.3 and Fig. 4.

Table.1. Composition of formulations and optimized formulations generated by Box Behnken design and measured responses

Formulation	Oil A	Smix B	Water C	Size (nm)	ZP (mV)	Flux (µg/cm²/h)	PDI	ER	K_P*10⁻³ (cm/h)
ME1	5	45	65	147.9	-31.3	288	0.147	3.79	28.8
ME2	10	30	65	193.2	-22.7	193.2	0.216	2.55	19.32
ME3	5	30	45	156.4	-28.5	286	0.241	3.77	28.6
ME4	15	45	65	195.3	-21.2	135	0.194	1.78	13.5
ME5	10	45	45	186.8	-23.8	217.3	0.135	2.86	21.73
ME6	15	45	25	201	-20.5	122.1	0.249	1.61	12.21
ME7	10	45	45	188.5	-23.3	229.2	0.169	3.02	22.92
ME8	15	30	45	213.7	-19.2	90.1	0.127	1.19	9.01
ME9	10	45	45	185.3	-24.9	210.1	0.235	2.77	21.01
ME10	10	45	45	184.6	-25.2	224.3	0.178	2.96	22.43
ME11	10	30	25	207	-19.8	172	0.239	2.27	17.2
ME12	10	45	45	184.3	-25.8	219.3	0.167	2.89	21.93
ME13	5	45	25	142.2	-31.6	271.2	0.288	3.57	27.12
ME14	10	60	65	185.4	-24.3	250.1	0.176	3.30	25.01
ME15	5	60	45	148.2	-30.9	262.1	0.235	3.45	26.21
ME16	10	60	25	165.6	-27.3	235	0.154	3.10	23.5
ME17	15	60	45	193.5	-21.4	157	0.189	2.07	15.7
ME 18	**5**	**41.96**	**65**	**150.2**	**-29.6**	**291.7**	**0.137**	**3.84**	**29.17**
MME18	**5**	**41.96**	**65**	**176.4**	**12.1**	**323.1**	**0.21**	**4.26**	**32.315**
Drug Solution (Drug dissolved in Water)						**75.9**		1	

Note: Rasagiline mesylate equivalent 5 mg of rasagiline is common in all formulations, **ME:** Microemulsion; **ZP**: Zeta potential. Data shown as mean ± SD (n=3)

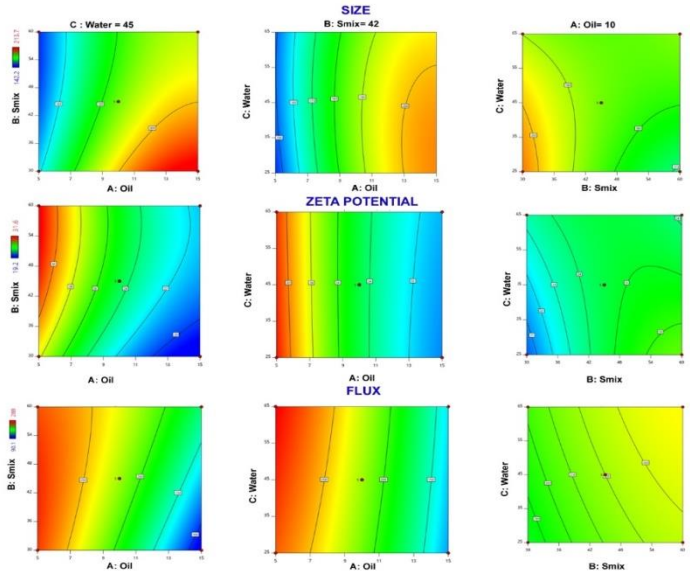

Fig.3. Contour plots showing effects of factors on responses

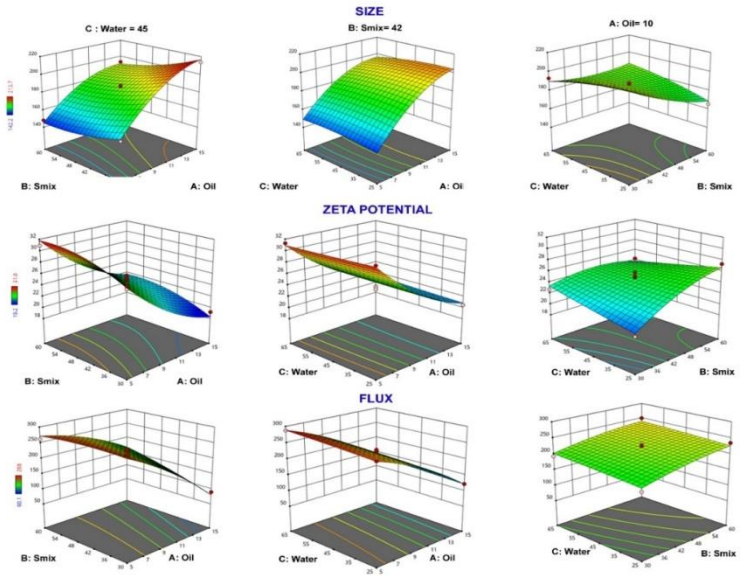

Fig.4. 3D response surface plots showing effects of factors on responses

Microemulsion characterization

The experimental measured values of mean globule size, Zeta potential and PDI of the formulations were shown in Table 1. The mean globule size of microemulsions varied between 142.2 nm to 213.7 nm, Zeta potential between -19.2 mv to -31.6 mv and PDI between 0.135 to 0. 288. The pH of the ME 18 formulation was 6.3±0.4. The viscosity of ME 18 was 89±0.2 cP and MME18 was 142 cP. PDI value less than 0.2 indicates uniform size distribution of globules. Drug content of microemulsion formulation was within the limits.

***Ex vivo* permeation studies**

The *ex vivo* permeation profiles of all design (17) formulations were shown in Fig 5. The flux, permeation coefficient (K_P) and enhancement ratio (ER) values were shown in Table 1. The permeation profile of optimized microemulsion (ME18), Mucoadhesive microemulsion (MME 18) and Drug solution were shown in Fig 6.

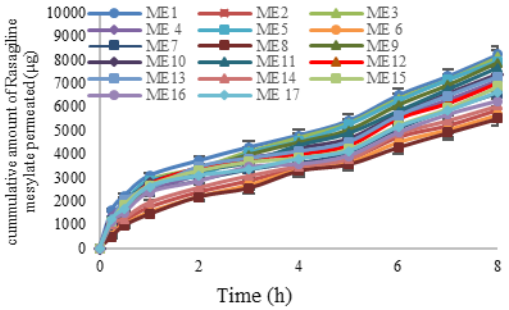

Fig.5.*Ex vivo* permeation profiles of rasagiline mesylate microemulsion formulations generated by Box Behnken design (mean ± SD, n=3)

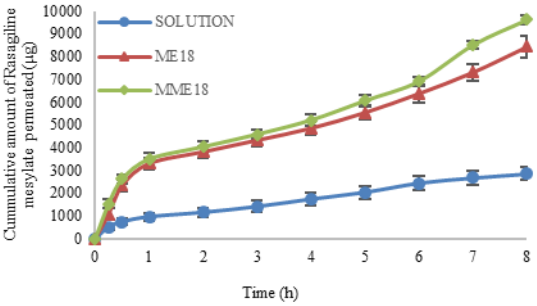

Fig.6.*Ex vivo* permeation profiles of microemulsion formulation (ME18), Drug Solution and Mucoadhesive microemulsion (MME18) (mean ± SD, n=3)

Flux values of 17 formulations were found to be 90.1 to 288 µg/cm²/h. The optimized formulation (ME18) showed flux of 291.7 µg/cm²/h. Mucoadhesive formulation MME18 showed flux of 323.1 µg/cm²/h which was significantly high ($p<0.0001$) compared to drug solution (75.9 µg/cm²/h) and the enhancement ratio of MME18 was 4.2 folds, and ME18 was 3.8 folds when compared with drug solution. The results clearly indicate the potential advantage of microemulsion formulation in improving the permeation of rasagiline mesylate through nasal mucosa. MME18 formulation containing chitosan improved flux by 10%.

Data analysis

The experimental values were analyzed using ANOVA and the results were shown in Table 2. The confirmation of model fit was by the difference between adjusted R^2 value and predicted R^2 value and P-Value (probability) greater than F-value [18]. The P-values for the responses size, zeta potential and flux were significantly high. Low PRESS value and high P-Value indicate the significance of the Quadratic model. The high F-value and non-significant lack of fit values of all the three responses indicate the model fitness. The difference between predicted R^2 value and Adjusted R^2 value was below 0.2 for all the three responses, indicating the validity of the model. Adequate Precision was used to measures the signal to noise ratio. It was greater than 4 for all the three responses, indicating a sufficient signal to noise move. The effect of independent factors on the dependent responses was quantified by polynomial equations, Contour plots and 3D response surface plots were shown in Fig.3 and Fig.4

Effect of formulation variables on response Globule size (Y1)

The polynomial equation for the quadratic model in coded factors for the response size was given below

$$Y1=+185.9+26.10A-9.70B+0.750C-3.0AB-2.85AC+8.40BC-12.08A^2+4.12B^2-2.22C^2$$

The A, BC, B^2, B and A^2 are significant model terms influencing globule size in the decreasing order. The oil (A) has higher positive effect on globule size, Smix (B) has negative effect on size and water has less positive effect on size. Higher concentration of oil at constant level of Smix leads to higher size of globules due to increase in interfacial tension. Increase in the Smix leads to reduction in interfacial tension that result in decrease in globule size [19].

Effect of formulation variables on response zeta potential (Y2)

The polynomial equation in coded factors of quadratic model for the zeta potential was given below

$$Y2= +24.60-5.00A+1.7B+0.0375C-0.0500AB+0.2500AC-1.47BC+1.51A^2-1.11B^2+0.0375C^2$$

In this case A, B, BC and A^2 are significant model terms. Smix (B) showed positive effect as Smix content increased zeta potential also increased and oil (A) showed negative effect on zeta potential as oil increases zeta potential decreased [20].

Effect of formulation variables on response Flux (Y3)

The polynomial equation in coded factors of quadratic model for the flux was given below

$$Y3 =+220.04-75.39A-20.36B+8.25C+22.70AB-0.9750AC-1.52BC-14.87A^2-6.37B^2-1.09C^2$$

In this case A, B, AB and A^2 are significant model terms. Oil (A), Smix (B) had negative

effect on formulation flux. As Oil and Smix increased within the studied range, formulation flux range was decreased. This is due to size of the formulation increased as a result nasal mucosa permeability was decreased and thermodynamic activity of the drug in the formulation was low which results in the reduction of flux respectively. Water has positive effect on flux, as water increased within the studied range flux also increased. This is due to hydration effect of water on the nasal mucosa leads to increase in the flux [21].

Check point analysis

The Quadratic model was validated by check point analysis. The compositions measured and predicted responses of check point formulations were shown in Table 3. The predicted values of size, zeta potential and flux were compared with the experimental values and the percentage prediction error was calculated. The percentage prediction error of all the check point formulations was below 5%, which indicates validity of the response surface model.

Formulation optimization

The desirability of the optimized formulation was 0.976. The composition of the optimized formulation (ME18) was oil (Capmul MCM), Smix (Tween 20: Transcutol P; 1:1) and water in the ratio of 5: 42: 65. The measured responses globule size, zeta potential, flux were 150.2 nm, -29.6 mV, 291.7 µg/cm²/h respectively. Flux and enhancement ratios of formulations were shown in Table 1. The formulation with chitosan as mucoadhesive agent showed significant increase (323.1 µg/cm²/h) in the flux. Optimized formulations ME18 and MME18 showed significantly higher flux compared to drug solution ($P < 0.0001$). Enhancement ratio of MME18 was 4.2 folds higher compared to drug solution [22].

Table.2. ANOVA and Regression values for quadratic model

Parameter	Source	D.F	S. S	M.S	F-value	P-value	Adeq. Pre	%C.V	PRESS
Size	Model	9	7253.08	805.90	77.94	<0.0001	30.3498	1.78	982.23
	Residual	7	72.38	10.34					
	Lack of fit	3	60.20	20.07	6.59	0.0500			
	Pure error	4	12.18	3.05					
ZP	Model	9	246.59	27.40	25.56	0.0002	16.9077	4.17	59.11
	Residual	7	7.50	1.07					
	Lack of fit	3	3.28	1.09	1.04	0.4664			
	Pure error	4	4.22	1.05					
Flux	Model	9	52570.97	5841.22	42.87	<0.0001	23.2720	5.57	12243.19
	Residual	7	953.71	136.24					
	Lack of fit	3	744.80	248.27	4.75	0.0831			
	Pure error	4	208.91	52.23					
R^2 Analysis	R^2			R^2 Adjusted			R^2 Predicted		Adj R^2-Pred. R^2
Size	0.9901			0.9774			0.8659		0.1115
ZP	0.9705			0.9325			0.7674		0.1651
Flux	0.9822			0.9593			0.7713		0.188

Note: DF: Degrees of freedom; **SS**: Sum of squares; **MS**: Mean squares; **CV**: Coefficient of Variation; **Adeq.Pre**: Adequate Precision; **PRESS**: Predicted Residual Error Sum of Squares; **ZP**: Zeta potential.

Table.3. Check point analysis of formulations

Formulation number	Formulation composition (A:B:C)	Response variable	Experimental value	Predicted value	Percentage prediction error
1	**5:30:57**	Y1	156.76	154.70	1.31
		Y2	-28.9	-29.037	-0.47
		Y3	280.43	282.78	-0.84
2	**5:58:65**	Y1	149.95	148.85	0.73
		Y2	-30.68	-31.14	-1.52
		Y3	286.36	284.49	0.65
3	**5:38:65**	Y1	147.31	149.20	-1.28
		Y2	-31.41	-30.55	2.70
		Y3	290.78	289.09	0.57
4	**5:48:57**	Y1	152.84	148.85	2.60
		Y2	-30.89	-31.14	-0.83
		Y3	287.8	284.49	1.14
5	**5:60:49**	Y1	149.8	147.46	1.56

		Y2	-32.29	-31.42	2.67
		Y3	271.62	273.34	-0.63
6	**5:53:65**	Y1	152.73	151.19	1.00
		Y2	-31.8	-30.77	3.23
		Y3	285.8	284.78	0.35

Note: Y1 – Size (nm), **Y2** – Zeta potential (mV), **Y3** – Flux (µg/cm²/h)

Stability studies

The optimized formulations ME18 and MME 18 were physically stable for three months and the data was shown in Table 4. There was no significant change observed in mean size, zeta potential and PDI values, after 3 months.

Table.4. Stability studies of ME18 and MME18.

S.No.	Size (nm)	Zeta potential (mV)	PDI
ME18 (1st day**)**	150.2	-29.6	0.137
ME18 (3rd month**)**	152.6	-28.2	0.143
MME18 (1st day)	176.4	+12.15	0.21
MME18 (3rd month)	180.3	+13.18	0.23

Conclusion

The rasagiline microemulsion formulations for brain targeting via nasal route were developed by Box Behnken statistical design. The flux of microemulsion (ME18) and mucoadhesive microemulsion (MME18) was found to be 291.7 µg/cm²/h and 323.1 µg/cm²/h respectively. Enhancement ratio of MME18 was 4.2 folds and ME18 was 3.8 folds compared to drug solution. The present study demonstrated the potential advantage of mucoadhesive microemulsion and microemulsion for the intranasal delivery of rasagiline mesylate.

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