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# Development of rasagiline mesylate loaded solid lipid nanoparticles in a thermosensitive mucoadhesive gel: Formulation design using DoE, *in-vitro* and *ex-vivo* characterization

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**ABSTRACT**: Rasagiline mesylate (RM) is a selective irreversible MAO-B inhibitor used in the treatment of Parkinson's disease. This study was designed to prepare and optimize RM loaded solid lipid nanoparticles (RM-SLNs) in a thermosensitive mucoadhesive gel (RM-SLNs-GEL). RM-SLNs were prepared combining Gelucire 50/13 (10%), Labrasol (0.3%) Cremophor RH40 (12%) with a mixing rate and time of 500 rpm, 45 min. Mucoadhesive gels were prepared combining Poloxamer 407 and HPMC E5 (15.5% + 0.25%). Optimized formulation (RM-SLNs-GEL) was evaluated for sol-gel transition temperature, viscosity, mucoadhesive force, particle size and distribution, SEM imaging, *in-vitro* drug release and *ex-vivo* drug permeation. It was found that optimal formulation had a suitable gelation temperature at 31°C ± 0.2°C. It was observed that the system was fluid during nasal application at 25°C and viscous at nasal temperature at 32°C. RM-SLNs-GEL has shown particle size, polydispersity index (PDI), % encapsulation efficiency (EE%); 253 nm, 0.282, 37.8% respectively. Ex-vivo permeation study exposed significant enhancement of permeability of RM-SLNs-GEL across mucosa than RM loaded thermosensitive gel (RM-GEL). Our results show that RM-SLNs-GEL formulation could be a potential drug delivery system for the treatment of Parkinson's disease.

**KEYWORDS**: Rasagiline mesylate; solid lipid nanoparticle; thermosensitive mucoadhesive gel; full factorial design; *ex-vivo* permeation.

## 1. INTRODUCTION

Parkinson's disease (PD) is a progressive, neurodegenerative disorder observed in the brain. 60% loss of dopaminergic neurons leads to the specific motor symptoms such as tremors, spasms, rigidity in the muscles, bradykinesia, hypersensitivity, disturbed posture and movement. Dopaminergic neurons are the main target of the drug treatment. There is no certain therapy to fix this dopamine loss but palliative treatments such as dopamine control are available. Dopamine receptor agonists such as levodopa, monoamine oxidase inhibitors (MAO-B) and catechol-o-methyl transferase (COMT) are used in the treatment of PD. It is known that 3% of the world population aged 50 and above have PD but the treatment is limited by the insufficiency in delivering therapeutic drugs to the brain [1, 2].

Rasagiline mesylate (RM) is a second-generation, selective, irreversible MAO-B inhibitor [3]. The inhibition of MAO-B activity leads to an increase in dopamine level [4]. RM is a BCS class III drug and has low permeability. There are only tablet formulations in the market and when taken orally, RM is rapidly absorbed but suffers from first pass metabolism and bioavailability decreases to 36% [5]. There are many studies to overcome this problem [6, 7].

The brain is separated from the bloodstream by a barrier. This structure known as blood brain barrier (BBB) which prevents the passage of many molecules to the brain. Therefore, the priority in the disorders observed in the brain is to exceed the BBB [8]. Nasal administration, one of the non-invasive methods for drug

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targeting to the brain, has been shown as an alternative way to the oral and parenteral routes. Nasal route provides direct access to the brain via olfactory and trigeminal nerve pathway [9]. Its advantages are that it eliminates the first pass effect, has large surface area for rapid absorption of the drug into the systemic circulation, enhances the bioavailability and targets drugs to the brain [10, 11]. However, through the anatomical condition of nasal cavity and mucociliary clearance, nasal formulations have a short residence time and absorption. This situation, which is a natural defense mechanism of the body against the foreign substance on the mucous membrane, can also expel the formulations applied to this area [12, 13]. One possible strategy to enhance nasal duration is to use formulations with higher viscosity and mucoadhesion.

Thermosensitive mucoadhesive *in-situ* gelling systems are fluid at room temperature. They are gelled due to the temperature on the application area [14]. This characteristic behavior makes them very popular to enhance residence time and improve the bioavailability of the formulations in the nasal mucosa. Nasal mucosa temperature varies between 30°C – 34°C (mean 32°C) depending on breathing [15]. The systems to be prepared are also required to gel between these temperatures. Since the application of the formulations can be done by drip or spray, dose can be adjusted easily [9, 16, 17].

Solid lipid nanoparticles (SLNs) are widely used drug delivery systems with a particle size of 10-1000 nm. They are bio-degradable and bio-compatible. Because of their lipid structure, both hydrophilic and lipophilic drug molecules can be encapsulated [18-20]. They can be applied parenterally, orally, topically and nasally [21-24].

The purpose of the study was to formulate RM loaded SLNs in a thermosensitive mucoadhesive gel system to eliminate hepatic first pass, enhance the bioavailability and targeting RM to the brain via intranasal route. In this study, RM loaded SLNs were formulated and these particles were combined with thermosensitive mucoadhesive *in-situ* gel system (RM-SLNs-GEL). The particle size, encapsulation efficiency, morphological properties, viscosity and mucoadhesive strength were investigated. *In-vitro* release, *ex-vivo* permeation studies were carried out.

## 2. RESULTS AND DISCUSSION

### 2.1. Preparation and optimization of SLNs

Formulations were optimized using full factorial design. During the cooling phase at 25°C, the system was mixed in a magnetic stirrer at a 500-750-1000 rpm for 30-45-60 min to prevent lipid particles from aggregation. Independent variables as mixing time and mixing rate were chosen on preliminary studies. As a result, it was observed that SLNs with the smallest particle size and polydispersity index (PDI) were obtained for three formulations at a rate of 500 rpm and a period of 45 min (Figure 1, Table 1).

**Table 1.** Particle size and distribution of SLN formulations (n = 3, mean ± s.d[a]).

| Formulations | Particle size (nm) | PDI |
|---|---|---|
| **F13** | 26.26 ± 2.888 | 0.511 ± 0.034 |
| **F35** | 79.21 ± 4.261 | 0.605 ± 0.033 |
| **F46** | 34.67 ± 2.102 | 0.687 ± 0.03 |

[a] Standard deviation.

F13 formulation was chosen due to the smallest particle size and smallest PDI for subsequent studies. RM was loaded into the formulation F13 and the particle size and PDI were re-determined. After loading RM, the particle size was found 166.3 nm ± 53.51 and PDI was found 0.276 ± 0.069.

### 2.2. Entrapment efficiency

The encapsulation efficiency of RM-SLN suspension was determined as 37.8% ± 0.596. Remaining RM was determined to be in the aqueous phase of the formulation. Encapsulation efficiency value was found to be 16-40% in SLNs prepared with conventional microemulsion technique [25, 26]. Our value is within these limits.

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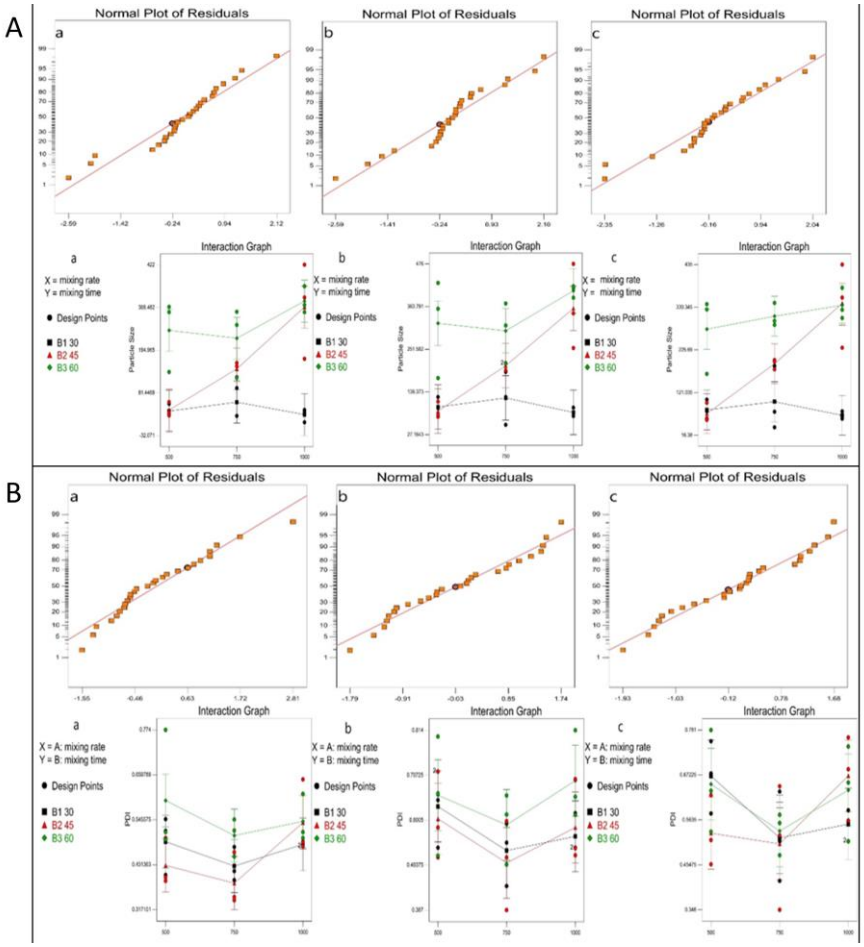


**Figure 1.** Effect of mixing time and rate on particle size (A) and PDI (B) of a) F13, b) F35, c) F46.

## 2.3. Lyophilization studies

Mannitol and trehalose were used as lyoprotectants. The study was carried out with SLN:lyoprotectant ratio of 1:0.1 and 1:0.2. Table 2 shows the particle size and PDI of the formulation before and after lyophilization.

**Table 2.** Particle size and distribution of RM-SLNs with mannitol and trehalose before and after the lyophilization (n=3, mean ± s.d).

| Formulation | Particle Size (nm) | | PDI | |
|---|---|---|---|---|
| | Before Lyophilization | After Lyophilization | Before Lyophilization | After Lyophilization |
| **A1 (SLN:Trehalose 1:0.1)** | 166.3 ± 53.5 | 439.8 ± 9 | 0.276 ± 0.069 | 0.274 ± 0.110 |
| **A2 (SLN:Trehalose 1:0.2)** | 166.3 ± 53.5 | 455.3 ± 14.4 | 0.276 ± 0.069 | 0.397 ± 0.005 |
| **A3 (SLN:Mannitol 1:0.1)** | 166.3 ± 53.5 | 183.1 ± 1.3 | 0.276 ± 0.069 | 0.301 ± 0.025 |
| **A4 (SLN:Mannitol 1:0.2)** | 166.3 ± 53.5 | 168.2 ± 3.6 | 0.276 ± 0.069 | 0.302 ± 0.014 |

The particle size of the formulation is important in targeting the particles to the brain, which is desired to be between 50-400 nm [18]. After the lyophilization process, it was determined that mannitol showed more suitable results in terms of particle size compared to trehalose. When the particle sizes were compared, it was observed that the particle size of the formulation lyophilized with mannitol (168.2 nm – 183.1 nm) was smaller than the particle size of the formulation lyophilized with trehalose (455.3 nm – 439.8 nm). For this reason, it was decided to continue the studies with mannitol. To determine the mannitol ratio, formulations were dispersed in distilled water. The time required for the dispersion of the formulation prepared with mannitol at a ratio of 1:0.2 was found to be shorter. It was decided to continue the study with a ratio of 1:0.2 (SLN:Mannitol). It has been observed that the lyophilization process slightly increases the particle size in SLNs [27, 28]. Findings in our study are consistent with this situation.

### 2.4. Preparation and measurement of sol-gel transition temperature of thermosensitive mucoadhesive gel

Solution containing 18% poloxamer 407 (P407) is gelled at approximately 32°C [29]. Since the optimum formulation will be applied to the nose, the gel system is desired to gel at the nasal temperature (30°C-34°C, mean 32°C). According to these results given in Table 3, the solution containing 17% P407 gels at the desired temperature.

**Table 3.** Gelation temperatures of poloxamer 407 in different percentage (n = 3, mean ± s.d).

| Poloxamer 407% | Gelation Degree |
|---|---|
| 15 | 39°C ± 0.5°C |
| 16.50 | 34°C ± 0.5°C |
| **17** | **32°C ± 0.5°C** |
| 17.50 | 29°C ± 0.5°C |
| 20 | < 25°C |

It is requested that the applied gel adheres to the mucosa and remains there. There are studies to increase the mucoadhesion power by adding HPMC E5 polymer to the prepared gel formulations [16,30]. HPMC E5 in different concentrations were added to the solution containing 17% P407. The gelation temperatures were determined (Table 4). As a result, the ratio of P407 17% and HPMC E5 0.25% polymers which gelled in the desired temperature range were determined.

**Table 4.** Gelation temperatures of mixed polymers (n = 3, mean ± s.d).

| Poloxamer 407% | HPMC E5% | Gelation Degree |
|---|---|---|
| 17 | - | 32°C ± 0.5°C |
| **17** | **0.25** | **31°C ± 0.5°C** |
| 17 | 0.50 | 29°C ± 0.5°C |
| 17 | 1 | 28°C ± 0.5°C |
| 17 | 2 | 27°C ± 0.5°C |

### 2.5. Preparation of the RM-SLNs-GEL

Lyophilized RM-SLNs were mixed with gel system (17% P407 + 0.25% HPMC E5) for an hour at 300 rpm and RM-SLNs-GEL was obtained. It was observed that RM-SLNs-GEL was a viscous system at room temperature and did not show fluidity. Therefore, P407 and HPMC E5 ratios were re-arranged and the gelation temperature was re-determined. It is known that the solid substances added to the thermosensitive mucoadhesive gel systems could change the gelation temperature of the formulations [31, 32]. This situation was also observed in our study. The reason is thought to be that the lyophilized SLNs added to the system enhance the hydrophobicity of copolymers in the gel system. Particle size and PDI of RM-SLNs-GEL were found as 253 nm ± 16.59; 0.282 ± 0.02 respectively.

### 2.6. DSC studies

In DSC studies, the melting degree of the substance varies as a result of a possible interaction which is an indication that the physicochemical properties of the substance are changing. Pure substance gives a single peak at the melting point. If the substance is decomposed, the location of the peak will change or more than one peak may be observed due to the decomposition product. The melting point of RM has been reported as 155°C – 158°C in the literature [33-35]. In our study, RM gave an endothermic peak at 158°C, indicating the melting point. RM was mixed with other substances used in the formulation at a ratio of 1:1 and examined in DSC. It was observed that the physicochemical properties of the substances were preserved. Additionally, on lyophilized RM-SLNs thermogram, the peak of the RM was observed which is an indication that not all of the RM was dissolved, a system with a heterogeneous matrix structure has been prepared and the active substance is re-crystallized during the cooling phase while SLNs are formed [36] (Figure 2).

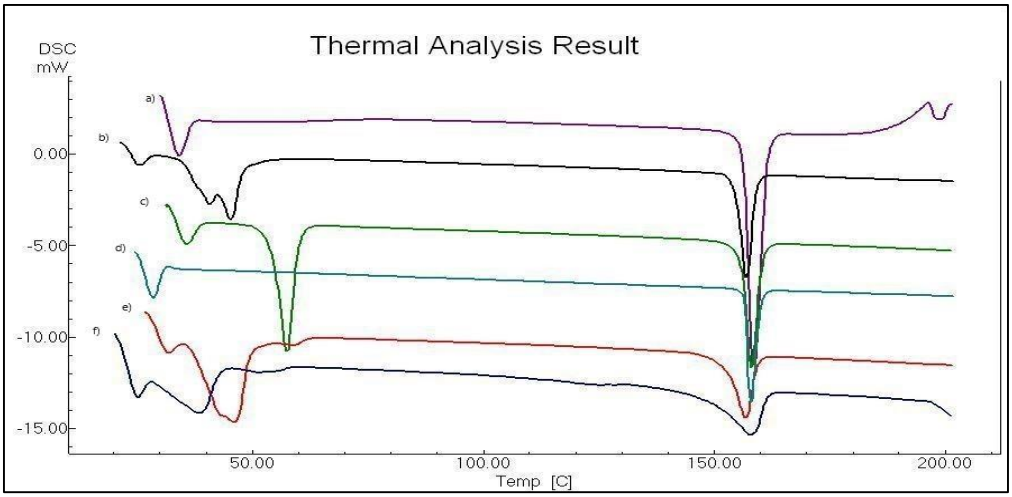


**Figure 2.** DSC thermograms of a) RM + HPMC E5, b) RM + P407, c) RM+ Gelucire 50/13, d) Physical mixture, e) RM + Mannitol, f) Lyophilized RM-SLNs.

### 2.7. Determining of flow properties and viscosity

Flow properties and viscosity of gel systems are important in terms of applicability. It is important that the formulations to be applied nasally are fluid at room temperature in terms of their ability to be sprayed or dropped. In contact with the nasal mucosa, it is desired to gel and increase in viscosity due to the temperature difference. Thus, the formulation is desired to remain in the nasal mucosa for a long time and help the diffusion of the active substance.

After the polymer ratios were arranged, flow properties and viscosities at 25°C and 32°C were investigated in RM-GEL and RM-SLNs-GEL (Table 5).

**Table 5.** Power law constant (K) and flow density index (n) of formulations.

| | Formulation | K (mPa.s) | n | $r^2$ |
|---|---|---|---|---|
| **25 °C** | **RM-GEL** | 353.4 | 0.853 | 0.906 |
| | **RM-SLNs-GEL** | 348307.029 | 0.261 | 0.997 |
| **32 °C** | **RM-GEL** | 114999.735 | 0.095 | 0.999 |
| | **RM-SLNs-GEL** | 654693.529 | 0.396 | 0.989 |

Pseudoplastic flow is observed in systems whose viscosity decreases as shear rate increases. This type of flow is generally observed in gel and emulsion systems. In this type of flow, a constant viscosity cannot be mentioned [31]. Since all gels showed pseudoplastic flow (Figure 3), Ostwald de Waele equation was used to calculate viscosity values. The n values obtained was found to be less than '1'. This indicated that the viscosity of the gel formulations decreases as they mix. Formulations showed shear thinning at both temperatures.

Viscosity of gels were higher at 32°C than 25°C, which is due to the thermosensitive property of gels. In addition, solid molecules added to the thermosensitive gel systems, can change the viscosity and gelation temperature of the formulations. There are studies showing that SLNs added to the gel systems increase the viscosity of the gels [31, 32]. This is thought to be due to the added particles settling in the spaces between the polymer chains and making polymer movement difficult. After the lyophilized SLNs were added to the gel system, increase of the viscosity in the systems confirms this phenomenon.

### 2.8. Measurement of adhesion properties

Mucoadhesive strength of RM-GEL and RM-SLNs-GEL were determined at 25°C and 32°C. Results are given in Table 6.

The absorption of drug molecules depends on the contact with the mucosa. Due to mucociliary clearance, the durations of the formulations are shortened, and the desired efficiency cannot be achieved [37]. Thanks to the mucoadhesive systems, the duration of the formulations in the nose can be increased and desired efficiency can be achieved.

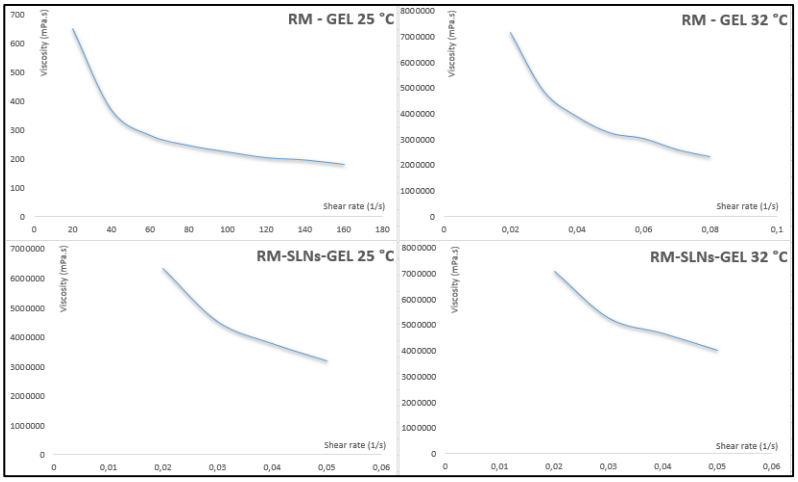


**Figure 3.** Shear rate versus shear stress profiles of gel formulations at 25°C and 32°C.

**Table 6.** Work of Adhesion of the gels at 25°C and 32°C.

| | Work of Adhesion (mJ/cm²) | |
|---|---|---|
| **25 °C** | **RM-GEL** | **RM-SLNs-GEL** |
| | 0.025 ± 0.002 | 0.357 ± 0.048 |
| **32°C** | **RM-GEL** | **RM-SLNs-GEL** |
| | 0.041 ± 0.003 | 3.511 ± 0.381 |

As a result of the studies a significant difference was observed between the formulations at both 25°C and 32°C ($p < 0.05$). It was observed that the work of adhesion increased, although viscosity increases with the temperature increase. It was determined that the presence of SLNs in the gel system increased the adhesion strength of the gel. This increase in high temperature was even more pronounced. RM-SLNs-GEL shows high muco-adhesion as expected. Increase in the work of adhesion was related to the concentration of the dispersed phase in the formulation. The dispersed phase leads to an increase of the particle – particle interaction, resulting more rigid structure. As a result, adhesion increases [38].

### 2.9. SEM imaging

The SEM image of the RM-SLNs-GEL was given in Figure 4. Spherical nanoscale particles were monitored.

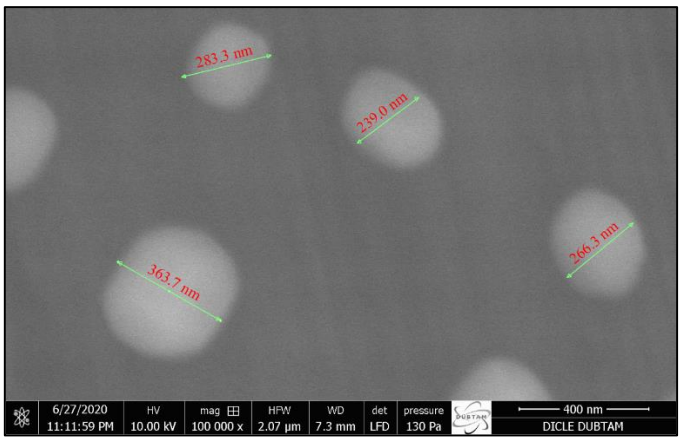


**Figure 4.** Scanning electron microscopy image of RM-SLNs-GEL.

### 2.10. *In-vitro* drug release studies

*In-vitro* release study was carried out in RM-GEL, RM-SLN suspension and RM-SLNs-GEL formulations. The release of RM from different formulations was given in Figure 5.

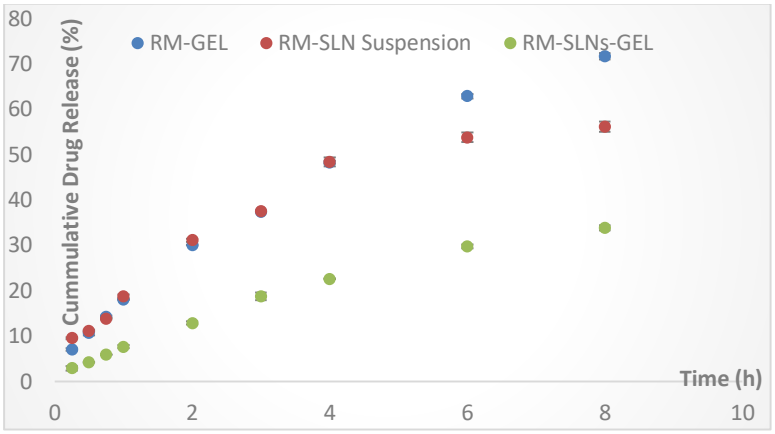


**Figure 5.** Drug release profiles of formulations (n = 3, mean ± s.d).

The system contains both gel and SLNs. In addition to the release of the active substance from spherical particles, delayed release and diffusion should be considered together. In such systems, generally Zero-order, First-order, Higuchi and Hixon Crowell models are examined [39, 40]. Considering all these, it has been determined that the kinetic model that expresses the formulations best is the Higuchi matrix kinetic model (Table 7). The SLNs loaded gels prepared by Kesharwani et al. for use in the treatment of arthritis showed Higuchi kinetics [40]. Similarly, SLNs prepared by Anjum et al. for use in HIV treatment also showed Higuchi kinetics [41]. It can be concluded that the formulation has diffusion controlled release.

**Table 7.** Higuchi kinetic parameters (n = 3, mean ± s.d).

| x = √hour<br>y = mg/cm² | RM-GEL | RM-SLN suspension | RM-SLNs-GEL |
|---|---|---|---|
| **Slope (mg/cm²/√hour)** | 10.506 ± 0.273 | 8.271 ± 0.546 | 5.123 ± 0.150 |
| **intercept (mg/cm²)** | -3.623 ± 0.459 | -1.075 ± 0.919 | -2.090 ± 0.253 |
| **r²** | 0.995 | 0.97 | 0.994 |

When released % amounts of RM for each formulation were examined, a ranking was obtained such as RM-GEL>RM-SLN suspension>RM-SLN-GEL. According to this ranking, viscosity was thought to affect the release rate. The solidification of the formulations due to the increase in viscosity limits the outflow of the RM form the formulation This affects the amount of RM diffused. Findings was also confirmed in the literature [42-44].

### 2.11. *Ex-vivo* drug permeation studies

The cumulative amount of RM permeated though the mucosa per time was given in Figure 6.

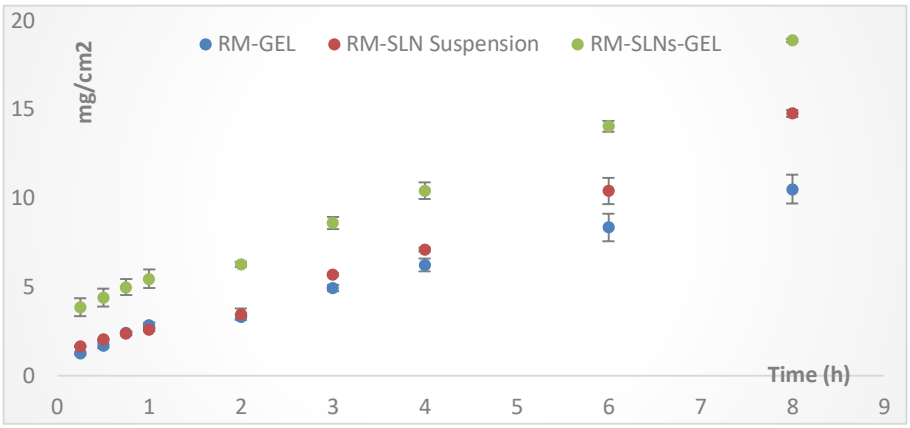


**Figure 6.** *Ex-vivo* permeation profiles of the formulations through nasal mucosa (n = 3, mean ± s.d).

It is important to calculate the permeability of the mucosa to the active substance. This provides information on whether RM crosses the mucosa in *in-vivo* experiments. Permeability and flux values of the formulations were calculated and compared (Table 8). A significant difference between 3 formulations was determined ($p < 0.05$). It was determined that the permeation of RM through the nasal mucosa increased with RM-SLNs-GEL.

**Table 8.** Permeability and flux values of the formulations.

| | RM-GEL | RM-SLNs Suspension | RM-SLNs-GEL |
|---|---|---|---|
| **Permeability (cm/hour)** | $58.4x10^{-3}$ | $82.6x10^{-3}$ | $93.6x10^{-3}$ |
| **Flux ($mg/cm^2$/hour)** | 1.168 | 1.651 | 1.872 |
| **$r^2$** | 0.993 | 0.990 | 0.992 |

It is known that *in-vitro* membrane permeability of the drug delivery system and drug molecules increases due to the increase in lipophilicity [45]. It is clear that oil, surfactant and co-surfactants in the structure of SLNs facilitate the permeation of the active substance into tissues [46, 47]. Furthermore, it has been reported that budesonide loaded SLNs show 3-4 times more permeability than the market preparation due to the small particle size of the SLNs, their mucoadhesive properties and their increased permeability [48]. In our study, higher rate of the RM permeation of RM-SLNs-GEL than RM-GEL and RM-SLN suspension can be explained by these conditions.

### 2.12. Statistical analysis

All results were statistically analyzed using one-way ANOVA through excel.

## 3. CONCLUSION

Oral drug delivery is the most desirable application route but it fails to deliver some therapeutics to the brain efficiently. However, there is a blood-brain barrier which localized in the brain and separates brain interstitial fluid from the blood, which basically protects the brain from neurotoxic substances, unwanted cells and also therapeutic drugs. Furthermore, intranasal drug delivery is becoming very popular. *In-vitro* studies, targeting efficiency and brain localization will be researched in further studies. It is obvious that RM-SLNs-GEL systems could be useful for promising strategy to targeting brain via nasal route for the treatment of Parkinson's disease.

## 4. MATERIALS AND METHODS

### 4.1. Preparation and optimization of SLNs

In this study, based on Gasco's patent [49], modified microemulsion method was used to create SLNs. Briefly, Gelucire 50/13 was heated to 55°C. RM was added to this melted lipid (oil phase). Cremophor RH40, Labrasol and distilled water were mixed together (aqueous phase) and heated to 55°C. Phases were mixed. Hot liquid mixture was stirred at 500 rpm for 45 min at 25°C with the magnetic stirrer (IKA-RCT, Germany) until the system cooled. As a result, SLN suspension was created.

Design Expert program 6.0.6 (Stat-Ease Inc. Minneapolis, MN) was used to create SLNs. Three factor (oil, surfactant, co-surfactant), four-five level full factorial design (FFD) was employed for the optimization of SLNs using Gelucire 50/13 (10-20-30-40%), Cremophor RH40 (12-15-20-25-30%) and Labrasol (3-5-10-15%) as independent variables. Total 80 experimental runs were acquired with different independent levels. The formation of SLN systems were selected as dependent response. Table 9 shows the formulations that can be formulated into SLN.

Two factor as mixing time and rate and three level (30-45-60 min, 500–750-1000 rpm) with 3 replicated FFD were employed for the optimization of mixing time and mixing rate of SLN formulations. The particle size and PDI were selected as dependent responses. The diagrams were created by the software and used to evaluate the independent factor of each response. The mixing rate and mixing time, giving the lowest particle size and PDI were determined.

**Table 9.** Contents of SLN suspensions.

| Formulations | Oil% | Surfactant% | Co-surfactant% | Water% |
|---|---|---|---|---|
| **F13** | 10 | 12 | 3 | 75 |
| **F35** | 10 | 15 | 3 | 72 |
| **F46** | 10 | 12 | 5 | 73 |

### 4.2. Particle size and distribution

Dynamic light scattering was employed to measure the particle size and distribution of SLN suspension with the Malvern Zetasizer (Malvern industries, UK).

### 4.3. Encapsulation efficiency

Analytical method linearity is an indication that the response obtained is proportional to the sample concentrations in the specified concentration range. Stock solutions of RM were prepared to demonstrate the linearity parameters. Studies were carried out in UV spectrophotometry (Shimadzu UV-1600, Japan) at different concentrations 0.125 µg/ml – 300 µg/ml of RM. Equation was determined by linear regression. As a result of these studies, limit of detection (LOD) and limit of quantification (LOQ) were found to be 13.482 µg/ml and 44.941 µg/ml respectively.

RM loaded and unloaded SLN suspensions were prepared and immediately were centrifuged (Hitachi CS 150 GLX, Japan) at 18000 rpm for 1 hour at 25°C. After the centrifugation, SLNs were stuck to the centrifuge tube walls and supernatants were taken. In UV spectrophotometry, absorbances were read at 265 nm against blank SLN suspension supernatant (n = 3, mean ± s.d). Entrapment efficiency was calculated by the equation (Eq. 1) given below [50].

$$(X_1 - X_2)/X_1 \; x\, 100 \qquad \text{(Eq. 1)}$$

$X_1$ is the amount of RM used in SLNs and $X_2$ is the amount of RM detected in supernatant.

### 4.4. Lyophilization studies

Lyophilization process was carried out in a lyophilizer (Christ Alpha 1-2 LD Freeze Dryer, Germany) at a pressure of 0.021 atm at -55°C for 48 hours. Mannitol and trehalose were examined as lyoprotectant. After the procedure, lyophilized powder was dispersed in 30 g of distilled water. The effect of lyoprotectant on particle size and PDI was investigated.

### 4.5. Preparation of thermosensitive mucoadhesive gel

Poloxamers are copolymers of polyoxypropylene surrounded by 2 hydrophobic polyoxyethylene chains. It is used safely in formulations and its aqueous solutions become thermosensitive gels according to its concentrations. These features make it frequently used in the development of formulations with different properties such as extended release, delayed release or thermosensitive gels [14, 51]. HPMC is also non-toxic ingredient and exhibits a thermal gelation property as Poloxamers [9].

This study was performed by using cold method [52]. Briefly, P407 and HPMC E5 polymers were added to distilled water and mixed at 300 rpm. Mixing was carried out in a refrigerator at +4°C for overnight. Thus, a clear and transparent gel was obtained.

### 4.6. Measurement of sol-gel transition temperature

To determine sol-gel transition temperature, the magnetic bar method was used [53]. Briefly, polymer solutions were placed in a water bath on a magnetic stirrer with the heater at 50 rpm. Temperature was increased by 0.5°C/min. The point where the magnetic bar was halted during the rotation was determined as the gelling temperature.

### 4.7. Preparation of the RM-SLNs-GEL system

Thermosensitive mucoadhesive gel system and lyophilized RM-SLNs were mixed at room temperature and RM-SLNs-GEL was obtained. The gelation temperature, flow property, viscosity, mucoadhesive strength and particle size studies were carried out.

### 4.8. DSC studies

Studies were carried out in DSC at 10°C/min scanning speed, up to 200°C. Thermogram of the mixtures of plane substances were taken. For this, samples were weighted and put into aluminum sample containers and placed in the heating cell of DSC. Experiments were carried out in the temperature range of 25-200°C.

### 4.9. Determining of flow properties and viscosity

RM-GEL, RM-SLN suspension and RM-SLNs-GEL viscosities were determined with a rotational viscometer (Brookfield DV-III Rheometer, UK, Spindle no: 52). Tests were performed at both 25°C and 32°C. the flow curves defined by Ostwald de Waele equation (Eq. 2) were given below:

$$\eta = K\gamma^{n-1} \qquad \text{(Eq. 2)}$$

η is power law constant, K is flow density index (mPa.s) and γ is shear rate (1/h).

### 4.10. Measurement of adhesion properties

Adhesive properties of RM-GEL and RM-SLNs-GEL were determined by TA.XT plus texture analyzer (Stable Micro Systems, UK). Nasal mucosa was obtained from slaughterhouse and stored at -80°C. on the experiment day, mucosa was removed from the freezer and kept in simulated nasal fluid (SNF, pH = 5.5) at 25°C [54]. 2 mm thick section of mucosa was attached to the lower end of the cylindrical probe with the cyanoacrylate glue. The surface area of attached mucosa was 1.1 $cm^2$. The experimental working speed was 1 mm/s and the force applied to nasal mucosa surface was determined as 0.2 N. The contact time of the mucosa with the formulation was determined as 30 seconds [55]. Measurements were made at both 25°C and 32°C. Work of adhesions were calculated by the instrument software.

### 4.11. SEM imaging

SEM imaging was used to monitor the morphological properties of RM-SLNs-GEL. Images were taken at Dicle University DÜBTAM SEM Laboratory. For imaging, the formulation was diluted 10000 times, dropped on aluminum foil and dried at 25°C for 24 hours. Dried formulation was fixed on the grid with double sided carbon tape. Measurements were made under low vacuum with 40000-100000 magnification at 10 kV.

### 4.12. *In-vitro* drug release studies

In this study, RM-SLN suspension, RM-GEL and RM-SLNs-GEL formulations were used. Franz diffusion cells with a volume of 16 ml and surface area of 1.1 $cm^2$ were used to examine the diffusion of the RM through the dialysis membrane. Briefly, the donor cells were filled with formulations containing 40 mg of RM. Dialysis membrane with the cut-off value of 12000 was used as diffusion membrane. Receptor cell was filled with 16 ml buffer of SNF. Sink condition was maintained during the drug release studies. Studies were carried out at 32°C with stirring rate of 300 rpm. Samples of 1-2 ml, with instant replacement of equal amount of fresh SNF, were collected at specified time intervals (15, 30, 45, 60, 120, 180, 240, 360, 480 min). The concentration of RM in samples was determined with the UV spectrophotometer. For the determination of the release kinetic model; Zero-order, First-order and Higuchi models were analyzed. The highest coefficient value ($r^2$) was referred as the kinetic model of drug release (n = 3, mean ± s.d).

### 4.13. *Ex-vivo* drug permeation studies

RM permeation through nasal mucosa from RM-SLN suspension, RM-GEL and RM-SLNs-GEL were determined using the same conditions described in *in-vitro* studies. Sink condition was maintained during the drug permeation studies. Bovine nasal mucosa, obtained from the slaughterhouse, was cleaned and stored at -80°C freezer. At the time of the experiment, nasal mucosa was taken out, kept in SNF, cut in appropriate size and placed in the Franz diffusion cells. Flux (J) was determined by plotting the cumulative amount of the RM permeated versus time. The permeability coefficient (P) was calculated. Equations (Eq. 3, Eq. 4) were given below:

$$M/S = (D.K.Cd/h)\ x\ t \qquad \text{(Eq. 3)}$$

$$P = D.K/h \qquad \text{(Eq. 4)}$$

Where M is the amount of RM diffusing through the mucosa (mg), S is the surface area of the membrane where the transition occurs ($cm^2$), D is the diffusion coefficient of RM ($cm^2$/time), K is the partition coefficient of RM, Cd is the concentration of RM in donor cell, h is the thickness of the membrane, t is the time (hour), (n = 3, mean ± s.d).

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