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Preparation of nanoparticle and nanoemulsion formulations containing repaglinide and determination of pharmacokinetic parameters in rats

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ABSTRACT

Repaglinide (RPG) belongs to the class of drugs known as meglitinides and is used for improving and maintaining glycemic control in the treatment of patients with Type 2 diabetes. RPG is a Class II drug (BCS) because of its high permeability and low water solubility. It also undergoes hepatic first-pass metabolism. The oral bioavailability of RPG is low (about 56 %) due to these drawbacks. Our aim in this study is to prepare two different nano-sized drug carrier systems containing RPG (nanoparticle: RPG-PLGA-Zein-NPs or nanoemulsion: RPG-NE) and to carry out a pharmacokinetic study for these formulations. We prepared NPs using PLGA and Zein. In addition, a single NE formulation was developed using Tween 80 and Pluronic F68 as surfactants and Labrasol as co-surfactant. The droplet size values of the blank-NE and RPG-NE formulations were found to be less than 120 nm. The mean particle sizes of blank-Zein-PLGA-NPs and RPG-Zein-PLGA-NPs were less than 260 nm. The C_{max} and t_{max} values of RPG-Zein-PLGA-NPs and RPG-NE (523 ± 65 ng/mL and 770 ± 91 ng/mL; 1.41 ± 0.46 h and 1.61 ± 0.37 h, respectively) were meaningfully higher than those of free RPG (280 ± 33 ng/mL; 0.72 ± 0.28 h) ($p < 0.05$). The $AUC_{0-\infty}$ values calculated for RPG-Zein-PLGA-NPs and RPG-NE were approximately 4.04 and 5.05 times higher than that calculated for free RPG. These nanosized drug delivery systems were useful in increasing the oral bioavailability of RPG. Moreover, the NE formulation was more effective than the NP formulation in improving the oral bioavailability of RPG ($p < 0.05$).

1. Introduction

Type 2 diabetes (T2D), which causes unusual blood glucose levels (elevated levels), is an increasingly common condition in adults and, in recent years, in children. Ineffective insulin use in T2D caused primarily by physical inactivity and obesity. Optimization of glycemic control is critical in the treatment of T2DM in minimizing the risk of diabetic complications (such as microvascular complications) (Kaplan et al., 2023).

RPG, a carbamoylmethyl benzoic acid derivative belonging to the class of drugs known as meglitinides, is used to improve and maintain glycemic control in the treatment of patients with T2D (Scott., 2012). RPG stimulates insulin secretion by blocking ATP-dependent potassium channels in pancreatic β-cells in order to lower blood sugar levels. RPG can be used alone (monotherapy) as an adjunct to dietary control and exercise or in combination with other antihyperglycemic agents (except sulfonylureas) (Milner and Akhondi, 2023).

RPG is classified as a "Biopharmaceutics Classification System (BCS)" Class II drug because of its high lipophilicity (logP 3.97), high permeability, and low water solubility (about 34 μg/mL at 37 °C). Drugs in Class II do not dissolve easily (Amidon et al., 1995; Albetawi et al., 2021) and, therefore, may not be adequately absorbed. RPG undergoes hepatic first-pass metabolism besides its high lipophilicity/low solubility, leading to poor oral bioavailability (about 56 %) (Kaplan et al., 2023; Milner and Akhondi, 2023). RPG has inconsistent pharmacokinetics, which makes it challenging to achieve optimal therapeutic outcomes. RPG is rapidly absorbed from the gastrointestinal tract (Milner and Akhondi, 2023). The hypoglycemic effect of RPG is rapid but short-lived because of its short plasma half-life (approximately one hour). Therefore, RPG is usually taken several times a day to improve glycemic control. However, increased frequency of dosing may result in poor patient compliance (Fouad et al., 2023).

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The oral route is considered one of the most convenient and still preferred drug administration route due to factors such as patient compliance and ease of use. On the other hand, the challenges encountered in oral drug delivery include poor drug solubility in water, first-pass metabolism, and poor drug bioavailability. To overcome these limitations, nano-sized drug delivery systems (such as nanoemulsion, and polymeric nanoparticles) have been developed (Shrivastava et al., 2020; Alqahtani et al., 2021). In particular, the particle size (PS) and surface properties of nano-sized dosage forms have made them attractive because these properties provide them with advantages such as improving the solubility of poorly water-soluble drugs and enhancing their bioavailability (Demirtürk et al., 2022). Polymeric nanoparticles (NPs), colloidal particles with sizes less than 1000 nm, are prepared using natural polymers (chitosan, Zein, etc.) or synthetic polymers [poly (lactic-co-glycolide) (PLGA), poly(glycolic acid) (PGA), etc.]. The use of nanoparticles offers several advantages, including improved drug solubility, increased drug stability, sustained drug release, improved bioavailability, reduced side effects, and enhanced therapeutic efficacy (Zielińska et al., 2020; André de Almeida Campos et al., 2023).

Lipidic carrier systems (liposomes, microemulsions, nanoemulsions (NEs), etc.) have also been developed for oral administration of drugs with poor water solubility (BCS classes II and IV drugs) because these systems primarily increase the dissolution rate and solubility of these drugs in the gastrointestinal tract (Alqahtani et al., 2021).

NE formulations or polymeric NPs containing RPG have been prepared for oral use in the literature to overcome the drawbacks as mentioned above of RPG (Kaplan et al., 2023; Wadhwa et al., 2023; Akhtar et al., 2016; Karami et al., 2020). Wadhwa et al. (2023) prepared RPG-loaded poly(ethylene glycol)-poly- ε-caprolactone (PEG-PCL) NPs with a particle size of 112.5 nm and zeta potential of about -6 mV. They also carried out a pharmacokinetic study in Wistar rats. Compared to free RPG (at a dose of 4 mg/kg, p.o.), the use of NPs containing RPG (equivalent to 4 mg/kg RPG, p.o.) resulted in a 2.46-fold and 1.25-fold increase in t_{max} and C_{max}, respectively (Wadhwa et al., 2023).

In another study, RPG-NEs [average droplet size (DS): 86.5 nm and zeta potential (ZP): -33.8 mV) or chitosan-coated RPG-NEs (average DS: 149.3 nm and ZP: + 31.5 mV] formulations were developed and pharmacokinetics parameters for these formulations after oral administration to Sprague–Dawley rats were evaluated. It has been emphasized that the use of chitosan-coated RPG-NEs may be a promising approach for the oral delivery of RPG (Karami et al., 2020).

In this study, we aimed to develop RPG-NEs and RPG-Zein-PLGA NPs and to carry out a pharmacokinetic study of these formulations. We prepared RPG-Zein-PLGA NPs using PLGA (an FDA-approved biodegradable polymer) and Zein (a protein of natural origin that is "generally recognized as safe-GRAS" by FDA) polymers.

2. Materials and methods

2.1. Materials

The materials used in this study were as follows: RPG (as a generous gift; İlko İlaç San. Tic. A.S., Türkiye), PLGA (50:50, Resomer RG 502 H, Ave. Mw 7000-17000 Da; Sigma-Aldrich, Germany), Zein (Sigma-Aldrich, USA), Polyvinyl alcohol (PVA, MW 30000-70000; Sigma-Aldrich, USA) Labrasol (as a generous gift; Gattefosse, France), Ethyl oleate (Merck, Germany), Tween 80 (Merck, Germany), Pluronic F68 (BASF, Germany). All other materials used were analytical grade.

2.2. Methods

2.2.1. Preparation of the RPG-NE

RPG-NE was prepared using an oil phase [ethyl oleate (10 %), Labrasol (5 %), and RPG (10 mg)] and aqueous phase [Tween 80 (2 %), Pluronic F68 (1 %) and ultrapure water]. First, to prepare the coarse emulsion, the aqueous phase was added to the oil phase on a magnetic stirrer. Later, this emulsion was homogenized firstly using an Ultraturrax T10 (25000 rpm, 5 min; IKA, Germany) and then sonicated using a probe-type ultrasonicator (40 % power, 15 min; "Sonoplus HD 2070, Bandelin Electronics, Germany") to obtain nano-sized droplets.

Blank NE (B-NE) was prepared using the method mentioned above without RPG.

2.2.2. Preparation of the RPG-Zein-PLGA NPs

A slightly modified emulsion-solvent evaporation method was used to prepare RPG-Zein-PLGA NPs. Briefly, PLGA (75 mg) and RPG (25 mg) were dissolved in dichloromethane. Zein (25 mg) was dissolved in dichloromethane:ethanol (1:1, v/v). Afterwards, these two solutions were mixed to obtain the organic phase (OP). The OP was added dropwise to PVA aqueous solution (3 %, w/v) and homogenized using the Ultraturrax T10 (25000 rpm, 5 min). Then, the mixture was sonicated using a probe-type ultrasonicator (60 % power, 5 min). The RPG-Zein-PLGA NPs dispersion was centrifuged (12500 rpm, 40 min, 15 °C) after removing the organic solvents under reduced pressure. The prepared RPG-Zein-PLGA NPs were lyophilized for 24 h (Martin Christ, Alpha 1-2 LD Plus, Germany).

Blank Zein-PLGA NPs (B-Zein-PLGA-NPs) were prepared according to the above-mentioned method without RPG.

2.2.3. Characterization studies for NEs and NPs

The TEM (Hitachi HighTech HT7700, Japan) images of NEs and NPs were obtained. "Zetasizer Nano ZSP (Malvern Ins. Ltd, UK)" was used to measure the PS of NPs and the DS of NEs (at a scattering angle of 173°) as well as the ZP values of these formulations after appropriate dilutions.

The RPG content in the RPG-NE and RPG-Zein-PLGA-NPs was determined as follows: 1). After weighing 0.5 g of RPG-NE, the volume was made up to 10 mL (in a volumetric flask) with methanol and then stirred on a magnetic stirrer for 15 min. Later, the mixture was filtered (PTFE membrane filter; 0.45 μm). 2). 20 mL DCM:ethanol (1:1, v/v) mixture was added to the lyophilized RPG-Zein-PLGA-NPs and kept in an ultrasonic bath for 15 min. Then, the mixture was stirred on a magnetic stirrer for 3 h and filtered (PTFE membrane filter; 0.45 μm). The RPG contents of all the samples were determined using a validated HPLC method (Kaplan and Cetin, 2023). Entrapment efficiency percent (EE %) values were calculated for RPG-NE and RPG-Zein-PLGA-NPs [Used equation: (RPG amount in the formulation/initial RPG amount) x 100].

2.2.4. In vitro release studies

The release studies were performed in HCl (pH 1.2) with 0.1 % Tween 80 or phosphate buffer (PB, pH 6.8) with 0.1 % Tween 80 using the dialysis bag method. Free RPG or NPs (NPs were dispersed in 1 mL of release medium) or NEs (1 mL) were placed in a dialysis bag (MWCO 14000 Da; Sigma-Aldrich, USA). The dialysis bags were immersed in the release media (50 mL) in vials at 37 ± 0.5 °C and shaken at 50 rpm in a horizontal shaking water bath. 1 mL of sample was withdrawn and replaced with the same volume of fresh release medium to maintain "Sink Condition" at specified time intervals. All samples were filtered (PVDF membrane filter; 0.45 μm). The RPG contents of all samples were determined using a validated HPLC method (Kaplan and Cetin, 2023).

2.2.5. Pharmacokinetic study

The animal study was approved (Date and number 22 April 2022 and 3/12) by the local ethics committee of Çukurova University. In the current study, male rats (Albino Wistar; 310 ± 25 g, 4–6 months), which were obtained from the "Medical Experimental Application and Research Center of Çukurova University", were randomly divided into three groups (6 rats/group; total 18 rats) and housed in customary lab setups. Before the pharmacokinetic study, the rats were fasted overnight (with free access to water) to reduce the impact of food on the pharmacokinetic profile. Following an overnight fast, drug administration (the suspension of free RPG or RPG-Zein-PLGA-NPs in 0.5 % sodium carboxymethyl cellulose solution or RPG-NE formulation) to all healthy

rats was made orally by gavage at a dose equivalent to 2 mg/kg RPG. The rats were fed again half an hour after drug administration. Following drug administration, blood samples were collected from the femoral vein of the rats into heparinized microcentrifuge tubes at various time intervals (0.25, 0.5, 1, 2, 4, 8, 12, 24 and 48 h) and centrifuged (5000 rpm; 5 min). We kept the plasma samples at -80 °C until analysis.

2.2.6. HPLC method for the determination of RPG in rat plasma

For the pharmacokinetic study, we used the HPLC method developed by Pattanaik et al. (2013) with some modifications. This method was validated on the parameters (specificity, sensitivity, linearity, accuracy and precision) described in the ICH guideline (ICH Topic Q 2 (R1), 2021; FDA Guidance, 2022). We gave the optimum chromatographic conditions in Table 1. The RPG stock solution was prepared in methanol (100 μg/mL). The stock solution was diluted appropriately with the mobile phase to obtain the working solution (0.5 μg/mL). The certain amounts of the RPG working solution were added to the blank plasma samples collected from RPG-free rats to prepare calibration curve samples (40-200 ng/mL). The calibration curve was created by plotting the measured peak areas (mAU) versus the RPG concentrations, and it was then fitted using least squares linear regression analysis ($n = 6$). The HPLC method selectivity was assessed by contrasting the chromatograms of the blank plasma samples collected from RPG-free rats with the chromatograms of the plasma samples (6 repetitions) spiked with RPG standard solutions (50 ng/mL, 100 ng/mL and 150 ng/mL). Internal standards were not necessary since the amount of material analyzed remained stable, and the instrument response remained constant from run to run. In addition, the limit of detection [LOD=3.3x(σ/S); S= the slope of the calibration curve and σ= the standard deviation (SD) of the intercept] and limit of quantification [LOQ=10x(σ/S)] were calculated (ICH Topic Q 2 (R1), 2021; Shabir, 2003). The standard solutions of RPG prepared at three different concentrations (50 ng/mL, 100 ng/mL, and 150 ng/mL) were analyzed six times within the same day (intra-day) and six times on three different days (inter-day) to determine intra- and inter-day precision (as percent relative standard deviation, RSD%) and accuracy (as relative error, RE) (Shabir, 2003).

2.2.7. Plasma sample pretreatment and pharmacokinetic analysis

A minor modification was made to the study by Zhang et al. (Zhang et al., 2011) plasma sample pretreatment technique. This approach is recommended because it is practical in terms of equipment and solvents utilized, and the active substance can be readily extracted from the plasma. The plasma samples stored at -80 °C were defrosted at room temperature and vortexed (30 sec). Then, 3 mL of diethyl ether-dichloromethane (60:40, v/v) and 25 μL of water-methanol (50:50, v/v) were added to 25 μL of the plasma samples taken into a glass tube and vortexed (30 sec). The mixture was centrifuged at 12000 rpm (5 min) after vortexing. Then, the supernatant was transferred to a clean glass tube and dried at 40 °C under a moderate stream of nitrogen. HPLC analysis was performed (by injecting 20 μL) after the residue was reconstituted in 100 μL of mobile phase.

Utilizing the Kinetica software ("v4.4; Thermo Fisher Scientific, Waltham, MA, USA"), pharmacokinetic analysis was carried out. Experimental observations were used to derive these values, including $t_{1/2}$, t_{max}, C_{max}, and $AUC_{0-\infty}$. The following equation was used to compute the relative bioavailability (Frel).

$$F_{rel}(\%) = \frac{AUC_{for\ RPG-containing\ formulation\ (RPG-NE\ or\ RPG-Zein-PLGA-NPs)}}{AUC_{for\ free\ RPG\ suspension}} x100$$

Table 1
The optimum HPLC conditions for RPG analysis in rat plasma.

Device, detector and column	Shimadzu Nexera 2, Japan; Diode Array Detector (PDA/DAD); C18 ("4.6 × 250 mm, 5 μm, GL Sciences")
Mobile phase and wavelength	Methanol:0.01 M ammonium acetate buffer, pH: 4 (80:20, v/v); 242 nm.
Injection volume, flow rate and temperature	20 μL; 1 mL/min; 25 °C

3. Statistical analysis

The experimental results were presented as mean±standard deviation (SD). The "Independent t-test" (SPSS Statistics Version 22.0 software; SPSS Inc., USA) was used to compare the results obtained for PS or ZP in the *in vitro* characterization study. $AUC_{0\text{-tlast}}$ was determined using the linear-log trapezoidal rule in the pharmacokinetic study. $AUC_{0\text{-tlast}}$ and the extrapolated area were added together to create $AUC_{0\text{-}\infty}$. An estimate of the terminal disposition rate constant (λ_z) was created by regressing the terminal log-linear plasma concentration time points.

4. Results

In this study, we successfully prepared NP and NE formulations and first carried out *in vitro* characterization studies. We took the TEM images of the RPG-NE and RPG-Zein-PLGA-NPs formulations (Fig. 1a and 1b). The TEM images showed that nano-sized droplets (for NEs formulation) were almost spherical, and nanoparticles (for NPs formulation) were almost spherical. We also performed the ZP and PS/DS measurements of the formulations and gave the measurement results in Table 2. The DS values of NE formulations (blank-NE and RPG-NE) and the PS values of NPs formulations (blank-Zein-PLGA-NPs and RPG-Zein-PLGA-NPs) were in the nanometer range (Table 2). The results were supported by the TEM images (Fig. 1a and 1b). No significant difference was seen between the DS values of Blank-NE and RPG-NE ($p>0.05$). However, there was a significant increase ($p < 0.05$) in the PS of NP formulation in the presence of the active substance. The PDI values determined for both formulations were lower than 0.3, indicating a narrow PS/DS distribution. The ZP values of RPG-Zein-PLGA-NPs and RPG-NE were determined to be around -10 mV and approximately -31 mV, respectively (Table 2). In addition, the EE% values of RPG-Zein-PLGA-NP and RPG-NE were 94.83 ± 0.94 and 98.307 ± 0.348 ($n = 6$), respectively.

4.1. In Vitro RPG release from the formulations

In vitro release graphs are given in Fig. 2a and 2b. The percent dissolution values for free RPG were around 70 % at 12 h and about 87 % at 24 h in HCl pH 1.2 with 0.1 % Tween 80, and around 31 % at 12 h and approximately 38 % at 24 h in PB pH 6.8 with 0.1 % Tween 80. The percent RPG release values for RPG-Zein-PLGA-NPs were about 87 % at 12 h and approximately 92 % at 24 h in HCl pH 1.2 with 0.1 % Tween 80 and around 50 % at 12 h and about 58 % at 24 h in PB pH 6.8 with 0.1 % Tween 80. In addition, the percent RPG release values for RPG-NE were approximately 90 % at 12 h and about 98 % at 24 h in HCl pH 1.2 with 0.1 % Tween 80, and around 62 % at 12 h and around 79 % at 24 h in PB pH 6.8 with 0.1 % Tween 80 (Fig. 2a and 2b).

4.2. HPLC method for the determination of RPG in rat plasma

We developed and validated an HPLC method for RPG analysis in rat plasma samples in accordance with the ICH guidelines (ICH Topic Q 2 (R1), 2021; FDA Guidance 2022). The method's linearity was assessed at the beginning. Fig. 3 shows the calibration curve in the RPG concentration range of 40-200 ng/mL. R^2 was 0.9996 (Fig. 3). The fact that R^2 is close to 1 indicates the linearity of the developed method.

Specificity is the ability of the analytical method to assess the analyte of interest unequivocally in the presence of matrix components. The representative chromatograms of blank plasma and the mobile phase are given in Fig. 4a and 4b, respectively. Additionally, Fig. 4c shows the chromatograms of the plasma samples spiked with RPG standard solutions (50 ng/mL, 100 ng/mL and 150 ng/mL). The chromatograms of

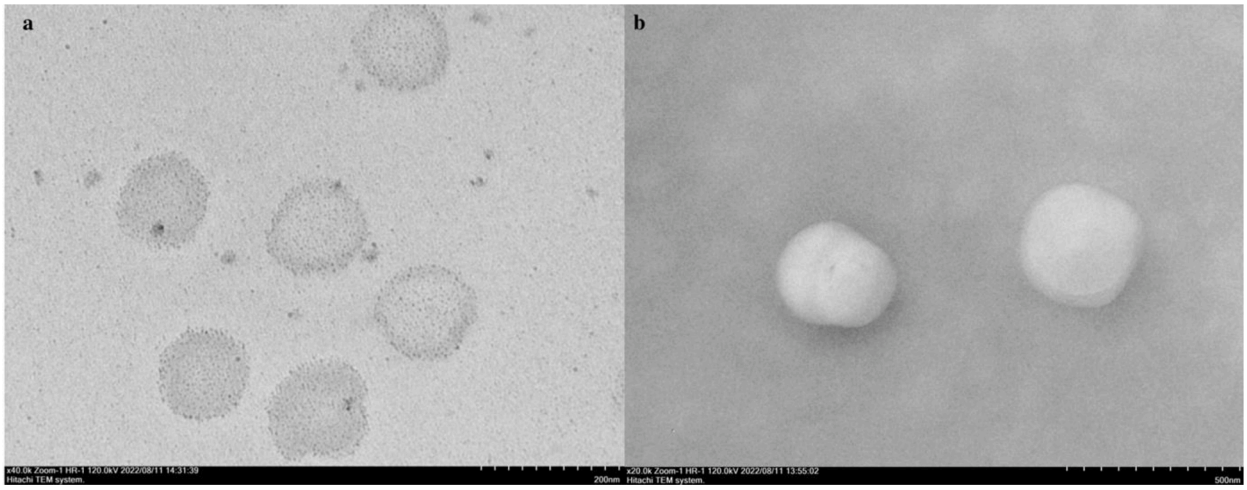

Fig. 1. The TEM images of RPG-NE (a) and RPG-Zein-PLGA-NPs (b).

Table 2
The PS/DS, PDI and ZP values of the NP and NE formulations ($n = 9$).

Formulation	PS/DS (nm)	PDI	ZP (mV)
Blank-Zein-PLGA-NPs	210.878 ± 7.100[a]	0.146 ± 0.043	-8.557 ± 0.352[c]
RPG-Zein-PLGA-NPs	257.489 ± 5.314[a]	0.166 ± 0.022	-10.077 ± 0.619[c]
Blank-NE	118.225 ± 1.935[b]	0.179 ± 0.015	-30.589 ± 0.822[d]
RPG-NE	116.188 ± 2.272[b]	0.155 ± 0.016	-30.811 ± 2.040[d]

[a and c]: The difference between the PS or ZP values Blank-Zein-PLGA-NPs and RPG-Zein-PLGA-NP is significant ($p < 0.05$)
[b and d]: The difference between the DS or ZP values Blank-NE and RPG-NE is not significant (p>0.05).

blank plasma and the mobile phase were compared with the chromatograms of the RPG standard solutions to evaluate the specificity of the HPLC method (Fig. 4). There was a peak at 11.25 min (the retention time of RPG) in Fig. 4c. However, no peaks were observed close to the retention time of RPG in the chromatograms of the blank plasma and the mobile phase (Fig. 4a and 4b). This indicates the specificity of the HPLC method.

Furthermore, the LOD and LOQ for the HPLC-UV method were determined to be 8.35 ng/mL and 25.31 ng/mL, respectively.

The method's intra- and inter-day accuracy (measured as RE) and precision (measured as RSD%) were assessed at 50 ng/mL, 100 ng/mL, and 150 ng/mL of RPG. The calculated RSD% and RE values are in Table 3.

4.3. Pharmacokinetic parameters

The oral dose (2 mg/kg single dose) of free RPG suspension or RPG-NE or the suspension of RPG-Zein-PLGA-NPs was administered to healthy rats, and the pharmacokinetic characteristics of RPG were evaluated using the validated HPLC-UV method. The mean plasma concentration-time profiles acquired for free RPG, RPG-Zein-PLGA-NPs, and RPG-NE are given in Fig. 5. Furthermore, Table 4 provides a summary of the pharmacokinetic parameters obtained from non-compartmental analysis. The $AUC_{0-\infty}$, C_{max}, and t_{max} values obtained for RPG-Zein-PLGA-NPs and RPG-NE were found to be considerably greater than those for free RPG suspension (Table 4).

5. Discussion

We evaluated the pharmacokinetic properties of RPG-containing NPs and RPG-NE formulations. We prepared the nano-sized systems first and then characterized them *in vitro*. The DS values of the blank-NE and RPG-NE formulations were found to be less than 120 nm. In addition, the PS values of blank-Zein-PLGA-NPs and RPG-Zein-PLGA-NPs were less than 260 nm. Both drug delivery systems were prepared in the nanorange. Furthermore, we found the ZP value of around -10 mV for RPG-Zein-PLGA-NPs. This is due to the PVA adsorption on the surface of particles, the presence of terminal carboxylic groups in PLGA and the rearrangement of Zein (Hillaireau et al., 2011; Gagliardi et al., 2021). The physical stability of colloidal dispersion can be predicted using the ZP values. Nanocarrier systems with a ZP between -10 and +10 mV are considered approximately neutral (Clogston and Patri, 2011). The ZP value of RPG-NE was about -31 mV. This result is compatible with the results in the literature (Karami et al., 2020). When ZP is ≤ -30 mV or $\geq +30$ mV, it is considered sufficient for the physical stability of the nanocarrier system. (Kaplan et al., 2023; Clogston and Patri, 2011). In addition, the release profiles showed that RPG was released more slowly from the NP and NE formulations in PB pH 6.8 with 0.1 % Tween 80 than in the acidic medium. RPG is a hydrophobic compound and shows a pH-dependent solubility (the solubility of RPG at pH 6.8 was less than that at pH 1.2) (Kaplan et al., 2023; He et al., 2015; Purvis et al., 2008).

In our pre-formulation study, the results of which were previously presented at a congress (Ugur Kaplan and Cetin, 2021), the organic phase was prepared by mixing the solution of RPG (5 mg) and PLGA (35 mg) in DCM with the solution of Zein (15 mg) in ethanol in order to prepare RPG-loaded PLGA-Zein nanoparticles. The particle size, PDI, and zeta potential values of the prepared nanoparticles were determined as 224.5 nm, 0.209, -22.63 mV, and 36.54%, respectively (Ugur Kaplan and Cetin, 2021). On the other hand, in our current study, we used different amounts of the polymers and RPG than those used in our previous study and also Zein was dissolved in DCM:ethanol (1:1, v/v) mixture as stated above (in the method section). Although the mean particle sizes of the obtained RPG-loaded PLGA-Zein NP formulation increased, its PDI and zeta potential values (approximately neutral NPs) decreased in the current study using this method. In addition, a very significant increase was achieved in EE (%) value.

In our correct study, we also developed and validated the HPLC-UV method for the determination of RPG in rat plasma. This method has been effectively applied in the pharmacokinetic study. Separating the analyte from matrix components and selectively analyzing them are essential steps in developing an assay method (Demirtürk et al., 2022). There were no interference peaks in the eluting position of RPG in the blank plasma samples (Fig. 4). Moreover, the obtained RSD% (<8 %; Table 3) and RE (<1 %; Table 3) values demonstrated the accuracy and precision of the analytical method developed for RPG analysis in rat plasma (RE is less than ± 15 % and RSD% is less than 15 % for *in vivo* studies) (FDA Guidance, 2022; Qin et al., 2020). As a result, one of the objectives of the study, the determination of RPG in rat plasma, was

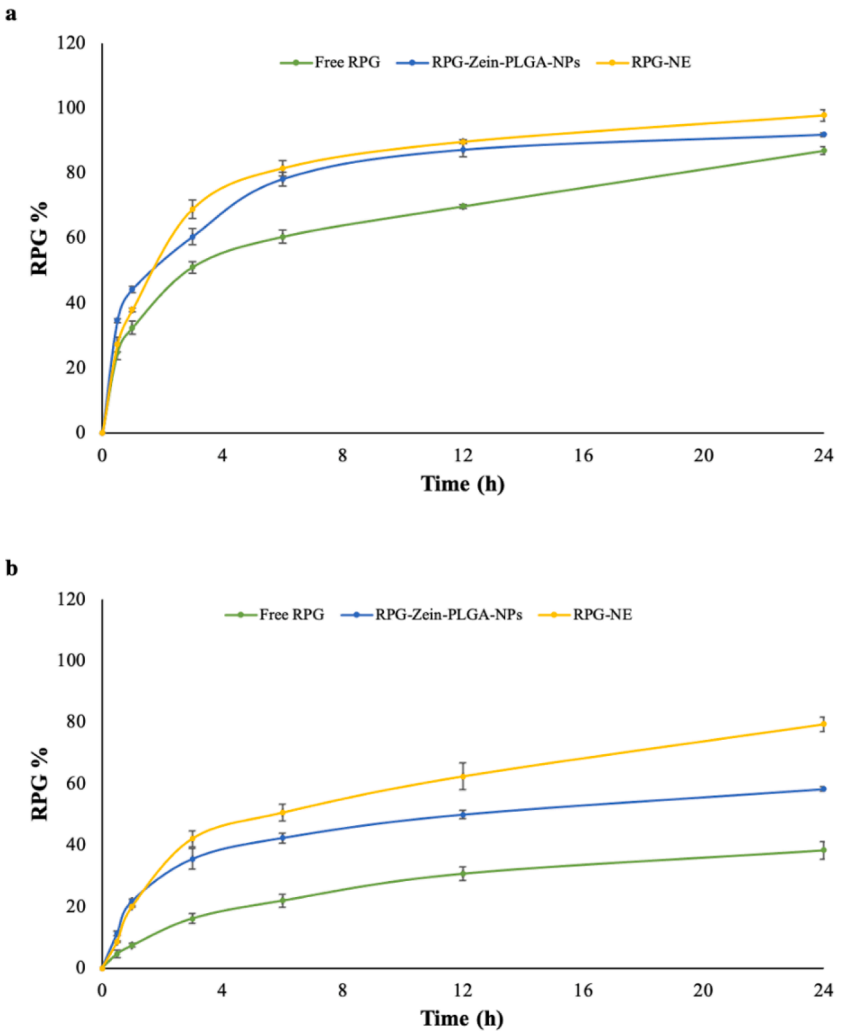

Fig. 2. The *in vitro* dissolution (for free RPG) and release (for the formulations) profiles (a: HCl pH 1.2 with 0.1 % Tween 80 and b: PB pH 6.8 with 0.1 % Tween 80) (Mean±SD; $n = 3$).

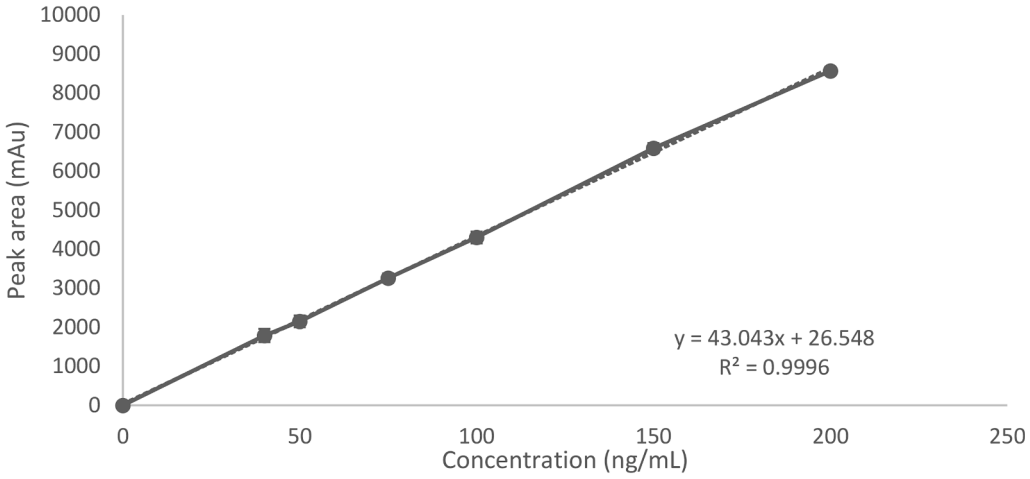

Fig. 3. Calibration curve for RPG.

achieved with a simple, accurate and precise HPLC-UV method.

Moreover, we set out to show how the NE and NP formulations containing RPG affect the bioavailability of RPG after oral administration. $AUC_{0-\infty}$, C_{max}, t_{max} and $t_{1/2}$ values for free RPG suspension, RPG-Zein-PLGA-NPs suspension and RPG-NE were estimated by non-compartmental analysis. The $t_{1/2}$ values of RPG in NPs and NE formulations were more prolonged than that of free RPG suspension (Table 4). The C_{max} and t_{max} values of RPG-Zein-PLGA-NPs suspension and RPG-

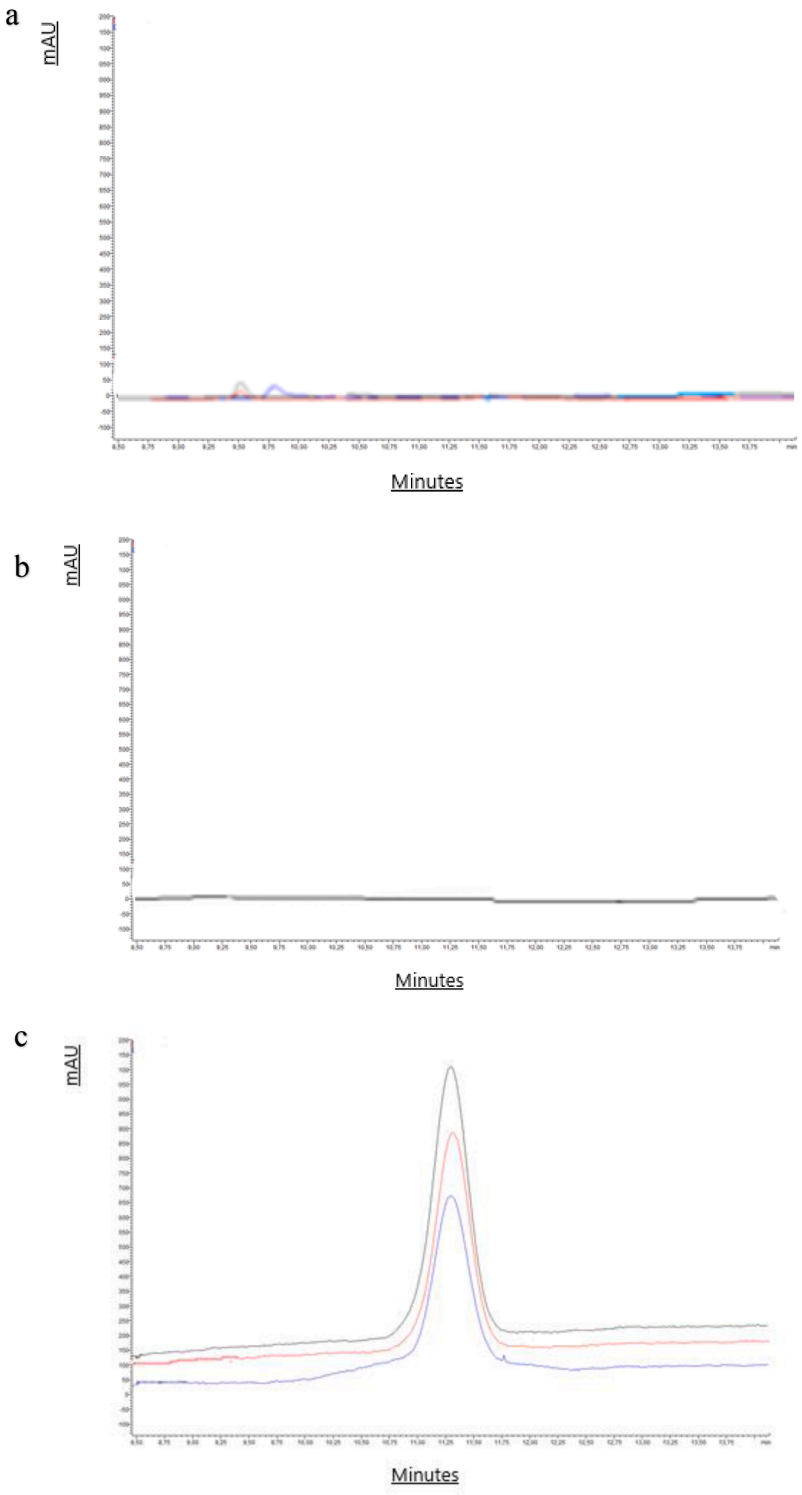

Fig. 4. Representative chromatograms of blank plasma (a), mobile phase (b) and RPG (50, 100 and 150 ng/mL) (c).

NE (523 ± 65 ng/mL and 770 ± 91 ng/mL; 1.41 ± 0.46 h and 1.61 ± 0.37 h, respectively; Table 4) were meaningfully higher than those of free RPG suspension (280 ± 33 ng/mL and 0.72 ± 0.28 h; Table 4) ($p < 0.05$). The $AUC_{0-\infty}$ values calculated for RPG-Zein-PLGA-NPs suspension and RPG-NE were approximately 4.04 and 5.05 times higher than that calculated for free RPG suspension, respectively ($p < 0.05$; Table 4, Fig. 5).

Nanoparticles provide sustained release of active compounds in the GI tract. They can increase the solubility of hydrophobic active compounds and their absorption. Moreover, NPs can improve the oral bioavailability of active compounds via lymphatic transport (Wang et al., 2020; Date et al., 2016). However, a slight change in the properties (such as the size, charge, and shape) of the NPs may alter the oral bioavailability of the active compounds. For instance, NPs with sizes below 200 nm are more effective in improving their oral bioavailability. The impact of NPs properties on oral bioavailability has been reviewed in the literature (Wang et al., 2020).

In a study, RPG-loaded PLGA NPs and RPG-loaded

Table 3
Intra- and inter-day accuracy and precision values obtained for the HPLC-UV method developed for RPG analysis in rat plasma (mean ± SD).

Added Concentrations (ng/mL)	Intra-day (n = 6)		Inter-day (n = 6)	
	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)
50	0.076	5.66	0.098	7.95
100	0.067	4.56	0.086	5.41
150	0.052	3.98	0.079	4.77

methoxypolyethylene glycol-PLGA NPs were prepared, and it was shown that blood glucose levels of STZ-diabetic rats treated (orally) with both NP formulations were significantly lower compared to that of untreated STZ-diabetic rats. However, RPG-loaded methoxypolyethylene glycol-PLGA NPs had a longer duration of action (Shelesh and Swarnlata, 2009).

Liu et al. (2021) prepared docosahexaenoic acid containing Zein-PLGA NPs with high EE% (84.6 %) and investigated the effect of zein-PLGA NPs on the bioavailability (as bioaccessibility) of docosahexaenoic acid by dynamic *in vitro* gastrointestinal experiment. They stated that the solubility of hydrophobic DHA in water can be significantly improved by the use of Zein-PLGA NPs, and the NPs could significantly increase the bioaccessibility of docosahexaenoic acid (Liu et al., 2021).

Low drug solubility may result in low oral bioavailability. NE, a lipid-based formulation, is one of the particularly preferred drug delivery systems for hydrophobic compounds to overcome this drawback. This formulation may significantly increase the solubility of these compounds. It also promotes the lymphatic transport of highly hydrophobic compounds. Additionally, the surfactants used in the NE formulation may change the permeability of the GI membrane and facilitate the passage of the active compound into the systemic circulation (Demirtürk et al.,2022).

Therefore, we also prepared an RPG-containing single NE formulation (O/W) using Tween 80 and Pluronic F68 as surfactants and Labrasol as co-surfactant in our current study. On the other hand, in our previously published study, multiple NE formulations (W/O/W) containing metformin HCl and RPG alone or in combination were prepared and evaluated *in vivo* (Kaplan et al., 2023). Only Tween 80 as surfactant and Labrasol as co-surfactant were used in the preparation of metformin HCl and/or RPG-containing multiple NE formulation in the study mentioned above. The DS, ZP and EE% values of only RPG-containing multiple NE formulation were about 110 nm, -21.95 mV and 96%. This above-mentioned *in vivo* study had shown that NE formulations containing metformin HCl and RPG alone or in combination caused a significant reduction in the blood glucose levels of diabetic rats after oral administration, compared to the control group (untreated diabetic rats) (Kaplan et al., 2023).

Karami et al. (2020) prepared RPG-containing NE formulation (uncoated), or RPG-containing chitosan-coated NE formulation and found that the C_{max}, $t_{1/2}$ and $AUC_{0-\infty}$ values calculated for uncoated and chitosan coated-NE formulations after oral administration to rats (17.58 ng/mL and 25.07 ng/mL; 2.38 h and 3.45 h; 78.86 ng.h/mL and 147.08 ng.h/mL, respectively) were significantly higher than those calculated for free RPG (C_{max}: 10.22 ng/mL, $t_{1/2}$:1.55 h, $AUC_{0-\infty}$: 20.42 ng.h/mL) (Karami et al., 2020).

In our study, it was seen that the F_{rel} (%) value calculated for RPG-NE formulation was about 1.25 times higher than that calculated for RPG-Zein-PLGA-NPs suspension (Table 4).

Jeong et al. (2021) conducted a study to reveal the pharmacokinetic difference that may occur with the use of two different nanosized carriers containing methotrexate (1. Methotrexate-containing NE formulation with an average DS of approximately 174 nm and ZP of about -36 mV. 2. Methotrexate-loaded PLGA NPs with an average PS of approximately 164 nm and ZP of around -20 mV). In this study, the researchers used a population pharmacokinetic modeling approach to compare the pharmacokinetics between these formulations. They identified

Table 4
The pharmacokinetic parameters obtained for free RPG, RPG-Zein-PLGA-NPs, and RPG-NE groups (Mean ± SD, $n = 6$).

Parameters	Free RPG suspension	RPG-Zein-PLGA-NPs suspension	RPG-NE
$AUC_{0-\infty}$(ng.h/mL)	458 ± 66	1854 ± 234[a]	2314 ± 324[ab]
C_{max} (ng/mL)	280 ± 33	523 ± 65 [a]	770 ± 91 [ab]
t_{max} (h)	0.72 ± 0.28	1.41 ± 0.46 [a]	1.61 ± 0.37 [ab]
$t_{1/2}$ (h)	1.28 ± 0.36	2.23 ± 0.62 [a]	3.09 ± 0.46 [ab]
F_{rel} %	-	404.80	505.24

[a] $p < 0.05$ compared with free RPG suspension group;
[b] $p < 0.05$ compared with RPG-Zein-PLGA-NPs suspension group.

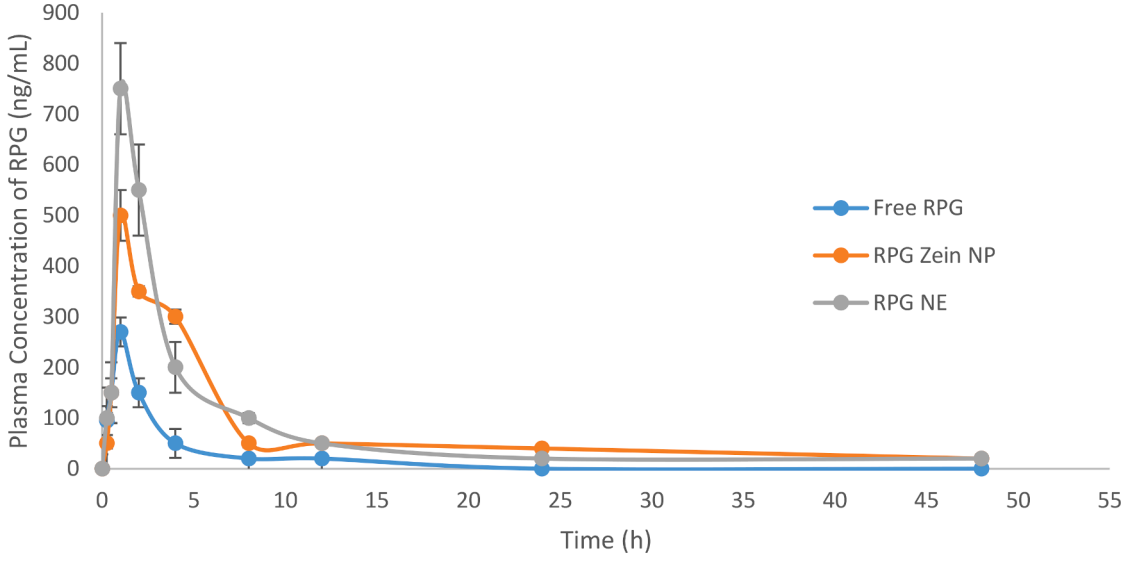

Fig. 5. The mean plasma concentration–time profiles obtained for free RPG, RPG-Zein-PLGA-NPs, and RPG-NE after oral administration to the healthy rats (Mean ± SD; n = 6).

significant pharmacokinetic differences between these formulations using this approach. They reported that after oral administration of NE formulation, the plasma concentration of methotrexate was significantly higher than that observed with oral administration of NPs. Its bioavailability tended to increase by 19 % compared to that of NPs. Possible reasons for this difference were explained as follows by the researchers: a. NE, a lipid-based formulation, can be easily absorbed and enter the systemic circulation through a process similar to the absorption of fat from the GI tract. However, NPs can enter the systemic circulation by passive diffusion (through some limited pathways) or be absorbed through Peyer's patches, which have a limited distribution in the GI tract. b. NE, which is a relatively flexible formulation, diffuses and moves more easily in the intracellular space than NPs, depending on the flexibility of these formulations. c. NE has a better capacity than NPs in delivering the active compound to lymphatic tissues (especially with the contribution of the fat absorption process) (Jeong et al., 2021).

6. Conclusion

In this study, RPG-NE and RPG-Zein-PLGA-NPs formulations were successfully prepared and characterized *in vitro* and *in vivo*. The results of the pharmacokinetic study indicated that NE and NP formulations were useful nano-sized carrier systems in increasing the oral bioavailability of RPG. In addition, the NE formulation was found to be more effective than the NP formulation in improving the oral bioavailability of RPG. Nanosize is a crucial factor in overcoming the dissolution rate barrier in order to increase the oral bioavailability of poorly water-soluble RPG. Moreover, the type of nano-sized carrier system may also have an impact on its bioavailability.

CRediT authorship contribution statement

Esra Demirturk: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Formal analysis, Data curation. **Afife Busra Ugur Kaplan:** Methodology, Writing – original draft, Investigation, Formal analysis, Data curation. **Meltem Cetin:** Conceptualization, Writing – review & editing, Visualization, Validation, Supervision, Methodology. **Meltem Dönmez Kutlu:** Methodology, Formal analysis, Data curation. **Seda Köse:** Visualization, Methodology, Formal analysis. **Kübra Akıllıoğlu:** Methodology, Investigation, Formal analysis, Data curation.

Data availability

Data will be made available on request.

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