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Diazepam Loaded Solid Lipid Nanoparticles: *in vitro* and *in vivo* evaluations

Running title: Diazepam Solid Lipid Nanoparticles

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Abstract:

Purpose: To overcome side effects of repetitive administration of Diazepam (Dzp) besides gaining benefits from sustaining release (SR) of the drug, which contributes to patient compliance, we concentrated on designing and preparing Dzp Solid Lipid Nanoparticles (SLNs).

Methods: Using cholesterol (CHOL), stearic acid (SA) and glycerol monostearate (GMS), SLNs were prepared by high shear homogenization technique coupled with sonication. Polysorbate 80 (Tween 80) was used as a nonionic surfactant. After modification of prepared SLNs, particle size, zeta potential, drug-loading efficiency, morphology and scanning calorimetry as well as release studies were conducted. To increase the stability of desired particles, freeze-drying by cryoprotectant was carried out. In the final stage, *In-vivo* study was performed by oral (PO) and intraperitoneal (IP) administration to Wistar male rats.

Results: Results indicated that optimized prepared particles were in average 150 nm diameter in spherical shape with 79.06 % loading efficiency and release of more than 85% of loaded drug in 24 hours.

In-vivo investigations also illustrated differences in blood distribution of Dzp after loading this drug into SLNs.

Conclusion: Based on the findings, it seems that drug delivery using SLNs could be an opportunity for solving complications of Dzp therapy in future.

Key words: Blood distribution, Diazepam (DZP), Drug delivery, Solid Lipid Nanoparticles (SLN), Sustained release (SR).

Introduction:

Dzp belongs to a class of drugs called benzodiazepines, which enhances the effect of Gamma-aminobutyric acid (GABA), a neurotransmitter that moderates the activity of nerve signals in the brain. Dzp has some side effects including drowsiness, fatigue, muscle weakness and clumsiness.¹ This may lead to reduced patient compliance when repetitive administration is needed. To overcome this problem, numerous studies have been carried out for sustaining release of Dzp. Sharma *et al.*² tried to optimize Dzp-loaded poly (lactic-co-glycolic acid) nanoparticles to achieve delivery to the brain through intranasal administration. In their study, Dzp nanoparticles (DNP) were formulated by nano-precipitation technique and drug release was reported about 61% in 24 hours. Researchers have tried to solve a problem regarding the poor solubility of Dzp.³ Despite being lipophilic, Dzp is rapidly redistributed out of the brain. On account of its fast distribution, serum levels of Dzp falls down quickly in the brain resulting in repeated dosing, accumulation in the body, and serious complications.^{2,4} Dzp is poorly soluble in water, and its intravenous formulation has to be prepared using co-solvents such as propylene glycol (40%) and ethanol (10%) which, in turn, may result in adverse effects, so lipid nanoparticles could be an interesting option in resolving this issue.^{2,5} Therefore, nanoemulsion of Dzp was formulated with this approach by Dordevic *et al.*⁶ In one of the other researches, Bohrey *et al.*⁷ sustained the release of Dzp for 9 hours by formulating polymer nanoparticles.

Cyclodextrin conjugated magnetic nanoparticles were also fabricated and designed by Cai *et al.*,⁸ to prevent side effects of Dzp remaining in the body after multiday of usage. Some studies were reported on designing Dzp SLNs as well, in order to achieve Dzp prolonged release in suppositories.⁹

Instability is one of the most important challenges with SLNs. In the present study, Dzp SLNs were freeze-dried to increase the stability. Direct freeze drying of SLNs may cause to some problems including aggregation of particles and particle size enlargement. To overcome these restrictions, based on previous experiences, mannitol was used as cryoprotectant for lyophilization process.^{10,11}

Dzp absorption profile after different administration routes is one of the other fields that has been investigated by many researchers. Suksiriworapong *et al.*¹² tried to solve limited rectal absorption of Dzp by preparing miscellaneous drug delivery systems. Galletly *et al.*¹³ compared Dzp mixed micelles with Dzp in propylene glycol and midazolam, they found venous morbidity was 17% for Dzp mixed micelles, 26% for midazolam and 90% for Dzp in propylene glycol. Dzp mixed micelle formulation was suggested as a preferable alternative to the standard formulations.

There are various studies that have also been focused on designing novel drug delivery systems like Dzp nasal delivery to enhance brain delivery of drug. A case in point, is a new nasal drug delivery system of Dzp, that has been developed with a natural mucoadhesive agent from fenugreek (*Trigonella foenum-graecum*). This patient friendly, needle free dosage form may replace Dzp injections in the future.¹⁴ Efforts on preparing an oral patch of Dzp, composed of

the outer mucoadhesive carbopol 934 region, central drug region, and Tegaderm backing film, has been made as well.¹⁵

On the basis of literature reviewed, it was realized that various scientific researches have been conducted to beat the restrictions of Dzp therapy and presumably, SLNs could have the opportunity to be the desirable carrier based on the numerous pros which were discovered by researchers over the past decades.¹⁶ The aim of this research was to improve the delivery of Dzp using nanotechnology. *In-vitro* evaluations and *in-vivo* distribution of Dzp-SLNs after intraperitoneal (IP) and oral administration (PO) to Wistar rats were investigated as well.

Materials and Methods:

Materials:

Diazepam base (Cambrex, Italy), glycerol monostearate, stearic acid and sodium hydroxide (Sigma Aldrich, Germany), cholesterol, Tween 80, ethanol, acetone and mannitol (Merck, Germany).

Methods:

UV Detection of Diazepam:

Maximum absorbance wavelength (λ_{\max}) for Dzp determined using Shimadzu UV-vis, 2100, Japan, spectrophotometer. In addition, phosphate buffer solution (PBS, pH: 7.4) was used as blank. Calibration curve of Dzp was plotted for concentrations in range of 2-10 ppm. The plotted calibration curve was used for *in-vitro* studies.

Preparation of Solid Lipid Nanoparticles:

High shear homogenization technique was selected for SLNs preparation.¹⁷ Three different types of lipids with the possible capability of forming hydrogen bond with Dzp for developing of a sustained release drug, were selected as carrier including cholesterol, stearic acid and glycerol monostearate. In between different kinds of surfactants which were reported in publications, Tween80 was selected as a nonionic surfactant type with the concentrations of 0.5-1% w/w. The used drug concentrations were 0.12 and 0.24% w/w. The hot oily phase was prepared by heating the lipids in ethanol and acetone. Following this, the drug was added to the oily phase. Aqueous phase was prepared by adding the surfactant in water. Then the hot oily phase was added to the aqueous phase under homogenization. The homogenization time is a determining factor in the characteristics of particles. Hence, based on the obtained results, 10 minutes was selected as the best condition, after examining 5, 10 and 15 minutes for homogenization step and the amount of ethanol, acetone and water were fixed in volumes of 3, 1 and 25 ml, respectively. The nanoparticles were being formed while the mixture of aqueous and oily phases was cooling down to room temperature under homogenization followed by sonication. At first, nano lipid carriers were developed without drug to justify independent variables. In this step, seven carriers were prepared under reported conditions in the table 1. In all presented carriers, homogenization and sonication time were fixed on 10 minutes and concentration of Tween 80 was 0.5% w/w. In order to evaluate the effects of increasing surfactant amount, three best carriers in terms of particle size were selected and added 1% w/w of Tween 80. (S1-2, S3-2, S4-2)

Table 1. Composition of S1-S7 formulations without drug.

Formulation No.	Type and amount of lipid(s) %w/w	Visual stability results, during 2 weeks
S1	CHOL, % 1	Stable
S2	SA, % 1	Unstable
S3	GMS, % 1	Stable
S4	GMS, % 0.5 SA, %0.5	Stable
S5	GMS, %0.5 CHOL, %0.5	Stable
S6	CHOL 0.5+ SA 0.5	Stable
S7	GMS 0.33 CHOL 0.33 SA 0.33	Unstable

In the next step of this study, the drug was added to the optimized carriers and drug-loading efficiency was evaluated.

Table 2, demonstrates the designed and prepared formulations.

In all Dzp-SLNs which were mentioned in table 2 concentration of surfactant was 1% w/w. Moreover, for homogenization and sonication, 10 minutes was applied. Prepared samples were evaluated by measuring particle size, zeta potential and polydispersity index (PDI) as well as drug-loading efficiency.

Table 2. Composition of formulations containing drug.

Formulation No.	Type and amount of lipid(s) %w/w	Drug amount %w/w
SLN1	CHOL 1	0.12
SLN2	CHOL 1.4	0.12
SLN3	CHOL 1.4	0.24
SLN4	GMS 0.7+ SA 0.7	0.12
SLN5	GMS 1.4	0.12

Freeze-drying:

Freeze-drying of selected SLNs was done to increase the stability of particles. Mannitol was used as cryoprotectant of the lyophilization process to prevent probable aggregation of particles during freeze-drying.

Particle size determination:

Nanoparticles were evaluated before and after freeze-drying, with respect to their size, PDI, and zeta potential using Dynamic light scattering (DLS) and Zetasizer ZS (Malvern Co., UK).

Drug-loading efficiency:

Drug-loading efficiency (%LE) was determined by the reverse method. For this purpose, the prepared formulations were centrifuged at 20000 rpm for 45 minutes at -4°C . Nanoparticles precipitated and free drug remained in supernatant. Furthermore, supernatant were evaluated and the amount of free drug in supernatant was detected by UV detector. Finally LE% was calculated using the following equation.

$$\text{LE\%} = \frac{W_{\text{initial drug}} - W_{\text{free drug in supernatant}}}{W_{\text{initial drug}}} \times 100$$

Drug release study:

Release study was performed using dialysis sack method using Spectra/Por dialysis membrane which had 12,000–14,000 Mwt cutoff. In beginning, 5ml of prepared formulation was placed in a dialysis membrane and immersed in 400ml of PBS (pH 7.4). Then 4 ml of samples around the dialysis sack were withdrawn in desired time intervals and 4ml fresh PBS was added to the release vessel. Drug concentration was measured and analyzed by UV detector for each sample. Release study was carried out before and after freeze drying to ensure that lyophilization did not cause significant burst effect. In order to compare the release profile of free drug and the loaded drug, free DZP release test was executed as well.¹⁹⁻²¹

Morphology studies:

Morphology of the nanoparticles was characterized by scanning electron microscopy (SEM) using Philips XL30, Almelo, Netherlands instrument. The atomic force microscopy (AFM) photographs were taken after freeze-drying using NT-MDT Spectrum Instrument.

Differential Scanning Calorimetry (DSC) studies:

The thermotropic properties and possibility of forming hydrogen bonds between Dzp and lipids, were evaluated by differential scanning calorimeter, (METTLER TOLEDO, USA.) Samples of about 5 mg were sealed in a 50 μl aluminum pans at a heating rate of $10^{\circ}\text{C}/\text{min}$ throughout the analysis. Empty aluminum pans were used as references and the whole thermal behaviors were studied under a nitrogen purge.⁹

In-vivo studies

Adult male Wistar rats weighing 150-180 g were obtained from animal house of Experimental Medicine Research Center of Tehran University of Medical Sciences (TUMS). The animals were allowed to feed with standard pellet diet and water ad libitum at $20-25^{\circ}\text{C}$ under a 12 hour light/dark cycle. Food was withdrawn one day before the experiment, but water continued to be provided. All animal handling and experiment protocols complied with the guidelines of the Laboratory Animal Centre of the University of Tehran.

Ninety six rats were divided into 4 groups of twenty-four. Group A which received Dzp (31 μg PO), group B received 2.015 mg Dzp lyophilized SLNs containing 31 μg Dzp by oral rout, group C were given 31 μg Dzp by intraperitoneal injection (IP) and finally, group D received 2.015 mg of Dzp SLNs IP. At the next stage, rats were anesthetized by a cocktail containing a mixture (1:1 v/v; 1 ml/kg body weight) of xylazine 2% (10 mg/kg) and ketamine 10% (50

mg/kg) intraperitoneal (IP) and blood samples were collected from the animal hearts 8, 12 and 24 hours after the treatment protocol. All gathered blood samples were analyzed using High-performance liquid chromatography. For each time point, eight animals were studied.²²⁻²⁶

Results and discussions:

UV detection of Dzp:

The λ_{\max} of Dzp was determined on 231 nm and this wavelength was selected for Dzp detection in the *in-vitro* studies.

Calibration curve:

Dzp calibration curve in PBS was plotted for concentrations between 1-10 ppm. For this concentration range, R^2 was equal to 0.9998.

Particle size studies:

The particle size for ten designed formulations are reported in table 3. They were in the range of 147-483 nm. S2 and S7 formulations were unstable after 2 weeks and phase separation was observed, therefore the study was conducted with other formulations. The effect of increasing the Tween 80 percentage on the particle size of the formulations was evaluated on S1, 3, and 4 and they named S1-2, S3-2, and S4-2. In these formulations percentage of Tween 80 was increased to 1%.

Table 3. Particle size results for the SLNs before drug loading.

Formulation No.	Particle Size nm
S1	223
S2	162
S3	221
S4	167
S5	376
S6	483
S7	208
S1-2	147
S3-2	211
S4-2	223

Zeta potential and PDI evaluation of S1, S3, and S4:

Zeta potential and PDI evaluations of S1-2, S3-2, and S4-2 were done using the Malvern system. Zeta potential of studied samples were -18.1, -13.9 and -14.6 mv for S1, 2 and

3respectively. Previously, researchers reported that GMS is a fatty acid ester that may be expected to impart a negative surface charge on the lipid particles.²⁷ Subsequently, the PDI of the mentioned SLNs were 0.239, 0.239 and 0.508. Particle size, zeta potential, and PDI investigations were repeated for drug loaded formulations and results are reported in table 4. After further studies on formulation No.5 including changing the amount of drug, we found that the presented production conditions are good enough and the quality parameters were evaluated for this formulation. After freeze drying of the formulation No. 5, particle size and zeta potential measurements were assessed again to ensure that significant size enlargement did not happen. The zeta potential rose to -16.8 mv and the size of particles was 205 nm for lyophilized formulation.

Table 4. Results of prepared formulations with drug.

Formulation No.	Type and amount of lipid (%w/w)	Type and amount of surfactant (%w/w)	Drug amount (%w/w)	Homogenization (min)	Sonication (min)	Particle size (nm)	%Drug loading efficiency
SLN1	CHOL 1	Tween 80 1	0.12	10	10	267	19.82
SLN2	CHOL 1.4	Tween 80 1	0.12	10	10	195	25.08
SLN3	CHOL 1.4	Tween 80 1	0.24	10	10	135	23.9
SLN4	GMS 0.7 + SA 0.7	Tween 80 1	0.12	10	10	605	56.13
SLN5	GMS 1.4	Tween 80 1	0.12	10	10	130	75.38

Drug release profile:

The drug release profiles of the loaded drug on/in SLNs before and after lyophilization in comparison with free drug are presented in fig. 1. The free drug rapidly pass through the membrane. After 80 minutes more than 75% of dissolved drug was found in receptor phase. DZP SLNs shows slower drug release profile. More than 70% of loaded drug was found after 350 and 450 minutes for SLNs before and after lyophilization, respectively. Total percentage of drug release for lyophilized SLNs was more than initially prepared SLNs. Although mannitol was used to minimize the stress of lyophilization process on the SLNs, as freeze drying in vacuum stage, cause to stress to the particles and some porosities may performed on the SLNs, the differences between lyophilize and non- lyophilized SLNs, is reasonable.

Morphology studies:

SEM pictures confirmed the size of particles that had been detected by DLS. Fig. 2 shows the SEM photograph of lyophilized Dzp SLNs. Based on the photo all particles are spherical in shape.

AFM technique was used to evaluate particles shape and surface characteristics as well as size. Fig. 3 indicates the AFM photo in which the spherical shape of the particles was demonstrated.

DSC studies:

DSC was carried out to investigate the probability of forming hydrogen bond(s) between GMS and Dzp. The result which is shown in Fig. 4, proved that melting points of these compounds changed after SLN formations. Based on these outcomes, we hypothesized the formation of hydrogen bonding which can cause sustained drug release profile. Previous studies confirmed probability of hydrogen bond forming by lipid carriers for some drugs including curcumin and cholesterol as well as amikacin and ampicillin with the same lipid which can cause sustaining release profile of the drug.^{28,29}

In-vivo studies:

According to the findings which were obtained from HPLC and presented in fig. 5, it seems that for free drug, the blood concentrations were higher in each sampling time in comparison with SLNs. For samples taken at 4 and 8 hours after administration, the achieved data can be the result of sustained drug release profile. However, for 24 hours samples in which more than 85% of the drug was released, the lower concentration for SLNs, illustrates that drug may be distributed in other tissues of the body and probably in the brain. Clearly, more studies need to be carried out to ensure drug distribution profile. As a recommendation, radioisotope linking could help to track drug in the bodies.

Based on this research, it seems that using SLNs could help us to inhibit the excretion of Dzp from the brain and increase drug concentration in the site of action. The drug concentration profile in blood after oral administration demonstrated more significant differences by changing free drug to SLNs. In IP administration the most significant difference refers to 24 hours samples.

Conclusions:

As reported by research findings, it can be concluded that loading Dzp into SLNs could be an appropriate approach for Dzp delivery with fewer side effects due to increased administration intervals in the future. Moreover, it appears that the prepared SLNs, are suitable semi-finished products, which can be used in different dosage forms and subsequently, diverse routes of administration such as intravenous, intranasal, and oral, considering the size of nanoparticles and type of involved materials that are safe.

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Ethical issues: IR.NIMAD.REC.1397.328

The protocol of this experimental study was approved by the Ethical Committee of Tehran University of Medical Sciences and performed in accordance with the ethical guidelines for animal studies. Based on the International Guiding Principles for Biomedical Research Involving Animals (1985).

Conflict of interest:

Author declares that there is no conflict of interest.

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