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Preparation and characterization of estradiol-loaded PLGA nanoparticles using homogenization-solvent diffusion method

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ABSTRACT

Background: The inherent shortcomings of conventional drug delivery systems containing estrogens and the potential of nanoparticles (NPs) have offered tremendous scope for investigation. Although polymeric NPs have been used as drug carriers for many active agents, the use of appropriate polymer and method of NP preparation to overcome different challenges is very important.

Materials and methods: Poly lactide-co-glycolide (PLGA) NPs containing estradiol valerate were prepared by the modified spontaneous emulsification solvent diffusion method. Several parameters including the drug/polymer ratios in range of 2.5-10%, poly vinyl alcohol (PVA) in concentration of 0-4% as stabilizer and internal phase volume and composition were examined to optimize formulation. The size distribution and morphology of the NPs, encapsulation efficacy and in vitro release profile in phosphate buffer medium (pH 7.4) during 12 hrs were then investigated.

Results: The NPs prepared in this study were spherical with a relatively mono-dispersed size distribution. By adjustment of the process parameters, the size and the drug encapsulation efficacy as well as the drug release kinetics can be optimally controlled. The mean particle size of the best formula with encapsulation efficiency of 100% was 175 ± 19, in which release profile was best fitted to Higuchi's model of release which showed that release mechanism was mainly controlled by diffusion of the drug to the release medium.

Conclusion: According to the size and surface properties of the prepared particles, it may be concluded that they are a good formulation for non-parenteral routes of administration.

KEYWORDS: Estradiol, Drug delivery, PLGA, emulsification solvent diffusion method

INTRODUCTION

Hormone replacement therapy using estrogen alone or in combination with progestins is employed in postmenopausal women for prevention of osteoporosis. It has been demonstrated that short-term treatment with pregnancy levels of estradiol with or without progesterone is highly effective in decreasing the incidence of mammary cancer (1).

The inherent shortcomings of conventional drug delivery and the potential of NPs as drug delivery systems have offered tremendous scope for researchers in this field and is moving from concept to reality. NPs may be used for oral administration of drugs with low aqueous solubility (2).

These colloidal carriers have the ability to cross the mucosal barrier. In addition to the potential for enhancement of drug bioavailability via particle uptake mechanisms, nanoparticulate oral delivery systems have slower transit times than larger dosage forms, increase the local concentration gradient across absorptive cells, thereby enhance local and systemic delivery of both free and bound drugs across the gut. These colloidal carriers may develop adhesive interactions within the mucosa and remain in the gastrointestinal tract, while protecting the entrapped drug from enzymatic degradation, allow release of the loaded drug or their absorption in an intact particulate form (3).

PLGA is a highly biocompatible and biodegradable synthetic polymer, which is hydrolytically degraded into non-toxic oligomers and finally to lactic acid and glycolic acid (4). Increasing attention has also been paid to the colloidal particles of these polymers as injectable drug carriers, which would enable a long systemic circulation. The pharmaceutical application of such nanoparticles has been extended to the field

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of non-parenteral deliveries of drugs via pulmonary, nasal or oral routes (5). Under such circumstances, various methods have been proposed for the preparation of PLGA and PLA NPs (6-8). The technique mostly used, however, is the emulsification solvent evaporation method (8) and one promising technique is the spontaneous emulsification solvent diffusion (SESD) method (9), in which nanoparticles of PLGA or PLA can be effectively obtained by pouring the polymeric organic solution into an aqueous phase with moderate mechanical stirring. The technical characteristic of this method is the use of a binary mixture of a water-miscible organic solvent such as acetone and a water immiscible solvent such as dichloromethane (DCM) as the solvent of the polymeric solution and the particles are formed via an emulsification process and a subsequent solvent-evaporation process. This article reports the design of biodegradable NPs containing estradiol using SESD method and various process parameters influencing the size and encapsulation efficacy of NPs.

Ravi Kumar et al have prepared estradiol-loaded PLGA NPs (10-12) by a modified emulsion-diffusion method using ethyl acetate as solvent and didodecyldimethylammonium bromide (DMAB) as stabilizer. Although, DMAB, as an infrequently used surfactant (13), and produces smaller NPs, PVA as a frequently used emulsifier in NP preparations, is regarded as non-toxic and safe for the use in human drug delivery systems (14-15). They also reported encapsulation efficiency (EE) of 70%. In this study PVA was used for the NP preparation as emulsifier.

MATERIALS AND METHODS

Materials

Estradiol valerate (USP 27) was donated by Abureihan pharmaceutical Co, Tehran, Iran, PLGA (Resomer® RG 504H, MW 48,000) was purchased from Bohringer Ingelheim, Germany, PVA (MW 22,000) was from sigma-Aldrich, USA and DCM and acetone (analytical grade) were purchased from Merck, Germany. Acetonitrile and ammonium nitrate buffer used as mobile phase in high performance liquid chromatography (HPLC) were purchased from Merck, Germany. Deionized water was used throughout the experiment. The in vitro release measurement was carried out at pH 7.4 at 37 ºC in phosphate buffer medium. Sodium dihydrogen phosphate and disodium hydrogen phosphate, used for the preparation of buffer were purchased from Merck, Germany. All other chemicals were of reagent grade.

Preparation of estradiol valerate loaded PLGA NPs

The estradiol loaded NPs were fabricated by a modified emulsification/solvent diffusion method (16-17). Briefly, 200 mg of mass of polymer and different amounts of estradiol valerate powder (2.5-10% w/w of the polymer) were added into the mixture of DCM/Ethanol/Acetone, which was suitably stirred to ensure that all material are dissolved. The solution was slowly poured into the aqueous solution of PVA using a high speed homogenizer (IKA, Ultra Turrax, USA) at 24,000 rpm for 5 minutes. After evaporation of the internal phase under stirring, the polymer precipitated and the NPs were isolated by using a centrifuge (Sigma 3K30, Germany) at 21,000 g for 15 minutes and washed three times with deionized water. All the preparation steps carried out at room temperature (23-25 °C). The produced suspension was freeze-dried for 48 h at -40 °C (Lyotrap Plus, LTE Scientific, UK) to obtain a fine powder of NPs, which was then kept in a desiccator.

Encapsulation efficacy measurement

The drug entrapped in the NPs was determined in triplicates by HPLC (18). A reversed phase C_{18} column (25×0.46 cm i.d., pore size 5 µm, Teknokroma, Spain) was used. The mobile phase consisted of a mixture of ammonium nitrate buffer and acetonitrile (30:70 v/v), and delivered at a flow rate of 2 ml/min with a pump (Well Chrom, K-1001, Knauer, Korea). A 20 mg sample of NPs powder was dissolved in 1ml of acetonitrile followed by addition of 2 ml of methanol to precipitate the polymer. The sample was then centrifuged for 5min at 21,000 g and a 20 µl of aliquot taken from the supernatant was analyzed by the HPLC system. The column effluent was detected at 280 nm with a UV detector (WellChrom, K-2600, Knauer, Korea).

The calibration curve for the quantification of estradiol was linear over the range of standard concentrations of estradiol at 0-50,000 ng/ml with a correlation coefficient of R^2=0.999. The encapsulation efficacy was obtained as the mass ratio between the amount of estradiol incorporated in NPs and that which was used in the NPs preparation while drug content was determined as the ratio of the weight of the estradiol in NPs to the weight of the NPs.

Determination of NP morphology

Scanning electron microscopy (SEM, Philips XL 30 scanning microscope, Philips, the Netherlands) was employed to determine the shape of the produced NPs. Particles were coated with gold under vacuum before SEM.

Size and Size Distribution measurement

The particle size and size distribution of the NPs were measured by laser light scattering (Malvern Zetasizer ZS, Malvern UK). The samples were examined to determine the mean diameter and size distribution. The samples were prepared by suspending the freeze dried NPs in 10 ml of deionized water (10 µg/ml).

In Vitro Releases Study

Drug release from estradiol loaded NPs was carried out using a modified dissolution method (19). The media was a 0.05 M phosphate buffer solution. NP powder (2 mg) was suspended in tubes containing buffer solution of pH 7.4 (10 ml) to simulate physiological pH. Three replicates were used for each sample. The tubes were placed in a shaker bath (Memmert WB14, Germany) at 37 ºC and shaken horizontally at 90 cycles/min.

At selected time intervals, the tubes were centrifuged and an aliquot of 900 µl was taken from the supernatant. A volume of 100 µl of methanol was added and analyzed by HPLC. A calibration curve was prepared prior to the start of dissolution using a phosphate buffer methanol (9:1) media. The HPLC method used was the same as described above. After the aliquots were removed, the entire supernatant was replenished in order to maintain sink conditions. Drug release data was normalized by converting drug concentration in solution to a percentage of the cumulative drug release.

Statistical analyses

One-way analyses of variance were performed for comparison of the results. P values of < 0.05 were considered significant.

RESULTS AND DISCUSSION

The formulations of estradiol-loaded PLGA NPs prepared in this study are presented in table 1. A factorial design study was used for the parameters affecting the formulation characteristics including acetone and ethanol volume, PVA percentage, homogenization and mixing time, centrifugation time, polymer/drug ratio to reach the optimum formulation and it was found that the main parameter in the design of formulations is the DCM volume.

The physicochemical characteristics of the PLGA loaded estradiol NPs are shown in table 2. As it can be seen in this table, addition of a small volume of ethanol to the mixture of acetone/DCM leads to smaller estradiol NPs and this alteration can prevent the aggregation of particles effectively. The estradiol entrapment obtained in NPs suggests that the drug molecule tends to move in the bulk aqueous phase in spite of its low solubility. This process is favored by either the spontaneous diffusion of acetone or concentration of surfactant that is the main parameters affecting NPs preparation optimization. Other preparation parameters, such as PVA concentration and estradiol/PLGA ratio were modified to obtain NPs with higher EE. While the increase in the concentration of PVA from 1% to 4%, prevented the coalescence of fine droplets during stirring, it also reduced the EE of NPs dramatically from 55% to 9% ($p<0.05$) which is in agreement with the results of other studies (24-25).

The optimum ratio of the solvents used for the preparation of NPs (containing 200 mg PLGA/20 mg of estradiol valerate) was 10:5:25 (v/v) for DCM, ethanol and acetone respectively to ensure the production of particles of 150-200 nm in size with a maximum standard deviation of about 20 nm, indicating a quite narrow population (S_2 sample). A volume of 10 ml of DCM was required to dissolve the PLGA and estradiol and to make a homogenous solution. Lowering the amount of acetone or ethanol resulted in production of larger particles. The higher the amount of acetone in the internal phase, the smaller was the size of the particles (S_9). However; the higher the ratio of the acetone to DCM, the lower was the EE. Rapid diffusion of acetone into the aqueous phase in SESD method may be the reason for such lower EE.

Higher surfactant concentration causes the migration of drug molecules to the continuous phase. When no PVA was used in the external phase, no NP was formed. At 0.5% w/v of PVA, larger particles were produced. By increase in the concentration of PVA, the size of the NPs was reduced. Among other parameters which were investigated, the acetone/DCM ratio influenced the EE markedly (25). As expected, higher theoretical drug loading corresponded to higher drug content ($p<0.05$) which is in agreement with previous results (25).

Figure 1 shows the SEM photograph of NPs prepared in this study. As can be seen, the NPs were spherical with a relatively narrow size distribution.

Figure 2 shows the particles size distribution of NPs prepared in this study. While in the original SESD method (9) PLGA was dissolved in mixture of DCM and acetone, in this investigation, the polymeric solution was slowly poured into the emulsifier containing aqueous phase. By this procedure NPs are formed when the polymeric solution is added and emulsion droplets are formed in the aqueous phase. Then acetone quickly diffuses out from emulsion droplets, and the particle sizes reduce drastically. Consequently when DCM is removed from the system via

Table1. Various formulations of estradiol-loaded nanoparticles

Sample	Estradiol-PLGA (% w/w)	PVA (% w/v)	DCM/Ethanol/Acetone (ml)
S_1	10	0.5	10-5-25
S_2	10	1	10-5-25
S_3	10	2	10-5-25
S_4	10	4	10-5-25
S_5	10	0	10-5-25
S_6	5	1	10-5-25
S_7	2.5	1	10-5-25
S_8	10	1	10-5-50
S_9	10	1	10-10-100
S_{10}	10	1	10-5-15
S_{11}	10	2	10-5-15

Table 2. Physicochemical characteristics of the PLGA loaded estradiol nanoparticles (n=3)

Sample	Drug content (%) ± S.D.	Encapsulation Efficacy (%) ± S.D.	Mean particle Diameter (nm) ± S.D.	PDI
S_1	*	*	500 ± 12	0.340
S_2	4.8 ± 0.3	55.2 ± 1	186 ± 10	0.127
S_3	1.26 ± 0.01	13.8 ± 3	170 ± 20	0.198
S_4	0.9 ± 0.02	9 ± 0.5	167 ± 11	0.230
S_5	-	-	-	-
S_6	2.7 ± 0.2	63 ± 2	184 ± 18	0.178
S_7	1.5 ± 0.3	100 ± 3	175 ± 19	0.161
S_8	2.4 ± 0.2	24 ± 3	172 ± 12	0.21
S_9	0.6 ± 0.05	9 ± 5	164 ± 13	0.23
S_{10}	*	*	750 ± 16	0.287
S_{11}	*	*	624 ± 18	0.154

* Not measured; - Nanoparticles not formed; PDI: poly dispersity index

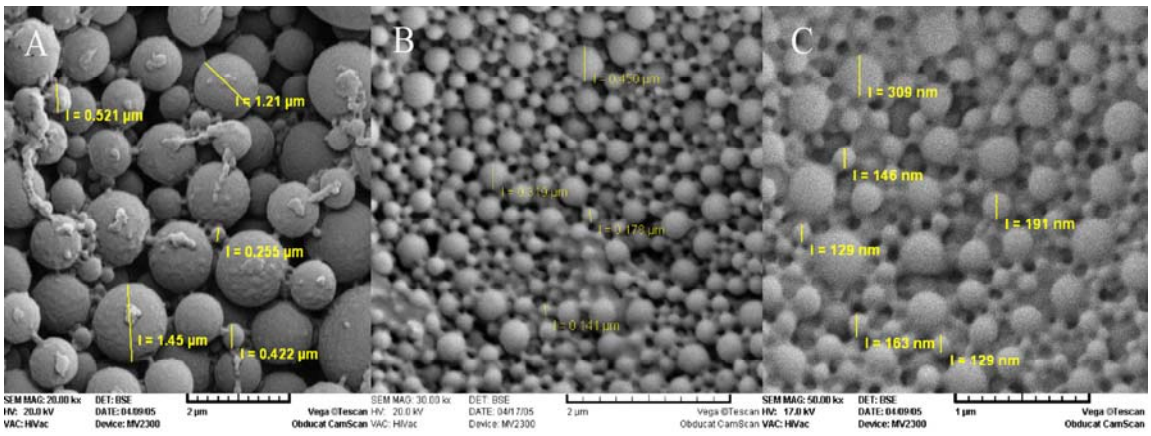

Figure 1. SEM micrographs of nanoparticles showing the shape and the surface characteristics: A (S_2), B (S_3), C (S_4)

evaporation, the droplets solidify and finally form polymeric NPs (20-21).

Murakami et al established a new method for the preparation of PLGA NPs by modification of the SESD method in which solvent system consisted of two water-miscible organic solvents (acetone and methanol or ethanol) to provide a good yield of NPs (22).

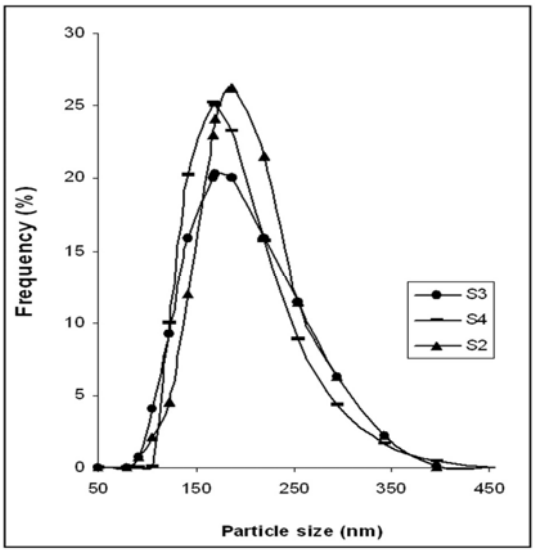

Figure 2. Size distribution of estradiol loaded PLGA nanoparticles determined by light scattering methods (n=3)

Since the size of the colloidal carriers is a key factor for the biological fate of the NPs, and NPs of small size (<100 nm) usually bypass the macrophages uptake, the goal of this study was to reduce the particle size of NPs. In the previous study it was shown that a combination of DCM and acetone with high speed homogenization technique is an appropriate method for making small particles of slightly water-soluble drugs (16-17). The impact of acetone on size reduction may be due to its diffusion to the aqueous phase and prevention of the particles aggregation. However in this study combination of DCM and acetone couldn't prevent the aggregation of the hydrophobic drugs-loaded PLGA particles and only when a combination of DCM, acetone and ethanol were used, NPs with small size (< 200 nm) were produced. It may be assumed that acetone has a higher affinity to PLGA than to PVA and ethanol has higher affinity to PVA than to PLGA. When the PLGA solution is dispersed into the aqueous solution of PVA, the disturbance of the interface spontaneously produces a larger interfacial area, which leads to nano-sized quasi-emulsion droplets of PLGA solution. This interfacial turbulence would be governed by the well-known Marangoni effect (23). Thus, the ethanol preferentially diffuses out of the droplets since the affinity of ethanol to PLGA is lower than that of acetone. Diffusion of, acetone out of the droplets continuously results in the coacervation of PVA by the increase in the concentration of acetone in the aqueous solution which leads to increase of PLGA concentration inside the droplets. The subsequent solidification of PLGA and PVA adsorption on the surface of NPs occurs simultaneously to produce uniform NPs.

The in vitro release behavior of the estradiol-loaded NPs is summarized in the cumulative percentage release shown in figure 3. Due to the constant release rate of estradiol from NPs, the release measurement was carried out over 12 hrs.

The initial burst release in figure 3, which was greater than 7%, was prominent for all formulations during the first hrs of drug release experiment. The release gradually decreased and remained constant even after 12 hrs. The initial burst could be due to the surface release of estradiol which was distributed near or at the surface of the NPs and can be explained by considering the mechanism of NPs preparation. In fact, estradiol has a good solubility in acetone which by diffusing toward the aqueous phase, not only enhances the drug leakage from NPs significantly but also promotes its distribution in proximity of the oil–water interface. Using 4% PVA solution caused the fastest drug release ($p<0.05$) which might be due to the effect of PVA in facilitating the migration of estradiol from the dispersed phase (droplets) to the continuous phase, hence leaving more drug molecules on the surface of the particles (25). The enhancement in the release of estradiol from NPs may be supported by the hydration process which occurs very rapidly due to the smaller size of particles. Afterwards, the matrix material would require time to erode in the aqueous environment.

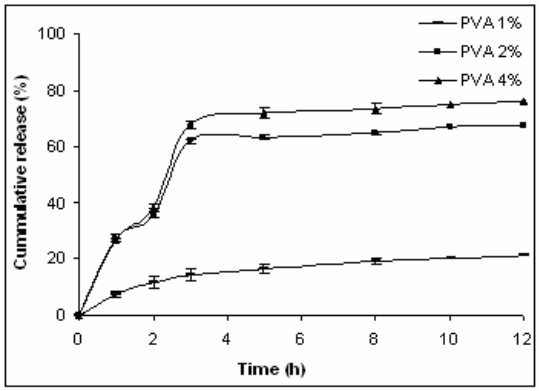

Figure 3. Effect of PVA concentration on the in vitro estradiol release from PLGA nanoparticles (n=3), error bars show the average amount of drug released ± SE

Table 3. Kinetic model for the formulations with various amount of the PVA stabilizer (S_2, S_3 and S_4)

Release Model		Formulation		
		PVA 1%	PVA 2%	PVA 4%
zero order	K	-3.3695	-0.0872	-0.0746
	R	0.8929	0.8351	0.8549
First order	K	-0.5762	-0.0423	-0.4714
	R	0.6179	0.6348	0.6350
Higuchi	K	-11.0670	-12.1010	-7.5229
	R	0.9878	0.9459	0.9511

The drug release from NPs may be due to mechanisms such as diffusion of drug molecules from polymeric matrix of NPs and the biodegradation of polymeric matrix of NPs (26). To determine the mechanism of release in this study, the cumulative drug release was fitted into different release models namely zero order, first order and Higuchi's square root plot (27). On the basis of results, it was found that the best curve fitting was obtained with Higuchi model of release (table 3) which describes the release mechanism largely on the basis of diffusion. PLGA degradation in neutral medium is very slow, and no degradation of the PLGA in the buffer medium is expected to be occurred within the period of the release experiment (28). Thus it may be reasoned that the release is mainly mediated through the diffusion process (a concentration gradient process) with very little or no involvement of degradation within the studied period.

The hydrophilic nature of the matrix caused by the surfactants may account for a more rapid entry of water into the NPs, eventually accelerating the release of estradiol in formulation when higher amount of the surfactant is used *($p<0.05$).*

CONCLUSION

The result of the present investigation proposes a novel formulation of estradiol NPs by application of a high speed homogenization-solvent diffusion method using a single oil/water emulsification. It was found that NPs with desirable size and high encapsulation efficacy can be produced and by adjusting the process parameters, the size and the drug encapsulation efficacy as well as the drug release kinetics can be optimally controlled. However to evaluate the in vivo efficacy of the produced estradiol NPs require further works.

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