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Formulation and Optimization of Aliskiren Loaded Poly(Lactide-Co-Glycolide) Nanoparticles

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Abstract

Aliskiren is a non-peptide, orally active renin inhibitor with poor absorption and low bioavailability (~2.6%). In order to improve the current drug delivery system, a commercially available, biodegradable copolymer, poly(lactide-*co*-glycolide) (PLGA), was employed for a nanoparticle (NP) reformulation of aliskiren. An emulsion-diffusion-evaporation technique was implemented where aliskiren and PLGA were dissolved in dichloromethane, ethyl acetate, or ethyl acetate/acetone. To an aqueous phase containing 0.25% w/v didodecyldimethylammonium bromide (DMAB) as stabilizer, the previously prepared organic phase was added drop-wise. Following sonication, NP diffusion was expedited with the addition of water, and the organic phase was evaporated to form a suspension. Centrifugation was performed at 10,000 rpm, and the supernatant was analyzed for drug entrapment efficiency via ultraviolet-visible spectroscopy as well as particle morphology with the use of a transmission electron microscope (TEM). Having the highest entrapment efficiency (82.68 ± 1.18 %), ethyl acetate was used as the organic solvent in further testing, such as examining the effects of variation in DMAB stabilizer concentration (0.10, 0.25, 0.50, or 1.00% w/v) and centrifugation speed (10,000 or 12,000 rpm). The optimum formulation was ascertained through observing certain NP characteristics, such as entrapment efficiency particle size, zeta potential, and polydispersity index (PDI). A NICOMP Particle Sizer was used to measure particle size, zeta potential, and PDI. The smallest NP size (67.27 ± 0.87 nm) was accomplished with 0.50% w/v DMAB concentration using a centrifugation speed of 12,000 rpm, while the highest zeta potential (18.73 ± 0.03 mV) was detected with the 1.00% w/v DMAB concentration and a 10,000 rpm centrifugation speed. Further, the best entrapment efficiency and PDI (82.68 ± 1.18 % and 0.15 ± 0.03, respectively) were accomplished with 0.25% w/v DMAB and centrifugation at 10,000 rpm. The most favorable formulation yielding the highest zeta potential (18.73 ± 0.03 mV) was observed when DMAB stabilizer was 1.00% w/v and centrifuged at 10,000 rpm. Particle size and entrapment efficiency for this formulation were 75.67 ± 0.89 nm and 71.62 ± 0.11 %, respectively.

1. Introduction

Worldwide, approximately 25% of people live with hypertension, a risk factor for cardiovascular disease; therefore proper treatment of this potentially serious condition is paramount [1]. A diagnosis of hypertension is conferred when a patient, exhibiting neither diabetes mellitus nor kidney disease, presents with a blood pressure in excess of 140/90 mm Hg. Blood pressure is mainly controlled by the renin-angiotensin-aldosterone system (RAAS) through angiotensin II which is produced via the angiotensin-converting enzyme (ACE) from angiotensin I [2]. Increased blood pressure is correlated with elevated blood levels of angiotensin II, a vasoconstrictor which restricts blood flow thereby increasing vascular resistance [3]. The rate-limiting step of RAAS is the process by which renin is secreted due to low plasma volume, decreased blood flow through the kidneys, or increased sympathetic central nervous system activity. When angiotensinogen, the only known renin substrate in plasma, is cleaved by renin, an inactive peptide, angiotensin I, is formed [2]. ACE then transforms angiotensin I into the active peptide angiotensin II, which interacts with angiotensin II type 1 (AT1) receptors inducing vasoconstriction and the release of catecholamines.

Although ACE inhibitors block the formation of angiotensin II, angiotensin I continues to be produced and may still be converted to angiotensin II via other metabolic pathways [4]. Angiotensin receptor blockers specifically block the AT1 receptors[2]. While multiple steps may be inhibited to prevent angiotensin II formation, the rate-limiting step is ideal for inhibition as renin is the first enzyme in the pathway [5]. Because angiotensin receptors are not activated when renin is inhibited, the specific metabolic pathway is not affected [2]. Inhibition at this step increases the amount of renin in circulation; however, inhibitors, such as aliskiren, block enzyme activity.

Aliskiren (Fig. 1) is a non-peptide, orally active renin inhibitor with attractive properties, such as low molecular weight, and was discovered using X-ray crystallographic structure analysis and molecular modeling [5]. On March 6, 2007, aliskiren became the first of its drug class to receive approval from the United States Food and Drug Administration (FDA) as an antihypertensive drug which reduces plasma renin activity through high affinity binding and specificity via aromatic side chains [3, 6]. The renin active site is able to accommodate seven substrate amino acid units with high affinity [7]. When the production of angiotensin I and angiotensin II is decreased, plasma renin concentration is increased, yet plasma renin activity (PRA) is decreased [6]. The reduction in PRA is beneficial to cardiovascular health through reducing cardiovascular risk factors [3]. Studies have shown that aliskiren is effective as either a monotherapy or in combination with other antihypertensive drugs such as hydrochlorothiazide [4, 6]. Aliskiren has a half-life of approximately 23 to 36 hours making it suitable for once daily dosing, typically 150 or 300 mg. Side effects including headache, diarrhea, and dizziness were reported in less than 3% of patients [8].

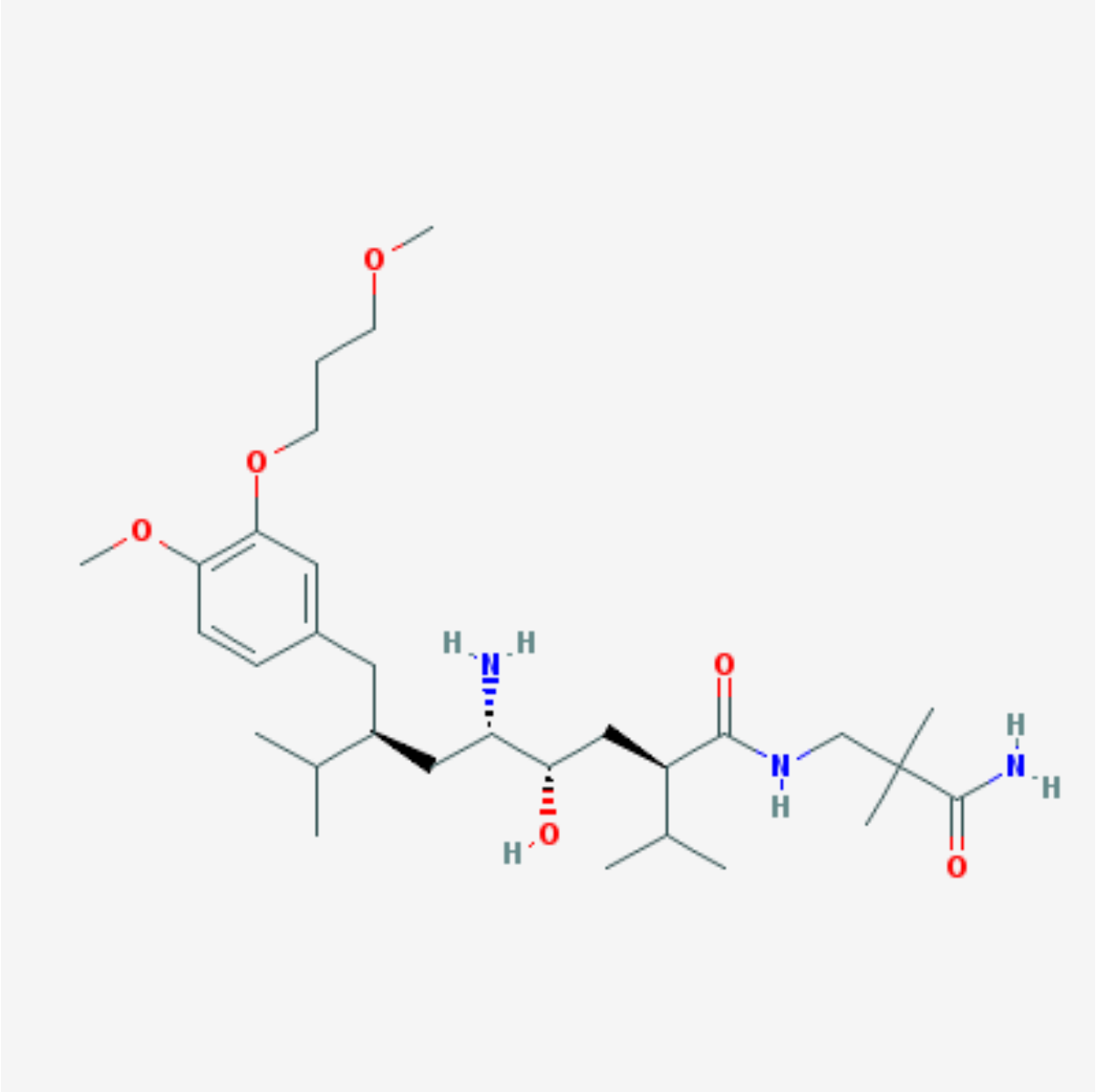

Figure 1. Chemical structure of aliskiren [9].

Although aliskiren has many positive attributes in regard to lowering blood pressure, such as reaching maximum plasma concentrations within 2 to 4 hours following administration, drug bioavailability has been found to be an extremely low (2.6%) with approximately 91% of the drug excreted unmetabolized from the body in feces and urine (> 0.6%) [2, 4, 5, 8]. Aliskiren exhibits high aqueous solubility (low lipophilicity) conferring greater resistant to

intestinal degradation [4, 6]. Synthetic polymer nanoparticle (NP) formulations are becoming prominent in nanomedicine in an attempt to enhance drug delivery systems through improving bioavailability, systematic absorption, and/or minimizing effective dosage [10, 11]. Since the current average wholesale cost of aliskiren is $70.20 for a 150 mg dose and $88.60 for a 300 mg dose, NP formulations could bring about a reduction in cost and become an appealing option for patients who suffer from hypertension [12].

NPs, defined by a range of size from 10 nm to 1000 nm, have the potential to modify particle size and surface characteristics which may in turn have a significant effect on drug pharmacokinetics and pharmacodynamics [13]. Nanoencapsulation can influence drug stability, specificity, efficacy, and/or tolerability [14, 15]. Further advantages include improvement of intracellular penetration and oral bioavailability along with the option of targeting drug delivery to specific tissues and organs. This is attained by either binding the drug to the exterior surface of the NPs or by encapsulating the drug internally [16, 17]. Particle size also plays a role in the modifiability of a NP such in the obtaining of certain surface properties which affect degradation time, elimination processes, and intracellular uptake. When the diameter of NPs are reduced to below 100 nm, degradation in the mononuclear phagocytic system (MPS) can be avoided, enabling them to stay in the circulatory system thereby increasing the probability of reaching the targeted delivery site [18]. Also, if the drug exhibits a hydrophilic surface, the chances of eluding the MPS is greatly decreased. If the MPS does not recognize a particle, macrophages will quickly attempt to rid the system of the particle [19]. By altering the NP characteristics, this important biological defense can be overcome.

Particle charge is a characteristic which has a significant effect on NP distribution [11, 20]. Adherence of cationic charged NPs to the negatively charged cell membrane essentially

increases cellular uptake. Lastly, particle shape consisting of short spheres with narrow size distribution exhibits effective delivery of the drug because as particle length increases, NP binding to the drug decreases [10, 17]. This approach is also advantageous because it can reduce drug toxicity and side effects associated with a drug due to the ability of targeting specific organs and tissues.

The FDA-approved, biodegradable, linear copolymer poly (lactide-*co*-glycolide) (PLGA) is favorable for NP formulation for many reasons, including commercial availability, exceptional biocompatibility, and low toxicity [11, 21]. Secondly, when this polyester copolymer undergoes hydrolysis (Fig. 2), water diffuses into the sample and causes the acidic end groups on each monomer to autocatalyze drug release [22, 23]. The mobility of the polymer chains is then greater due to a drop in PLGA molecular weight. The monomers are permitted to diffuse and water molecules occupy the newly accessible space causing polymer erosion. Degradation of PLGA continues releasing the drug through the porous features at a slow rate, and the acidic monomers are then consumed via the Krebs cycle with marginal toxicity [15, 22, 24]. Degradation time is affected by the ratio of lactic acid and glycolic acid where the optimum degradation time is achieved with a 50:50 ratio [23]. Typically, particle size ranges from 50-500 nm, which allows smaller size diameters to consistently be obtained [17]. With the use of PLGA and other biodegradable polymers, particle size, shape, and charge can be manipulated via specific synthetic processes. In order to increase bioavailability, a smaller particle size and increased surface charge are optimum characteristics.

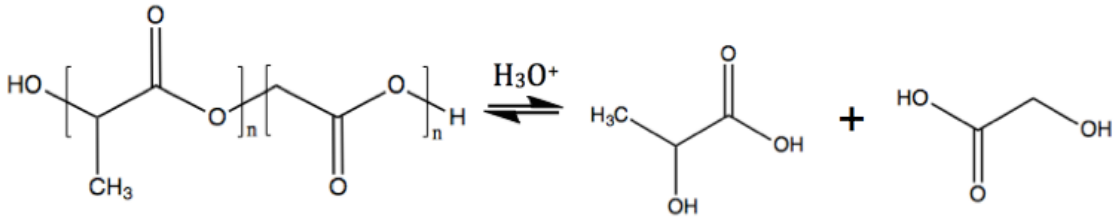

Figure 2. PLGA copolymer undergoing hydrolysis [14, 25-27].

The current study will employ an emulsion-diffusion-evaporation technique with an organic solvent, didodecyldimethylammonium bromide (DMAB) as the stabilizer, and PLGA as the synthetic copolymer. Variations in organic solvent, stabilizer concentration, or centrifugation speed will be employed and analyzed in order to assess optimum conditions. Following each formulation with various solvents, dichloromethane, ethyl acetate, or ethyl acetate/acetone, entrapment efficiency and morphology of NPs will be evaluated, while particle size, zeta potential, polydispersity index (PDI), and entrapment efficiency for each formulation with varying stabilizer concentration (0.10, 0.25, 0.50, or 1.00% w/v) and centrifugation speed (10,000 or 12,000 rpm) will also be studied.

2. Materials and Methods

2.1 Materials

Aliskiren hemifumarate powder was purchased from ChemScene, LLC (Monmouth Junction, NJ, USA). Dichloromethane, DMAB, and PLGA (50:50 copolymer compositions; MW 30,000–60,000 Da) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetone, ethyl acetate, high-performance liquid chromatography (HPLC) grade water and acetonitrile were purchased from Fischer Scientific Laboratory (Fair Lawn, NJ, USA).

2.2 Preparation of Nanoparticles

NP formulations were prepared based on a previously described method using a variety of solvents, stabilizer concentrations, or centrifugation speeds [28].

2.2.1 Effect of Varying Organic Solvents

The method of emulsion-diffusion-evaporation was employed with slight alterations for NP formulation. In brief, 50 mg of PLGA was completely dissolved in 3 mL of dichloromethane, ethyl acetate, or ethyl acetate/acetone. To the organic solvent, 10 mg of aliskiren was added under moderate stirring. Simultaneously, 0.25% w/v of DMAB was placed in water (6 mL) and allowed to fully dissolve under moderate stirring. While the aqueous phase was under moderate stirring, organic phase was added drop-wise. The suspension was sonicated for 5 minutes at 20 kHz, then the contents were allowed to stir under a hood for 30 to 40 minutes so evaporation of ethyl acetate could occur. Upon completion, each solution was centrifuged at 10,000 rpm using a Sorval Biofuge Stratos centrifuge equipped with a Heraeus fixed angle rotor (#3335)(Thermo Fisher Scientific Inc, Waltham, MA, USA) for 5 minutes. The supernatant was collected to measure entrapment efficiency as well as capture transmission electron microscope (TEM) images.

2.2.2 Effect of Varying Stabilizer Concentration and Centrifugation Speed

The variations of stabilizer concentrations for DMAB included 0.10, 0.25, 0.50, or 1.00% w/v, while the centrifugation speeds were 10,000 or 12,000 rpm. This allowed for detection of the optimum centrifugation speed and DMAB concentration in terms of zeta potential, particle size, and entrapment efficiency.

2.3 Nanoparticle Characteristics

Formulations were evaluated based upon various NP criteria, entrapment efficiency, particle morphology, size, zeta potential, and PDI.

2.3.1 Entrapment Efficiency

A 1:3 ratio dilution of collected supernatant to HPLC-grade acetonitrile was performed, and the corresponding absorbance values were measured employing the same instrumentation. These values were employed for the calculation of entrapment efficiency utilizing the following equation: Entrapment efficiency (%) = (Amount of aliskiren entrapped in NPs/Total amount of aliskiren used for formulation) × 100.

For all measurements of entrapment efficiency, a calibration curve was constructed using solutions of known aliskiren concentrations (10,000-1,000,000 ng/mL) and measuring their absorbance values using ultraviolet–visible spectroscopy (Eppendorf Biophotometer, Hauppauge, NY, USA) with a wavelength set at 260 nm.

2.3.2 Particle Morphology

To assess aliskiren loaded PLGA-NP shape and surface morphology under varied organic solvent conditions, TEM (Tecnai Philips Transmission Electron Microscope; FEI, Hillsboro, Oregon, USA) was employed. After vortex mixing, 2 μL aliquots of the NP suspensions were positioned on a 200 mesh copper grid containing Formvar film (Electron Microscopy Sciences, Hatfield, Pennsylvania). Examination was performed at 80 kV once the samples had air dried for 1 hour.

2.3.3 Particle Size, Zeta Potential, and Polydispersity Index

A NICOMP Particle Sizer (Particle Sizing Systems, Port Richy, FL, USA) was used to evaluate particle size via dynamic light scattering, while zeta potential was approximated under an electrical field with a foundation of electrophoretic mobility. PDI, as an indicative of particle size distribution, was also measured by NICOMP Particle Sizer.

2.4 Data Treatment and Statistical Analysis

Each experiment was carried out in triplicate, and the resulting data is presented as mean ± SD. Variation in centrifugation speeds were compared with the use of a Student's t-test, with a p-value < 0.05 indicating a significant difference. * indicates a significant difference between two groups.

3. Results and Discussion

3.1 Effect of Varying Organic Solvents

Different organic solvents were examined in order to determine which solvent would yield better results in terms of drug entrapment efficiency and particle morphology.

3.1.1 Entrapment Efficiency

For each known concentration of aliskiren formulation, the absorbance value was measured in triplicate, and the average value was plotted against the known concentration of aliskiren solution. With an R^2 value of 0.99716 (Fig. 3), this calibration curve was employed for all entrapment efficiency measurements.

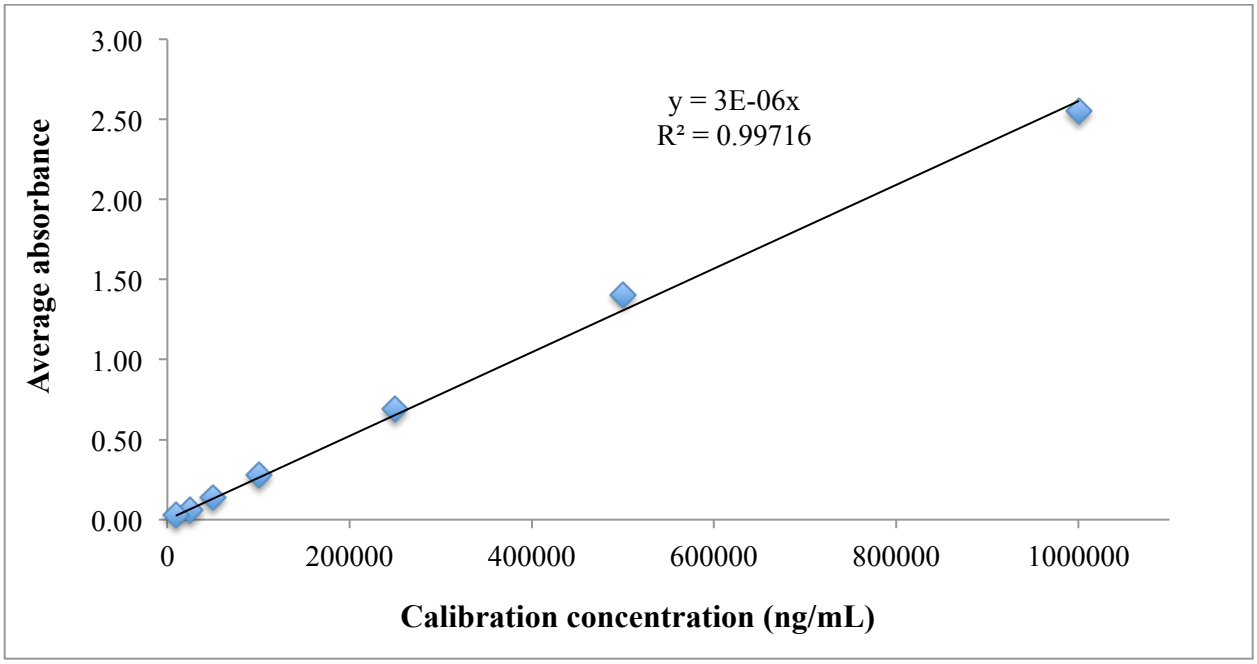

Figure 3. Standard calibration curve for drug entrapment efficiency.

It was imperative to choose an organic solvent, which could sufficiently dissolve hydrophilic aliskiren as well as produce a desired entrapment efficiency for the PLGA-NP formulation. The three readily available and cost effective choices were dichloromethane, ethyl acetate, and ethyl acetate/acetone due to their high solubility for many compounds. Additionally, solvent toxicity was considered, including the high volatility of dichloromethane as well as its toxic tendencies in humans; however, since the method of emulsion-diffusion-evaporation is inclusive of complete evaporation of the organic phase upon formation of the PLGA-NP, the toxicity is not an issue [29]. On the contrary, ethyl acetate and acetone both show lower toxicity than dichloromethane; however, each evaporates at a more rapid rate. Thus, when using these solvents, the method of preparing PLGA-NPs had to be performed in a timely manner [30, 31]. As shown in Table 1, entrapment efficiency was highest (82.68 ± 1.18 %) for PLGA-NPs that were formulated employing ethyl acetate as the organic solvent, 0.25% w/v DMAB, and 10,000

rpm centrifugation speed. The higher value for entrapment efficiency is indicative of an increased amount of aliskiren encapsulated in the particular PLGA-NP formulation, which is preferable.

Table 1. Effect of various organic solvents at 0.25% w/v DMAB and 10,000 rpm on aliskiren entrapment efficiency.

	Dichloromethane	Ethyl acetate	Ethyl acetate/acetone
Entrapment efficiency (%)	73.24	82.70	58.64
	72.45	81.50	58.12
	72.56	83.85	60.52
Average	72.75	82.68	59.09
S.D.	0.43	1.18	1.26

3.1.2 Particle Morphology

Fig. 4 displays PLGA-NPs (lighter areas of image) with employment of dichloromethane as solvent. Although many NPs are present, the poor NP morphology and increased particle size makes this formulation unfavorable as drug delivery to target cells would be inhibited due to reduced permeability through epithelial barriers in the body [32]. The shapes of the NPs appear to have pointed tips as opposed to the preferred spherical shape. Fig. 5 illustrates PLGA-NPs using ethyl acetate as solvent, and of the three images, this formulation exhibited the most ideal characteristics, which included spherical morphology, decreased particle size, and the highest entrapment efficiency. These conditions are less complimentary to the *in vivo* conditions of the vascular and lymphatic systems, therefore decreasing their chance of clearance from the circulatory system [33]. The decreased particle size is related to an increased surface area on the NPs which allows for more interaction with the solvent [28]. Lastly, Fig. 6 is representative of ethyl acetate/acetone as solvent, and it is observed to have similar physical features to the NP in Fig. 5. As opposed to Fig. 5, the entrapment efficiency is the least desirable of all three solvents.

The decreased entrapment efficiency with ethyl acetate/acetone and the poor morphology with dichloromethane indicate that, of those organic solvents tested, ethyl acetate is the best choice for PLGA-NP formulations in regard to spherical shape and increased entrapment.

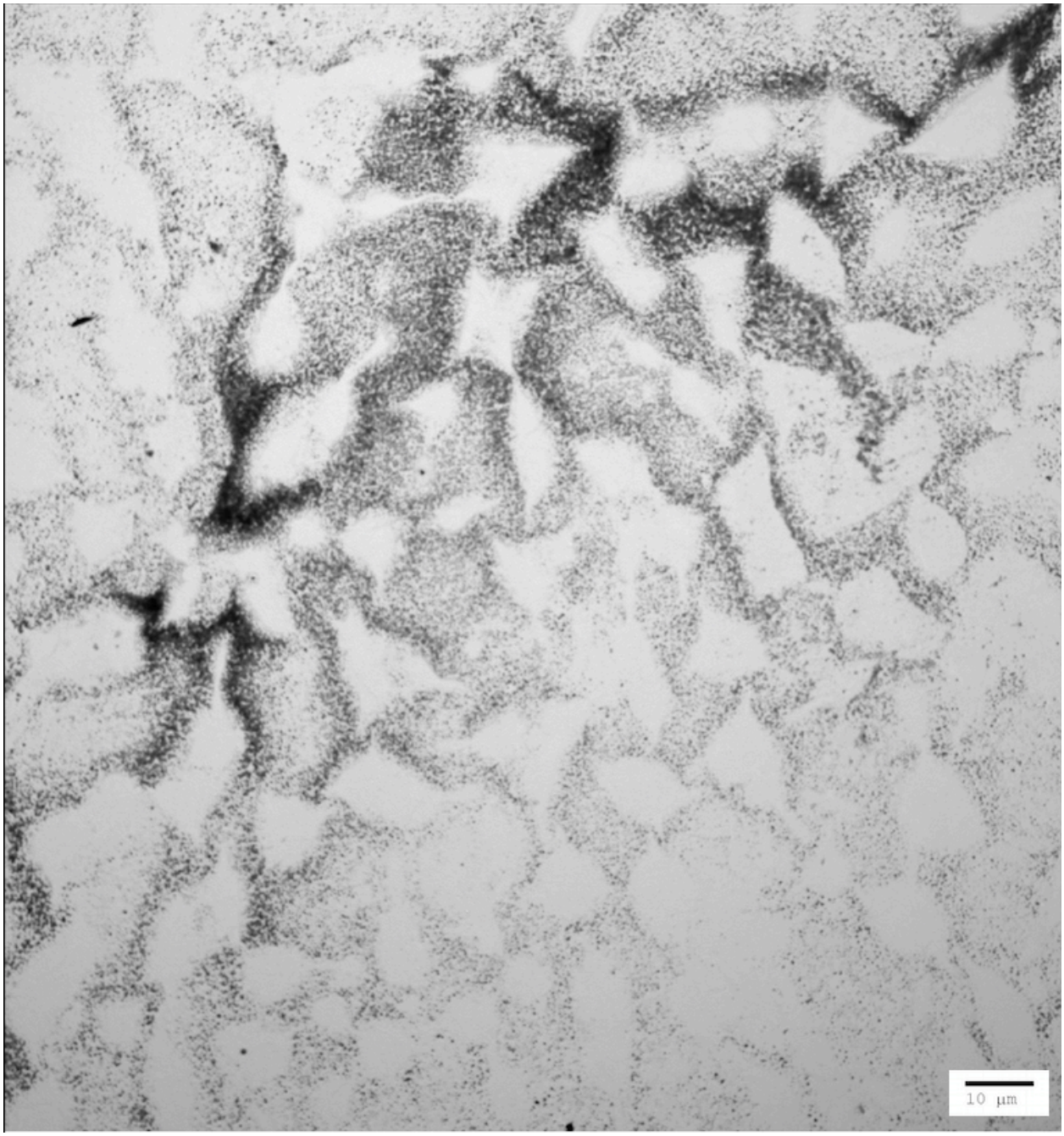

Figure 4. TEM image illustrating morphology of NPs formulated using dichloromethane.

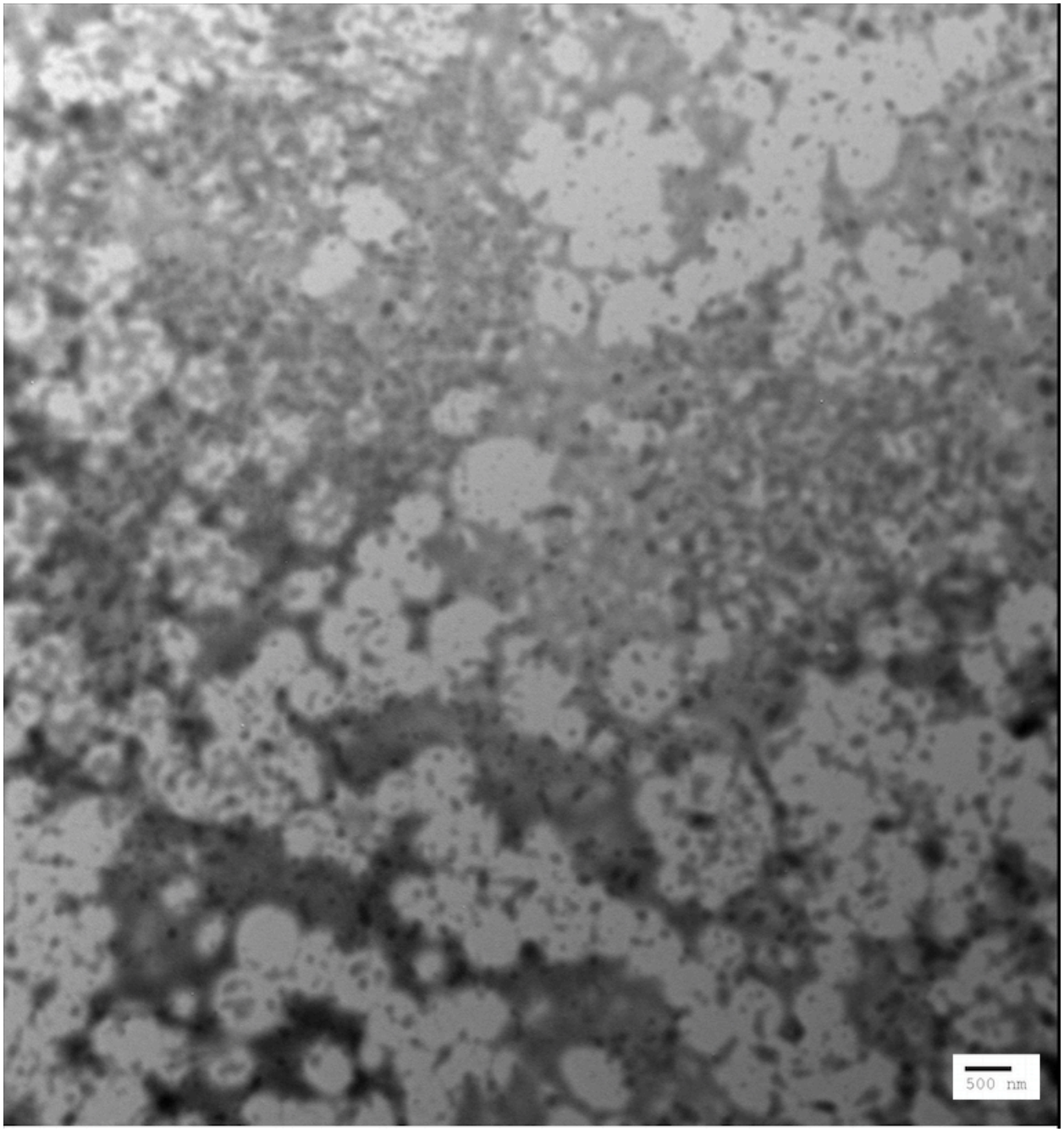

Figure 5. TEM image illustrating morphology of NPs formulated using ethyl acetate.

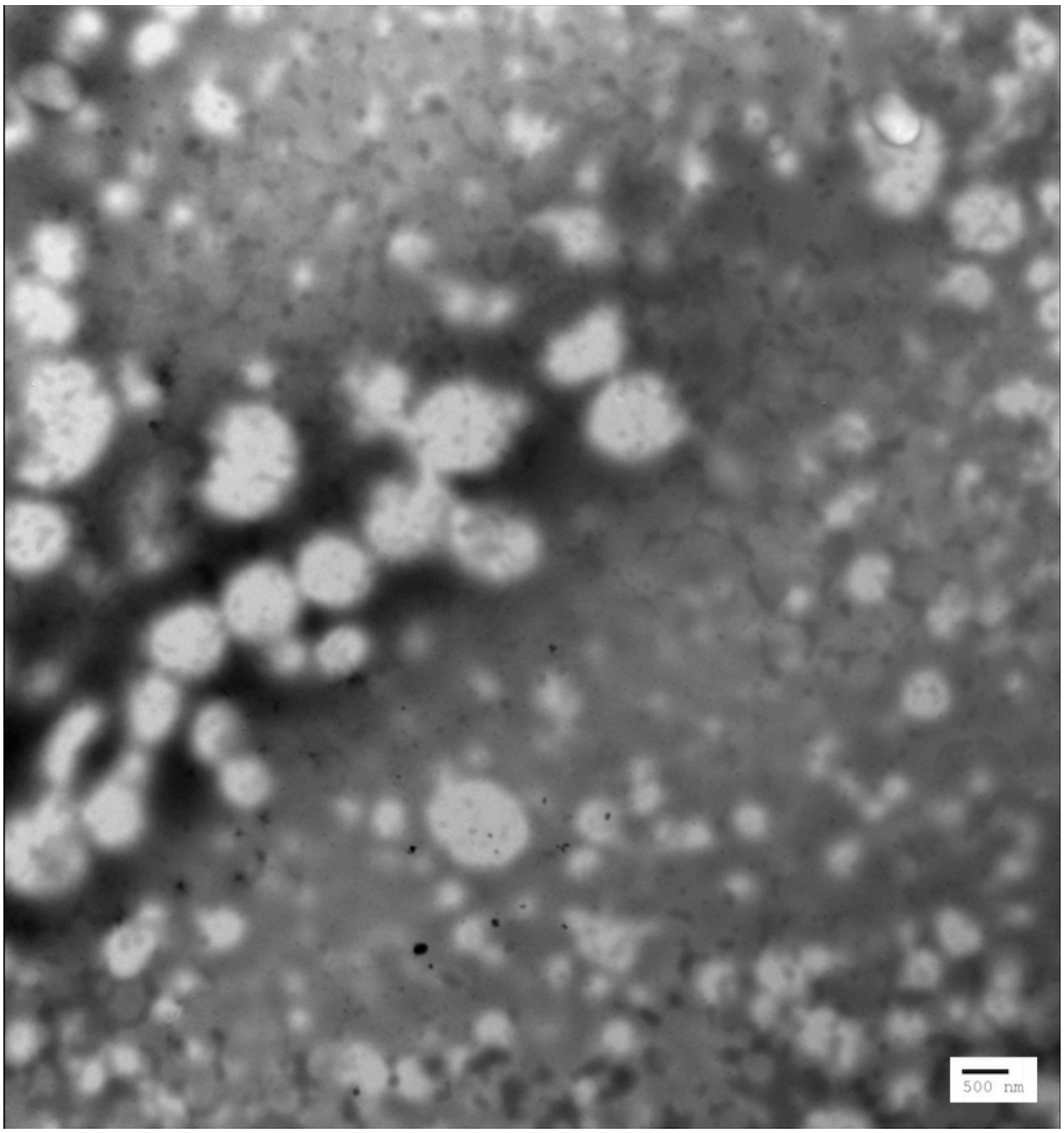

Figure 6. TEM image illustrating morphology of NPs formulated using ethyl acetate/acetone.

3.2 Effects of Varying Stabilizer Concentration and Centrifugation Speed

The use of increasing of concentrations of stabilizer along with variation in centrifugation speed allows for the optimization of NP formulation.

3.2.1 Particle Size

When DMAB stabilizer concentrations and centrifugation speeds were varied, the first characteristic analyzed was particle size. Centrifugation speed alteration produced significantly different particle sizes for the PLGA-NP formulations containing 0.10, 0.50, and 1.00% stabilizer concentrations. The smallest acquired particle size (67.27 ± 0.87 nm) was found with the use of 0.50% w/v DMAB stabilizer and a centrifugation speed of 12,000 rpm (Fig. 7). Particles that measure less than 100 nm are small enough to permeate sub-mucosal membranes, whereas particles greater than 100 nm remain in the epithelial lining and do not reach the target delivery site [15]. In turn, cellular uptake is affected since the number of NPs which reach the cell are minimized. Even though the 1.00% w/v DMAB stabilizer spun at 10,000 rpm (75.67 ± 0.89 nm) was the optimum formulation chosen, as opposed to the 0.50% w/v DMAB stabilizer with centrifugation speed of 12,000 rpm, the formulation still encompassed PLGA-NPs that were less than 100 nm in size. This made for NPs that also had efficient cellular uptake that bypassed the MPS.

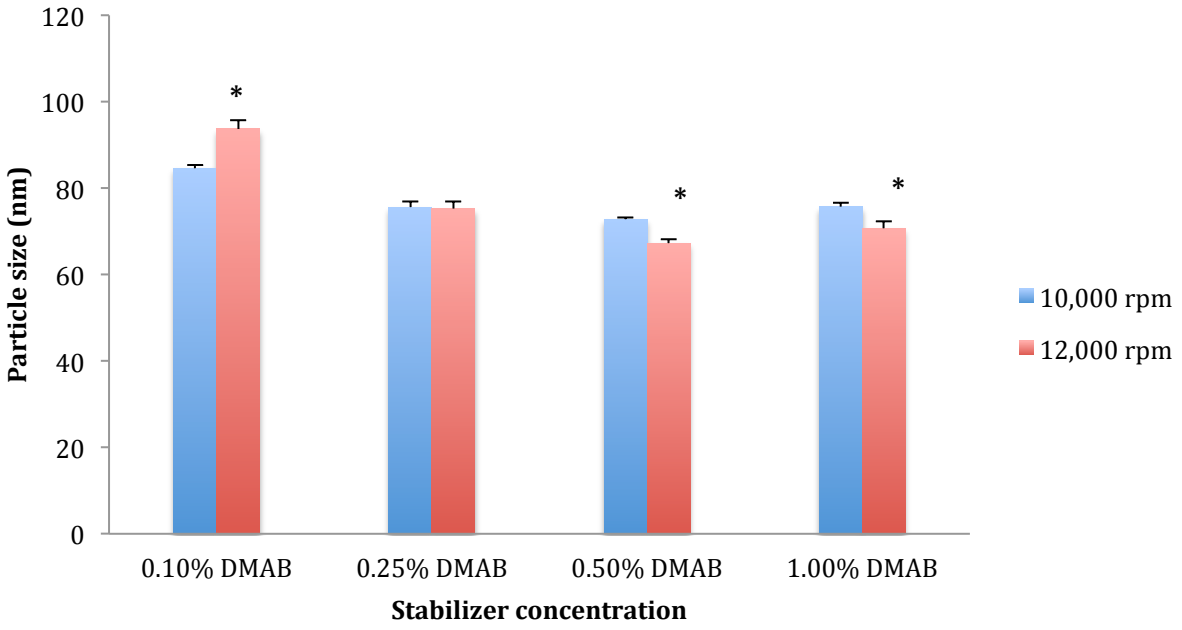

Figure 7. Effect of DMAB concentration at 10,000 or 12,000 rpm on NP particle size.

3.2.2 Zeta Potential

For zeta potential, variation in centrifugation speed produced a significant change within the formulations employing 0.50 and 1.00% stabilizer concentration. When analyzed, the highest zeta potential (18.73 ± 0.03 mV) was seen with 1.00% w/v DMAB stabilizer and 10,000 rpm centrifugation speed (Fig. 8). Zeta potential directly affects cellular uptake by creating an attraction between the positive surface charge of the PLGA-NP and the negative surface charge of the cell membrane. It is desirable to have a more positive zeta potential value, around 20 mV, so that the NPs may adhere to the negatively charged cell membrane more adequately [17]. Higher bioavailability will be achieved if the PLGA-NP is capable of permeating the cell membrane and transported to the primary endosomes within the cell [15]. From there, the PLGA-NPs can be separated, and selected NPs are recycled to the cell exterior in order to maintain a required concentration gradient of NPs in the outside medium of the cell. Without this balance,

exocytosis of the NPs will be activated. Inside the cell, the remaining encapsulated PLGA-NPs migrate to the cytoplasm where controlled release of aliskiren occurs. This characteristic is highly considered when choosing an optimum formulation due to its vital role in cellular uptake and drug release.

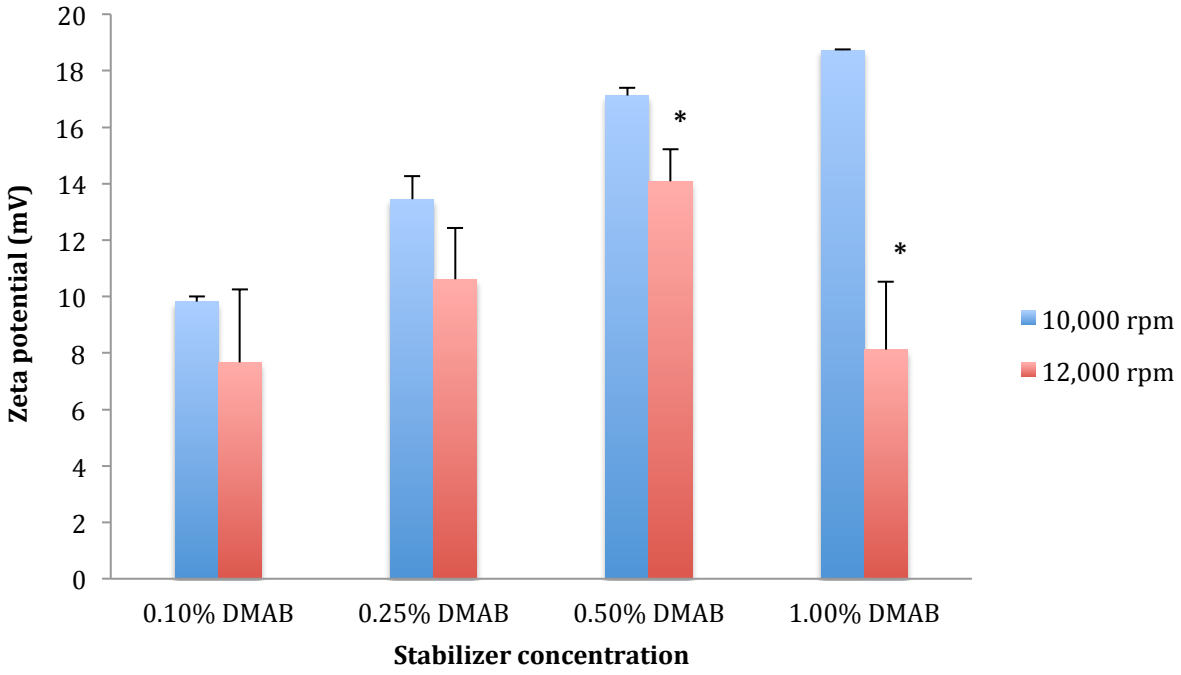

Figure 8. Effect of DMAB concentration at 10,000 or 12,000 rpm on NP zeta potential.

3.2.3 Polydispersity Index

When the PDI of a dispersion is less than 0.1, the particles are said to be narrowly dispersed in size. On the contrary, a value that extends beyond 0.3 has broadly dispersed particle sizes [10]. In this study, the best PDI (0.15 ± 0.03) value was observed when employing 0.25% w/v DMAB stabilizer and 10,000 rpm centrifugation speed (Fig. 9). However, the chosen formulation of 1.00% w/v DMAB stabilizer at 10,000 rpm centrifugation speed displayed a value

of 0.321 ± 0.01 for PDI. Though not the highest value, the formulation demonstrated sufficient consistently dispersed PLGA-NP in the final formulation. A significant change was observed in PDI only in the formulation using 0.50% stabilizer concentration.

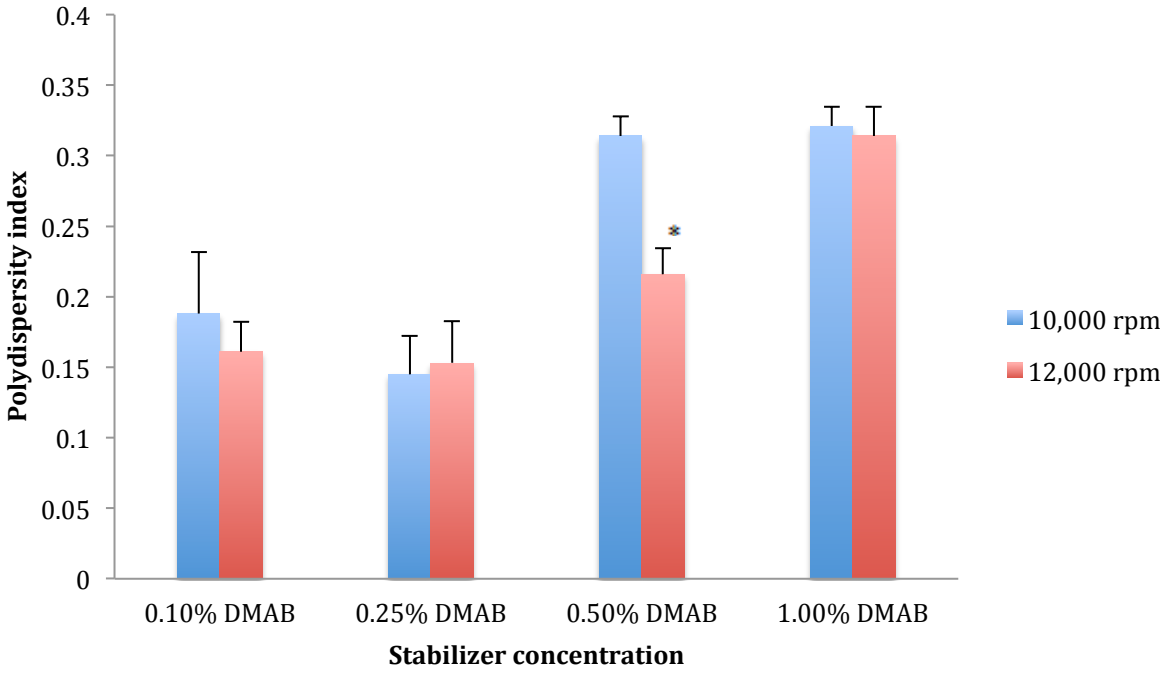

Figure 9. Effect of DMAB concentration at 10,000 or 12,000 rpm on NP PDI.

3.2.4 Entrapment Efficiency

Although the highest entrapment efficiency (82.68 ± 1.18 %) was achieved when 0.25% w/v DMAB stabilizer and 10,000 rpm centrifugation speed were used (Fig. 10), we chose formulation of 1.00% w/v DMAB stabilizer at 10,000 rpm centrifugation speed displaying 71.62 ± 0.11 % for entrapment efficiency, due to sufficient encapsulation of aliskiren and optimized particle size and zeta potential values of formulation. The importance of this value relates to the amount of drug bound to PLGA-NP present in the resulting formulation. In order for a drug

delivery system to be considered successful, it is essential to have a high loading capacity in order to curtail the overall dosage requirement. Drug loading of NPs is accomplished by adding the drug of interest, aliskiren, at the time of NP production, which was done in the methods described in previous sections [34]. The larger entrapment efficiency value indicated that there was a greater amount of aliskiren bound to the NPs per total amount of aliskiren used for NP production. Further, the amount of drug bound to the NPs was greatly influenced by the copolymer PLGA and the chemical structure of aliskiren [35]. Entrapment efficiency was significantly different between centrifugation speed in all formulations using 0.10, 0.25, 0.50 and 1.00% stabilizer concentration.

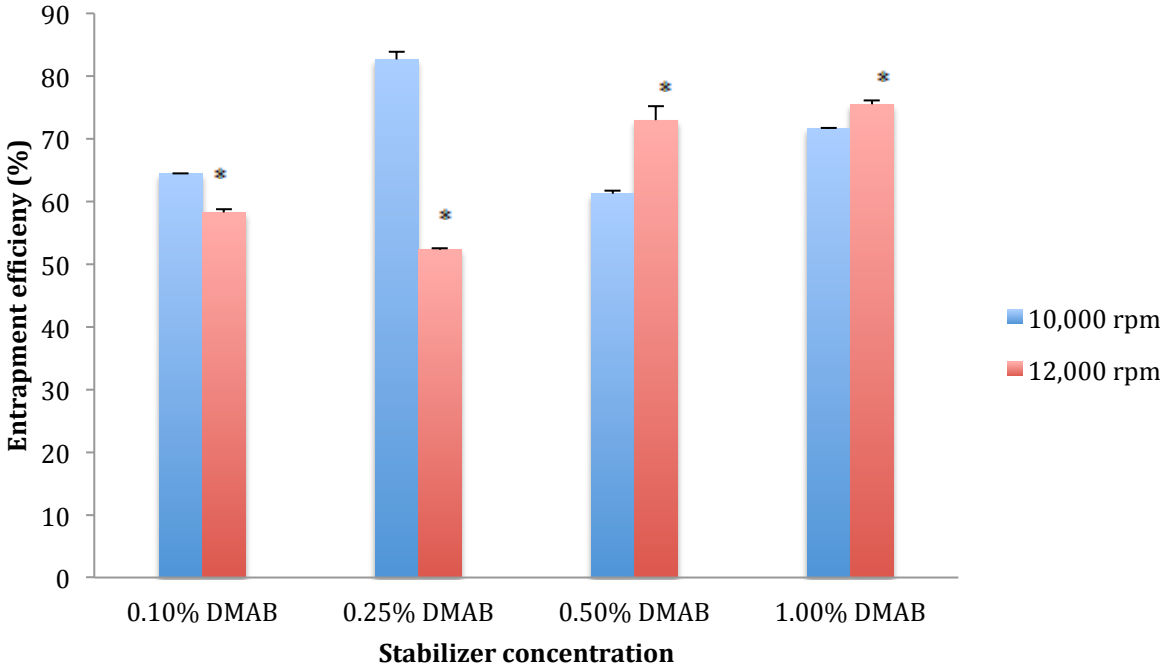

Figure 10. Effect of DMAB concentration at 10,000 or 12,000 rpm on NP entrapment efficiency.

4. Conclusion

It is conclusive that the optimum formulation for the aliskiren loaded PLGA-NPs includes ethyl acetate as the organic solvent, 1.00% DMAB stabilizer, and 10,000 rpm centrifugation speed. For all possible alterations, these conditions offered the highest zeta potential, reasonable particle size below 100 nm, agreeable entrapment efficiency, and a uniform distribution of NPs in the solution. With the reformulation of the aliskiren loaded PLGA-NPs, stability and structural improvement may have been achieved to permit distinct advantages over aliskiren without PLGA-NP encapsulation. Additionally, the basis formed by this optimum polymer synthetic NP formulation of aliskiren could be beneficial for the development of more effective oral dosing of aliskiren in terms of pharmacokinetic factors, such as increased bioavailability and systematic absorption, due to an enhanced drug delivery system. Further, patient compliance may be more favorable due an estimated reduction of cost for the current antihypertensive drug.

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