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Preparation and *in-vitro* Evaluation of Poly-ε-Caprolactone Nanoparticles Containing Atorvastation Calcium

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***Abstract*-**Preparation and evaluation of poly-ε-caprolactone (P-ε-CL) nanoparticles containing atorvastatin calcium (AC), a drug with poor solubility and poor bioavailability, is presented. The drug loaded nanoparticles were prepared by solvent displacement method. The influence of different formulation variables, such as type and concentration of stabilizers in the aqueous phase, concentration of polymer and surfactant in the organic phase, addition of an anti-oxidant, and others, on particle size, drug entrapment efficiency, *in-vitro* drug release along with other physico-chemical properties of the nanoparticles was investigated using several full factorial designs. The optimized AC nanoparticles showed an average particle size of less than 200 nm, a drug loading capacity of more than 70% and released the drug in a controlled fashion over 24 hour. Transmission electron microscope (TEM) revealed that the prepared AC nanoparticles were nearly spherical. Short-term stability results of selected formulae were satisfactory.

Keywords: Solvent displacement method; Nanosuspensions; Hyperlipidemia, Atorvastatin

1. Introduction

Nanotechnology has emerged as a powerful and promising tool to solve many of the problems encountered with the oral delivery of different types of drugs with poor absorption and bioavailability. Nanomedicines were reported to increase efficacy, specificity, tolerability and therapeutic index of corresponding drugs (Lai et al., 2009; Mishra et al., 2010). Atorvastatin, the leading drug among the statins which inhibits the enzyme HMG-CoA reductase resulting in reduced cholesterol synthesis (Law et al., 2003), suffers from poor water solubility, poor oral bioavailability, high presystemic clearance and dose-related adverse effects which limits its effective oral delivery (Wu et al., 2000; Lennernas, 2003). Therefore modification of the solid form and drug particle characteristics are expected to alter the dissolution rate, bioavailability, efficacy and safety of the drug especially when formulated as an oral nanoparticulate system (Anwar et al., 2011; Khan et al., 2012; Mantri et al., 2012). The aim of this work is to design AC nanoparticles for oral delivery with desirable physico-chemical properties that release the drug in a controlled fashion in order to enhance the drug oral absorption, reduce toxic effects associated with immediate-release marketed products, protect the drug from presystemic clearance in the GIT and/or liver and possibly alter the pharmacodynamics profile of the drug.

2. Methods

2.1. Selection of Formulation Variables for the Preparation of Drug-Loaded Nanoparticles

2.1.1. Effect of Using Different Polymer Concentrations

P-ε-CL at three different concentrations (0.5%, 0.8% and 1% w/v) and the drug were dissolved in 25 ml of acetone by sonication for 10 min to result in a drug: polymer ratio of 1:4 (w/w). The organic phase

was injected at a rate of 10 ml/min into the aqueous phase (pH 5.5) containing Pluronic F-68 at a concentration of 0.5% or 1% (w/v) using a syringe gauge 27G and a stirring rate of 800 rpm at room temperature. The ratio of the organic phase to the aqueous phase was kept constant at 1:2. Removal of acetone and a large proportion of water were done using a rotavapor at 150 rpm under reduced pressure and temperature of 45 ^{o} to 55 ^{o} C for 45 min. The final volume of the resulting suspension was adjusted to 10 ml using distilled water. The different formulae of AC nanoparticles are described in Table 1 and are coded A1 to A6. The prepared nanoparticles were evaluated for particle size. The individual and combined effects of the formulation variables on particle size were analyzed statistically.

Table1. Particle size of AC nanoparticles prepared using different P-ε-CL and Pluronic F-68 concentrations

Formula	F-68 (% w/v)	P-ε-CL (% w/v)	d (0.5) (nm)	d (0.9) (nm)	Span value
A1	0.5%	0.5%	133.6±1.5	246.6±3.0	1.36±0.03
A2		0.8%	152.7±2.5	326.0±5.2	1.67±0.00
A3		1%	141.6±1.5	313.3±3.0	1.72±0.01
A4	1%	0.5%	174.6±4.1	395.6±4.9	1.81±0.02
A5		0.8%	135.3±2.5	265.6±5.1	1.46±0.07
A6		1%	126.3±1.5	273.0±2.6	1.64±0.29

2.1.2. Effect of Adding Span 80 to the Organic Phase

Nanoparticles were prepared as described above except 0.25% w/v of Span 80 was added to the organic phase. The effect of using Span 80 on the physical stability and particle size of the produced nanoparticles was studied.

2.1.3. Effect of Using Different Drug to Polymer Ratios

The effect of using different drug to polymer ratios, namely 1:50, 1:20, 1:10 and 1:4, on particle size and drug entrapment efficiency (EE) was studied. In this study P-ε-CL was used at a concentration of 0.5% w/v, Pluronic F-68 at a concentration of 0.5% w/v and Span 80 at a concentration of 0.25% w/v.

2.1.4. Effect of Changing the Concentration of Surfactants in the Aqueous and Organic Phases

A $2^{1}3^{2}$ full factorial design was used to prepare AC nanoparticles using three formulation variables. The three variables evaluated were the type of the surfactant used in the aqueous phase at two levels, Tween 60 or Pluronic F-68, the concentration of the surfactant used in the aqueous phase at three levels, 0.125%, 0.25% or 0.5% w/v and the concentration of the surfactant (Span 80) used in the organic phase at three levels 0.125%, 0.25% or 0.5% w/v. In total, 18 experimental runs were carried out; these are detailed in Table 2. The formulae were coded ANF1-ANF18.The prepared nanoparticles were evaluated for particle size and drug EE.

2.1.5. Effect of Adding α-tocopherol Acetate to the Organic Phase

Atorvastatin is prone to oxidation. Formulae ANF6, ANF8, ANF15 and ANF16 were chosen to determine the effect of adding α-tocopherol acetate to the organic phase, as an antioxidant, on the particle size and drug EE. In these experiments α-tocopherol acetate was added to each of the formulae in the ratio of 1:1 and 1:2 drug to tocopherol.

2.2. Nanoparticle Characterization

2.2.1. Particle Size Analysis

Particle size and polydispersity were determined by laser diffraction particle size analyzer (Master seizer Hydro MU 2000, Malvern MU instruments, UK). d(0.9) and d(0.5) were used as qualitative parameters to characterize nanoparticle suspensions. The span value was used as an indication of the polydispersity of the preparations (index from 0-9). It was calculated from d (0.9), d (0.5) and d (0.1) according to the below equation:

$$Polydispersity\ (span\ value) = \frac{d(0.9) - d(0.1)}{d(0.5)}$$

where; d (0.9) corresponds to particle size above 90% of the sample, d (0.5) corresponds to particle size above 50% of the sample and d (0.1) corresponds to particle size above 10% of the sample.

2.2.2. Determination of Zeta Potential

The Zeta potential of selected nanoparticle formulae was determined by laser light scattering technique (Malvern Zetasizer ZS, Malvern, Worcestershire, UK) to study surface charges on the particles.

2.2.3. Determination of Entrapped Drug by HPLC

Both total and free (unentrapped) AC concentrations in each sample were determined using HPLC method (Zhang et al., 2009). Total AC was determined after full dissolution of 1 ml of drug loaded nanoparticles suspension in 25 ml of acetonitrile. The mobile phase consisted of 55:45(v/v) mixture of acetonitrile: 0.5% (v/v) glacial acetic acid in de-ionized water. The detection wavelength was 245.5 nm. The entrapment efficiency percent (EE%) was calculated from the following equation:

$$EE\% = \frac{total\ amount\ of\ AC - free\ AC}{total\ amount\ of\ AC} \times 100$$

2.2.4. Morphological Studies

The morphology of the nanoparticles was determined using transmission electron microscopic (TEM) technique.

2.2.5. *In-vitro* Release Studies

The *in-vitro* release of AC from the nanoparticles was determined using dialysis bag method (Calvo et al., 1996). Freshly prepared nanoparticles suspension corresponding to 10 mg of AC was placed in a dialysis bag previously soaked for 24 h in the dissolution medium. The bags were suspended in the dissolution vessel containing 1000 ml phosphate buffer (pH 7.4) in addition of 0.5% w/v Tween 80. All the vessels were kept at 37 ^{o}C ± 0.5˚C with a paddle rotation speed at 100 rpm (USP apparatus 2 dissolution). The percentage drug released in the medium was determined using HPLC method. Lipitor®10 mg (Pfizer, Egypt) was used as a reference tablet for the *in-vitro* release studies. Dissolution of the tablets was done following the above method but without using the dialysis bag.

2.3. Short-term Stability Studies

A short-term stability study was performed on four chosen formulae ANTF6, ANTF15, ANTF8, and ANTF18. The study was carried out at room temperature and at 4°C for 7 days. The vials containing AC nanoparticles suspensions were sealed and wrapped in aluminum foil and subdivided into two groups. One group is stored in refrigerator at 4°C and the other group is stored at room temperature 25°C for 7 days. At the predetermined time intervals, aliquots were taken and subjected to particle size and % drug entrapment studies. The change in appearance, particle size, span value, and % drug entrapped were recorded and compared to results obtained from freshly prepared nanoparticles.

2.4. Statistical Analysis and Experimental Design

All measurements were carried out in triplicate and values are presented as the mean ± SD. Statistical calculations were carried out using the software Minitab 16. Statistical comparisons between two groups were made using the two-tailed, unpaired Student's t-test. A p- value of less than 0.05 was considered statistically significant. The one-way analysis of variance (ANOVA) *F*-test for testing the equality of several means followed by Tukey's post t-test analysis was used for multiple comparisons. Full factorial designs were employed in the different studies to evaluate the individual and combined effects of the studied variables. The experimental trials were performed at all possible combinations with replication. Multiple regression analysis was performed in order to fit a second order polynomial equation, described below, to the data:

$$Y = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

Where Y is the response, β_o, β_1, β_2, β_3,……, β_{23} are the regression coefficients and X_1, X_2, X_3, are the studied factors at different levels.

3. Results and Discussion

3.1. Effect of Using Different Polymer Concentrations

The effect of using P-ε-CL at three concentrations levels in the organic phase and Pluronic F-68 at two concentrations levels in the aqueous phase, on the particle size is shown in Table 1. The ANOVA test showed a significant two-way interaction for the effect of P-ε-CL and Pluronic F-68 concentrations on the particle size ($p < 0.0001$). It was shown that at low Pluronic concentration increasing the polymer concentration caused a significant increase in particle size. This could be attributed to an increase in the organic phase viscosity with higher polymer concentration which provides higher resistance to the diffusion of polymer-solvent phase into the external aqueous phase and larger nanoparticles are thus formed. (Chorny et al., 2002; Mora-Huertas et al., 2011). On the other hand, results showed that increasing Pluronic concentration from 0.5% to 1% w/v resulted in a significant decrease in particle size at high polymer concentrations (0.8 and 1% w/v) but increased the particle size significantly at low polymer concentration (0.5% w/v). These results could be attributed to solubility difference of polymer in the acetone/water solvents in presence of high surfactant concentration compared to low surfactant concentration. Higher polymer concentration might also result in higher viscosity of the organic phase which might slow down the diffusion rate and might lower the rate of Ostwald ripening for the more viscous solutions thereby smaller particles were obtained (Beck-Broichsitter et al., 2010). All formulae (A1-A6) showed some degree of particle agglomeration after 24 h in refrigerator. This agglomeration phenomenon as a function of other formulation variables will be further investigated using formula A1 as it showed the smallest d (0.9) and span value.

3.2. Effect of Adding Span 80 to the Organic Phase

In an attempt to improve the physical stability of the formed nanoparticles and prevent particle aggregation, span 80 was added to the organic phase at a concentration of 0.25% w/v. Results showed that addition of Span 80 to the organic phase improved the physical stability of the nanosuspensions (using formula A1) showing no visible aggregates upon storage for one week in refrigerator. However, the mean particle size (d 0.5) of the nanoparticle increased upon addition of Span 80 to 182.33± 2.52 nm compared to 134.67±1.53 nm for particles prepared without the addition of Span 80.

3.3. Effect of Using Different Drug to Polymer Ratios

Results showed a significant increase in particle size and a significant decrease in % drug entrapped ($p<0.0001$) as the ratio of drug to polymer is increased. Formula AS3 (ratio 1:10), however, showed a significantly smaller particle size when compared to formulae AS1 (ratio 1:50) and AS2 (ratio 1:20)

which contain lower drug to polymer ratios. At this specific ratio of drug to polymer (1:10), a drug-polymer complex that exhibits minimum solubility in the aqueous phase could be formed resulting in higher rate of precipitation and decreased particle size. The decreased EE observed with increasing theoretical drug loading is in agreement with published work reporting that decreased drug EE is associated with a decrease in the mutual solubility of the drug and polymer in the organic solvent (Beck-Broichsitter et al., 2010).

3.4. Effect of changing the concentration of surfactants in the aqueous and organic phases

The results obtained from these studies are listed in Table 2 and are described in interaction plots in Figure 1.

Table .2. Determination of optimal surfactant concentration in the aqueous and organic phases by using a $2^1 3^2$ full factorial design

Type	Surfactant in aqueous phase (% w/v)	Span 80 (% w/v)	Formula	d (0.9) (nm)	E.E. (%)	Aggregation
Pluronic F-68	0.125	0.125	ANF1	164.0±1.6	29.37±1.10	present
		0.25	ANF2	164.0±4.6	37.00±1.73	present
		0.5	ANF3	203.3±17.2	44.50±1.50	present
	0.25	0.125	ANF4	168.0±0.0	55.05±2.00	absent
		0.25	ANF5	222.0±2.6	67.47±0.92	absent
		0.5	ANF6	192.3±2.5	67.70±0.79	absent
	0.5	0.125	ANF7	195.0±5.0	54.00±2.29	absent
		0.25	ANF8	202.0±16.0	67.55±1.61	absent
		0.5	ANF9	230.0±5.2	61.61±0.68	absent
Tween 60	0.125	0.125	ANF10	174.0±1.0	46.87±1.96	present
		0.25	ANF11	171.6±8.1	55.46±1.55	present
		0.5	ANF12	162.3±1.5	64.90±1.93	present
	0.25	0.125	ANF13	234.0±3.0	65.00±1.00	absent
		0.25	ANF14	171.3±8.0	71.78±1.17	absent
		0.5	ANF15	183.3±22.7	76.19±2.03	absent
	0.5	0.125	ANF16	361.0±4.6	47.17±2.02	absent
		0.25	ANF17	200.0±21.0	51.89±1.54	absent
		0.5	ANF18	163.6±1.5	62.86±1.49	absent

Results showed that particle aggregation occurred after one day storage in refrigerator for formulae containing low concentration (0.125%) of hydrophilic surfactants in the aqueous phase (ANF1, ANF2, ANF3, ANF10, ANF11 and ANF12) at all concentration levels of Span 80. This may be due to insufficient surface coverage of nanoparticles (Wu et al., 2011). On the other hand, formulae containing higher concentrations (0.25% and 0.5%) of surfactant in the aqueous phase showed no aggregation at all concentration levels of Span 80. The type and concentration of surfactant in the aqueous phase as well as the concentration of Span 80 in the organic phase had a significant effect ($p< 0.0001$) on EE based on the regression coefficient estimates obtained from the polynomial equation ($R^2= 0.9473$). Results also showed that the EE was increased on increasing Span 80 concentration, and this effect was more pronounced in presence of Tween than Pluronic. This could be due to specific interaction between the Spans and Tweens which lead to formation of stronger film around the particles that prevent leaching out of the drug. From the above results four formulae, ANF6, ANF8, ANF15 and ANF18 were taken to the next stage. The particle size distribution curves of the chosen formulae also showed unimodal distribution curves with

small polydispersibility index. Figure 2 shows the particle size distribution curve of one of the four chosen formulae.

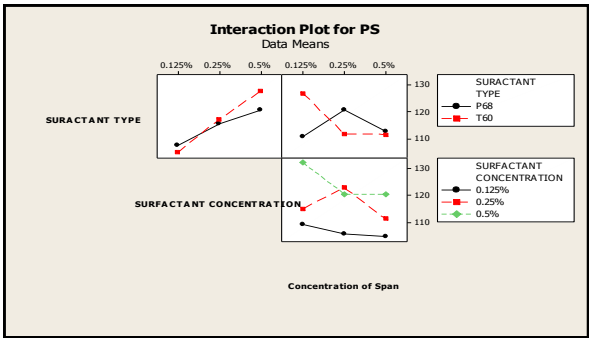

Fig. 1. Interaction plots showing the effect of the type and concentration of surfactant in the aqueous phase and the concentration of surfactant in the organic phase on particle size.

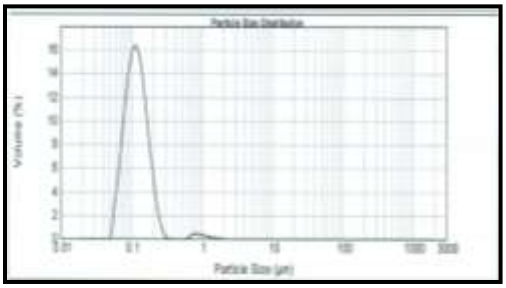

Fig. 2. Particle size distribution curve of formula ANF18.

3.5. Effect of adding α-tocopherol Acetate

Results show that incorporation of α-tocopherol acetate in the ratio 1:1 reduced the particle size, however the reduction in size was not statistically significantly different for all formulae. The EE was significantly increased for all formulae for the ratio 1:1 but the increase was not significant for all formulae in the ratio 1:2. The four formulae ANTF6, ANTF8, ANTF15 and ANTF18 containing α-tocopherol acetate in the ratio 1:1 drug to vitamin were taken to the next stage for nanoparticle characterization.

3.6. Characterization of AC Nanoparticles

3.6.1. Determination of Zeta Potential

Results showed that formulae ANTF15 (-21.2± 0.64 mV) and NATF18 (-23.5± 0.59 mV), containing Tween 60, had significantly higher zeta potential when compared to formulae ANTF6 (-11.7± 0.32 mV) and NATF8 (-12.4± 0.41 mV) which contain Pluronic F-68. It has been reported that adsorption of non-ionic surfactant onto the nanoparticles may partially screen the surface charge of the particles leading to an apparent reduction in the zeta potential (Fontana et al., 1998). For this reason, the zeta potential was remeasured after centrifugation of the nanosuspensions and redispersion in water. Results showed that the zeta potential of formula ANTF15 increased from -21.2± 0.64 mV to -30.5± 0.7 while that of formula ANTF6 increased from -11.7± 0.32 mV to -25.1± 0.40 mV indicating that these surfactants might have reduced the zeta potential of the nanoparticles due to screening of surface charge.

3.6.2. Morphological Studies

The TEM micrographs revealed that most nanoparticles were nearly spherical. The nanoparticle size observed by TEM correlated well with the particle size distribution measured by the Master seizer. Figure 3 show the TEM micrographs of ANTF6 nanoparticles.

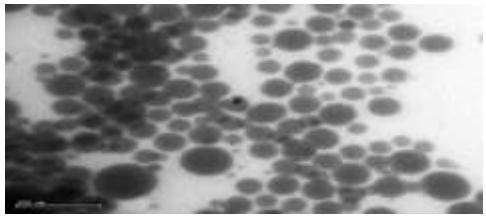

Fig. 3. TEM micrograph of ANF6 nanoparticles.

3.6.3. *In-vitro* Release Studies

The cumulative AC released as a function of time from ANTF6, ANTF8, ANTF15 and ANTF18 nanoparticles compared to AC from the marketed product Lipitor® is illustrated in Figure 4. Remarkable differences in the rate of drug release from the prepared nanoparticles and that of the drug from Lipitor are observed. The nanoparticles showed extended drug release patterns with about 50-60% of the drug being released during the first 6 h while 100% of the drug was released during the first 30 min from the commercial tablet. The release pattern was almost similar for the four formulae showing almost 100% drug released around 24 h.

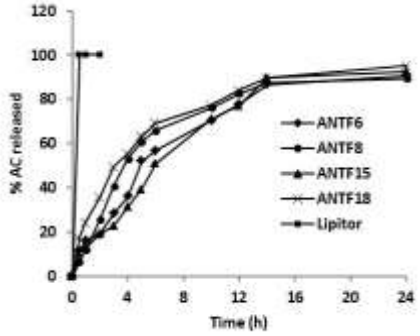

Fig. 4. In vitro release profiles of AC in prepared nanoparticles and AC in commercial tablets in phosphate buffer (pH 7.4) at 37°C (n=3).

3.7. Short-term Stability Studies

Results from particle size analysis showed no significant difference in the particle size for formulae ANFT8 and ANFT18 at room temperature and 4°C (p= 0.45 and p= 0.08 respectively) while formulae ANTF6 and ANTF15 showed significantly larger particle size when compared to as before storage ($p < 0.05$) at both temperatures. This could be due to Ostwald ripening which is known to be highly dependent on temperature (Van Eerdenbrugh et al., 2008). Results also showed that the EE was significantly increased after 7 days storage at room temperature for formulae ANTF6 and ANTF15 ($p<$ 0.05) while formulae ANTF8 and ANTF18 showed no significant difference in the % drug entrapped.

4. Conclusion

We demonstrated that several nanoparticle formulations containing AC investigated in this work are promising formulations that release the drug in a controlled fashion over 24 h when compared to AC oral conventional tablet. The nanoparticles showed mean particle size of less than 200 nm and % drug entrapped of more than 70%. The stability of selected nanoparticles aqueous dispersions was satisfactory.

References

Anwar M., Warsi M.H., Mallick N., Akhter S., Gahoi S., Jain G.K., Talegaonkar S., Ahmad F.J., Khar R.K. (2011). Enhanced bioavailability of nano-sized chitosan-atorvastatin conjugate after oral administration to rats. Eur. J. Pharm. Sci., 44, 241–249.

Beck-Broichsitter M., Rytting E., Lebhardt T., Wang X., Kissel, T. (2010). Preparation of nanoparticles by solvent displacement for drug delivery: A shift in the “ouzo region” upon drug loading. Eur. J. Pharm. Sci., 41, 244–253.

Calvo P., Vila-Jato J.L., Alonso M.J. (1996). Comparative in vitro evaluation of several colloidal systems, nanoparticles, nanocapsules and nanoemulsions, as ocular drug carrier. J. Pharm. Sci., 85, 530–536.

Chorny M., Fishbein I., Danenberg H.D., Golomb G. (2000). Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. J. Control. Release, 83, 389–400.

Fontana G., Pitarresi G., Tomarchio V., Carlisi B., San Biagio P.L. (1998). Preparation, characterization and in vitro antimicrobial activity of ampicillin-loaded polyethylcyanoacrylate nanoparticles. Biomaterials, 19, 1009–1017.

Khan F., Islam M.S., Roni M.A., Jalil R.U. (2012). Systematic development of self-emulsifying drug delivery systems of atorvastatin with improved bioavailability potential. Sci. Pharm., 80, 1027–1043.

Lai S.K., Wang Y.Y., Hanes J. (2009). Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Int. J. Pharm., 6, 158–171.

Law M.R., W

ald N.J., Rudnicka A.R. (2003). Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. Br. Med. J., 326, 1423–1427.

Lennernas H. (2003). Clinical pharmacokinetics of atorvastatin. Clin. Pharmacokinet., 42, 1141–1160.

Lin W.J. & Huang, L.I. (2001). Fabrication of porous poly(ε-caprolactone) microparticles for protein release. J. Microencapsul., 18, 577–584.

Mantri S.K., Pashikanti S., Murthy K.V. (2012). Development and characterization of self-nanoemulsifying drug delivery systems (SNEDDS) of atorvastatin calcium. Curr. Drug Deliv., 9, 182-196.

Mishra, B., Patel, B.B., and Tiwari, S. (2010). Colloidal nanocarriers: a review on formulation technology, types and applications toward targeted drug delivery. Nanomedicine, 6, 9–24.

Mora-Huertas C.E., Fessi H., Elaissari A. (2011). Influence of process and formulation parameters on the formation of submicron particles by solvent displacement and emulsification–diffusion methods critical comparison. Adv. Colloid. Interface Sci., 163, 90–122.

Wu L., Zhang J.,Watanabe W. (2011). Physical and chemical stability of drug nanoparticles. Adv. Drug Deliv. Rev., 63, 456–469.

Zhang H.X, Wanga J.X., Zhanga Z.B., Lea Y., Shenb Z.G., Chenb J.F. (2009). Micronization of atorvastatin calcium by antisolvent precipitation process. Int. J. Pharm., 374, 106–113.