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# Enteric coating of nanostructured lipid carriers (NLCs) and enteric coating of hard gelatin capsules filled with NLCs: Feasibility studies

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**Abstract**: Nanostructured lipid carriers (NLCs) of asenapine maleate (ASPM) were enteric coated with polymethacrylate polymers (Eudragit®) for oral delivery. The present study aimed to compare the feasibility of direct enteric coating of NLCs and enteric coating of hard gelatin capsules filled with lyophilized ASPM-NLCs. Organic solution of Eudragit® was prepared using acetone containing 3% v/v water, acetone or ethanol. Aqueous dispersion of Eudragit® was obtained by neutralization with base. Capsules were enteric coated by dip-coating method with 3:2 ratio of Eudragit® L100-55:S100 (7.5-12.5% w/v). ASPM-NLCs showed particle size of 84.91±2.14nm, polydispersity index of 0.222±0.026, entrapment efficiency of 86.9±1.8% and zeta potential of -4.83±0.29 mV. TEM images showed good sphericity of the particles with the size of ≈100nm. Non-aqueous enteric coating was not successful as NLCs were precipitated in organic solvent. Aqueous enteric coated ASPM-NLCs (lipid:coat=1:2) showed an increased size (150.8±16.7nm) and zeta potential (-23.5±2.2 mV) revealing the deposition of Eudragit®. However, aqueous enteric coated ASPM-NLCs and uncoated ASPM-NLCs showed higher drug release (18.3±3.1-22.3±3.2%) in HCl solution (pH 1.2) indicating no resistance offered by direct enteric coating of NLCs; whereas enteric coated capsules showed less drug release (4.7±0.8%) in HCl solution indicating sufficient gastric protection.

**Keywords**: Nanostructured lipid carriers, enteric coating, Eudragit®, Asenapine maleate, nano-carriers.

## INTRODUCTION

Asenapine maleate (APSM) is an antipsychotic drug used in the treatment of schizophrenia and bipolar disorder. It exhibits very low oral bioavailability because of extensive metabolism in the liver. But oral route is an ideal and much preferred over other routes for drug administration because of greater patient compliance. Presently nanotechnology is being applied to deliver the drugs to improve their oral absorption. Nanotechnology in today's world, is finding its distinct way in major areas such as healthcare, biomedical, textiles, environment, renewable energy, electronics, food agriculture and industry. In medicine, nanotechnology is gaining greater importance world-wide because of its desired properties and application in drug-delivery. Nanoparticles (NPs) are particle dispersions or solid particles with a size varying from 10-1000nm generally. The word "nano" means a "billionth" and is derived from Greek word "Dwarf". The drug is entrapped, encapsulated or attached to a nanoparticle matrix (Mohanraj and Chen, 2006; Shinde *et al*., 2012). Several nanotechnology based carriers such as liposomes, polymeric nanoparticles, solid lipid nanoparticles, nanostructured lipid carriers (NLCs), etc. have been trialed for oral delivery of drugs.

Among all these nano-carriers, NLCs have been more preferred due to many advantages when delivered orally as they easily gain access to intestinal lymphatic system (ILS). ILS serves as a pathway for bypassing the liver, and it drains the lymph from the *cisterna chyli* directly into systemic circulation via thoracic duct (Gambhire *et al*., 2011; Khan *et al*., 2013). Lipidic nature of NLCs will render them to be taken up into lacteal of villi in the intestine in the form of chylomicrons which finally drains into ILS. However, the NLC assisted drug absorption from intestine through ILS will happen only when NLCs will reach the intestine in intact form. On the contrary, the major hurdle encountered in oral delivery of nano-carriers is the destabilization leading to aggregation and consequent drug release in the harsh condition of stomach by which nano-carriers would fail to reach the intestine (Severino *et al*., 2012). Therefore NLCs should be protected from gastric content and deliver them in intact form in the intestine. To overcome acidic harsh stomach condition, enteric coating of the NPs is advantageous. To achieve gastric protection, we can either fabricate the NLCs of pH-responsive/ enteric polymers (Cetin *et al*., 2010; Hu *et al*., 2012; Mahalingam and Krishnamoorthy, 2015; Vineela and Krishna, 2014) or coat the preformed NLCs with enteric polymers (Hosny *et al*., 2013; Subudhi *et al*., 2015; Sun *et al.*, 2014). In recent years, techniques involving direct coating of nano-carriers such as liposomes and solid lipid nanoparticles with enteric polymers have been reported (Barea *et al*., 2010; Eskandari *et al.*, 2013; Tummala *et al*., 2015). pH-responsive NPs prepared with anionic polymers with carboxyl groups remain intact in stomach and release the drug in sustained manner in intestine (Yoshida *et al*., 2013).

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Another approach to achieve gastric protection of NLCs is by _ENREF_14making use of enteric coated capsules filled with lyophilized NLCs. There are hardly any lipids available with enteric coating properties. Therefore, in current research, attempts were made to i) enteric coat the preformed NLCs loaded with ASPM and ii) enteric coat the hard gelatin capsules which are filled with lyophilized NLCs of ASPM. In this study we used poly (methyl methacrylate-co-methacrylic acid), also called polymethacrylate, polymers which are commercially available with trademark Eudragit®. Since hardly any reports are available on enteric coating of preformed NLCs, the current research work was undertaken to coat the ASPM-NLCs directly using Eudrargit® and the gastric protection efficiency of these enteric coated NLCs was compared with enteric coated capsules filled with ASPM-NLCs.

Different grades of Eudragit® are available which can be specifically used to release the drug in specific part of intestine, such as Eudragit® L100-55 for release in duodenum (pH >5.5); Eudragit® L100 for release in jejunum to ileum (pH >6.0); Eudragit® S100 for release in colon (pH 6.5 to 7.5). Furthermore, mucus barrier in the intestine results in low absorption of drug into systemic circulation. Mucoadhesive nanoparticles are beneficial to alleviate the problem of mucus barrier as they adhere to mucus layer and release the drug in controlled manner near to epithelium which is then quickly absorbed by enterocytes (Pridgen *et al*., 2015; Shaikh *et al*., 2011). Eudragit® coatings are advantageous in this stage as they are also shown to possess mucoadhesive properties in addition to pH-dependent drug release. Karn *et al*. (2011) demonstrated superior mucoadhesion of Eudragit® polymer in freshly extracted pig intestinal tissue, compared to chitosan and carbopol polymer. Eudragit® L100-55, L100 and S100 dissolve at pH 5.5, 6.0 and 7.0, respectively. Khan *et al*. (1999) demonstrated that by combining Eudragit® S100 with Eudragit® L100-55, it is possible to modulate the drug release within the pH range of 5.5- 7.0. Therefore, 3:2 w/w ratios of Eudragit® L100-55 and S100 were used in the current research wherever combination is used.

## MATERIALS AND METHODS

### *Materials*

ASPM was gifted by MSN Organics Pvt. Ltd., Hyderabad, India and Orbicular Pharmaceutical Technologies Pvt. Ltd., Hyderabad, India. Glycerylmonostearate (GMS) and oleic acid were obtained from Fine Organics, Mumbai, India and Sigma Aldrich, St Louis, MO, USA, respectively. Polyethylene glycol 400 (PEG400) and triethylcitrate (TEC) were purchased from Ranbaxy Laboratories Ltd., Mohali, India and HiMedia Laboratories, Mumbai, India, respectively. Tween 80 was procured from Nice Chemicals Pvt. Ltd., Mumbai, India. Eudragit® L100-55 and Eudragit® S100 were obtained from Evonik, Germany. Acetonitrile (HPLC grade), methanol and potassium dihydrogen phosphate were purchased from SD Fine Chemicals, Mumbai, India. Ultra-pure water, obtained from a Millipore Direct-Q® water purification system, Millipore Corporation, Billerica, MA, USA, was utilized in formulation processing. All other chemicals used were of reagent or analytical grade unless otherwise specified.

### *Preparation of NLCs*

NLCs were prepared by ultrasound dispersion method as previously reported (Bose and Michniak-Kohn, 2013; Pathak and Nagarsenker, 2009)_ENREF_22 using glycerylmonostearate (GMS) and oleic acid as solid lipid and oil (liquid lipid), respectively. Briefly, mixture of lipid (1.2% w/v) and oil (20% of total lipids) was melted at 70ºC and accurately weighed ASPM (lipid/ drug: 20) was added to the molten lipid. To this, Tween 80 solution (2% w/v), maintained at 70ºC was added. The resultant coarse emulsion was probe sonicated (Probe sonicator VC 130, Sonics and Materials Inc., USA) for 10min at 60% amplitude and 6 sec pulse. Later, the mixture was cooled in ice bath for 15min for lipid solidification and rigidization of nanoparticles. The thermal stability of ASPM has been assessed previously at 80°C and drug did not show any instability at this temperature (Managuli *et al.*, 2016).

### *Enteric coating of NLCs*

*Non-aqueous enteric coating of NLCs*

Non-aqueous enteric coating of NLCs was carried out as per the previously reported method with little modification (Eskandari *et al.*, 2013). Polymeric solution (Eudragit® S100, Eudragit® S100+ Eudragit® L100-55, Eudragit® S100+PEG, Eudragit® S100+PEG+TEC) was prepared in a suitable solvent system (acetone containing 3% v/v water, acetone or ethanol). Then, the polymeric solution was added drop-wise to specified volume (2-5mL) dispersion of NLCs under stirring on a magnetic stirrer at room temperature for 1h. Later, the dispersion was placed in refrigerator overnight for stabilization. Several trials were taken by changing solvent system, volume of solvent system and different ratios of lipid to polymer as shown in table 1.

***Aqueous enteric coating of NLCs:*** Neutralization using 1M NaOH (6 mol%) and 1M $NH_4OH$ (15 mol%) was required to obtain aqueous dispersion of Eudragit® L100-55 and S100, respectively (Skalsky *et al*., 2011; Yang *et al*., 2010). This aqueous dispersion of Eudragit® L100-55 and S100 (3:2) was then added to the dispersion of NLCs under stirring. The resultant dispersion was stirred for 1h and then frozen for 8h at -80ºC followed by freeze-drying at -48ºC for 48h using freeze dryer (LFD-5508, Daihan Labtech Co. Ltd., Korea). The amount of Eudragit® polymer used in the formulation was in the weight ratio of

1:2 and 1:3 of total lipid to polymer to obtain formulations EC-NLCs (1:2) and EC-NLCs (1:3). Freeze drying of EC-NLCs was carried out in the presence of 5% w/v of cryoprotectant (sucrose). An additional EC-NLCs (1:2) formulation was also prepared without sucrose.

***Characterization of NLCs***

*Particle size, polydispersity index (PDI) and zeta potential*

The average particle size, PDI and zeta potential of the prepared NLCs were determined by Zetasizer (Nano ZS, Malvern Instruments, UK). Particle size was determined using a dynamic light scattering (DLS) technique. Zeta potential was assessed on the basis of particle electrophoretic mobility under an applied electric field using a combination of Laser Doppler Velocimetry (LDV) and Phase Analysis Light Scattering (PALS).

***Entrapment efficiency***

Any un-entrapped free drug in nanoparticle dispersion was separated from entrapped particles by gel chromatographic separation (Gu *et al.*, 2011; Liu *et al.*, 2014; Zhang *et al.*, 2014)_ENREF_25_ENREF_25 using Sephadex G-100 column (2.5cm × 1.0cm). The opalescent eluent containing nanoparticles was collected and ruptured using chloroform and methanol mixture (2:1% v/v). The resultant solution was diluted with mobile phase solution of HPLC and filtered through 0.22µm membrane syringe filter before injecting into HPLC. Entrapment efficiency was calculated using the formula:

$$\text{Encapsulation efficiency}(\%) = \frac{[\text{ASPM}]_{\text{E}}}{[\text{ASPM}]_{\text{T}}} \times 100$$

where, $[ASPM]_E$ represents the amount of encapsulated drug and $[ASPM]_T$ represents the total ASPM content in NLCs.

***Transmission electron microscopy***

To determine the shape and surface morphology of the NLCs, transmission electron microscope (TEM; CM200 supertwin system, Philip, Netherland) was used. A drop of sample was placed on a copper grid coated with carbon film and air dried for 1min. Excess sample was drained off from the side with the help of filter paper. Sample was then quickly stained with phosphotungstic acid solution (1% w/v, pH 6.0) and air dried for 1min followed by drying under IR lamp for 30min. Sample loaded copper grid was then examined in TEM instrument at voltage 200 KV and resolution 0.23nm.

***In vitro drug release study***

*In vitro* release of ASPM from NLCs was performed in hydrochloric acid (HCl) solution of pH 1.2 for 2h, ammonium acetate buffer of pH 4.5 for 1h, phosphate buffer of pH 6.8 for 6h and phosphate buffer of pH 7.4 for 24h by dialysis method (Li *et al.*, 2010; Shete and Patravale, 2013)_ENREF_28. The NLCs dispersion (equivalent to 2mg drug) was transferred in dialysis bag (MWCO: 12,000 Da) which was suspended individually in 100mL of each medium, kept on a magnetic stirrer with a speed of 100 rpm at 37±0.5ºC. Aliquots of 2mL were withdrawn and replaced the same volume with fresh media. The withdrawn samples were estimated by HPLC method for amount of drug released.

***Enteric coated capsules***

Empty capsules were weighed and then filled with lyophilized ASPM-NLCs. Weight of filled capsules was taken, from which empty capsule weight subtracted to get net weight of formulation filled. Capsules were enteric coated by dip-coating method using enteric coating solution, *i.e.* Eudragit® L100-55: Eudragit® S100 at 3:2 (7.5-12.5% w/v) dissolved in acetone, containing triethylcitrate (10% of polymer) as plasticizer.

***Dissolution study of enteric coated capsules***

Dissolution study of enteric coated capsules was performed in gradual pH-changing system *viz.*, HCl solution of pH 1.2 for 2h, ammonium acetate buffer of pH 4.5 for 1h and phosphate buffer of pH 7.4 up to 24h. Initial release was assessed in HCl pH 1.2 medium which was replaced after 2h with ammonium acetate buffer pH 4.5. After 1h of drug release study in pH 4.5 buffer, the dissolution medium was replaced with pH 7.4 phosphate buffer and dissolution study was continued till 24h. USP type II dissolution apparatus was used with paddle rotation speed of 50rpm at 37±2°C (Mutalik *et al.*, 2016).

***HPLC method for quantification of ASPM***

ASPM was quantified by following previously reported HPLC based method (Managuli *et al.*, 2016) wherein phosphate buffer: acetonitrile (80:20 %v/v, buffer pH 3.0±0.05) was used as mobile phase and Hyperclone BDS C18 (250mm×4.6mm id, 5µm particle size, 130 A) column was the stationary phase. Flow rate and injection volume was set to 1.0mL/min and 20µL, respectively, with a run time of 11min. Both column and auto-sampler temperature was maintained at 25°C. Detection wavelength was 230nm.

## STATISTICAL ANALYSIS

Data was analyzed statistically using Student's "t" test (to compare two groups) using Graph Pad VersionPrism software. A *p* value less than 0.05 was considered statistically significant.

## RESULTS

***Particle size, PDI, zeta potential and entrapment efficiency***

ASPM-NLCs showed an average particle size, PDI, zeta potential and entrapment efficiency value of 84.91±2.14nm, 0.222±0.026, -4.83±0.29mV and 86.9±1.8%, respectively.

**Table 1**: Process parameters in non-aqueous enteric coating of ASPM-NLCs

| Volume of formulation (Lipid amount) | Coating ingredients (mg) | Solvent and its volume (mL) | Problem |
|---|---|---|---|
| 5mL (75 mg) | 15 mg ES100 & L100-55 (2:3) | 2.5mL acetone (3% water) | Precipitation |
| 2mL (30 mg) | 15 mg ES100 + 0.3% PEG 400 | 1.5mL acetone (3% water) | Precipitation |
| 2mL (30 mg) | 15 mg ES100 + 0.3% PEG 400 + 10 µL TEC | 1.5mL acetone (3% water) | Precipitation |
| 2mL (30 mg) | 15 mg ES100 | 1mL acetone (3% water) | Precipitation |
| 2mL (30 mg) | 15 mg ES100 | 1mL ethanol | Precipitation |
| 2mL (30 mg) | 15 mg ES100 | 0.5mL acetone | Precipitation |
| 5mL (75 mg) | 12.5 mg ES100 | 1.25mL acetone | Precipitation |

ES100= Eudragit® S100; TEC= Triethylcitrate; ASPM-NLCs= Uncoated nanostructured lipid carriers of ASPM

**Table 2**: Results of characterization of ASPM-NLCs and EC-NLCs

| Formulations | Particle size (nm) | PDI | Zeta Potential (mV) |
|---|---|---|---|
| ASPM-NLCs | 84.91 ± 2.14 | 0.222 ± 0.026 | -4.83 ± 0.29 |
| EC-NLCs (1:2) | 150.8 ± 16.70 | 0.230 ± 0.087 | -23.5 ± 2.20 |
| Lyo-ASPM-NLCs | 252.1 ± 20.10* | 0.424 ± 0.081 | -28.1 ± 2.30* |
| Lyo-EC-NLCs (1:2) | 423.1 ± 27.30[#] | 0.654 ± 0.065 | -39.7 ± 3.70 |

ASPM-NLCs = Uncoated nanostructured lipid carriers of ASPM EC-NLCs = Enteric coated nanostructured lipid carriers Lyo-ASPM-NLCs = Lyophilized uncoated asenapine maleate loaded nanostructured lipid carriers Lyo-EC-NLCs= Lyophilized enteric coated nanostructured lipid carriers

* Significantly different ($p<0.05$) compared to ASPM-NLCs for respective parameters.

[#] Significantly different ($p<0.05$) compared to EC-NLCs (1:2) for respective parameters

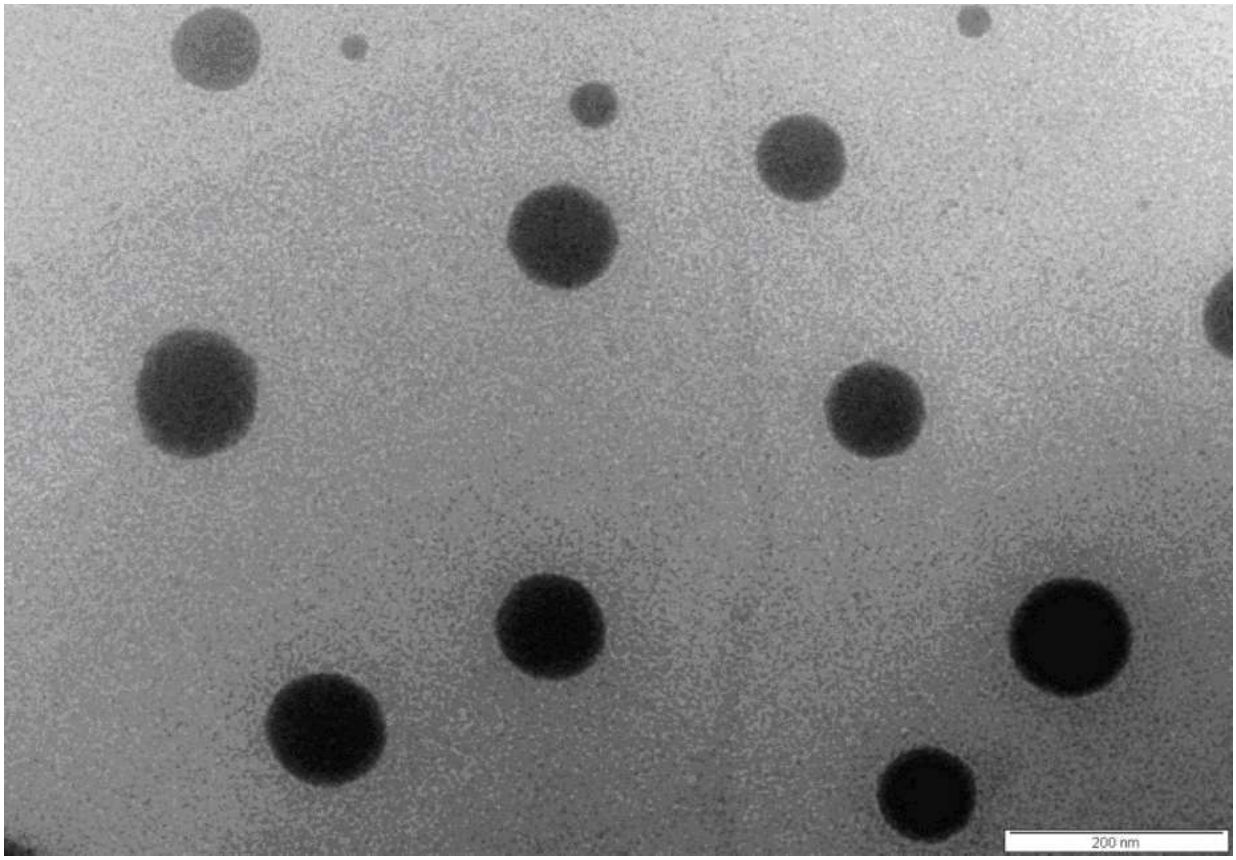


**Fig. 1**: Transmission electron microscopy image of optimized ASPM-NLCs.

***Enteric coating of NLCs***

With non-aqueous enteric coating method, lipids of NLCs were precipitated when organic solution of enteric coating polymers was used. On the contrary, with aqueous enteric coating approach there was no precipitation of the enteric coating polymer upon its addition to the dispersion of NLCs. Aqueous enteric coated NLCs (EC-NLCs) dispersion with lipid: coat ratio of 1:2 showed particle size of 150.8±16.7nm, PDI of 0.230±0.087 and a zeta potential value of -23.5±2.2 mV. After lyophilization, the zeta potential, PDI and size values of EC-NLCs were increased. The size, PDI and zeta potential values were observed to be 423.1±27.3nm, 0.654±0.065 and -39.7±3.7mV, respectively. Also the uncoated ASPM-NLCs showed the particle size of 252.1±20.1nm, PDI of 0.424±0.081 and zeta potential of -28.1±2.3mV after lyophilization (table 2).

***Transmission electron microscopy***

Photomicrograph of transmission electron microscopy of ASPM-NLCs showed spherical particles with an approximate particle size of ≈ 100nm (fig. 1).

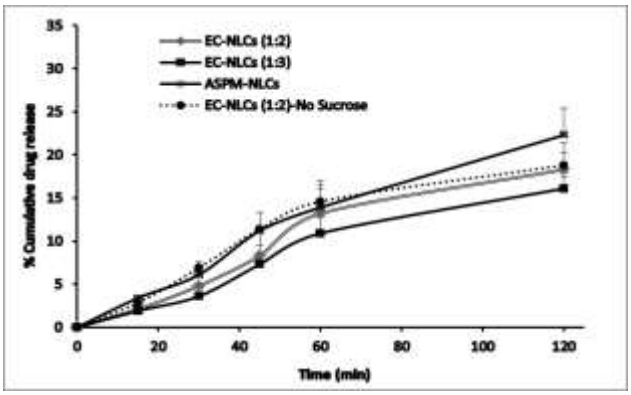


**Fig. 2**: *In vitro* drug release data of ASPM-NLCs, EC-NLCs (1:2 and 1:3) and EC-NLCs (1:2) containing no sucrose in pH 1.2 HCl

ASPM-NLCs= Uncoated nanostructured lipid carriers of ASPM EC-NLCs (1:2) = Enteric coated nanostructured lipid carriers with lipid: Eudragit® polymer 1:2% w/w
EC-NLCs (1:3) = Enteric coated nanostructured lipid carriers with lipid: Eudragit® polymer 1:3% w/w
EC-NLCs (1:2)-No sucrose= Enteric coated nanostructured lipid carriers with lipid: Eudragit® polymer 1:2% w/w and lyophilized without sucrose.

***In vitro drug release study***

*In vitro* drug release data of uncoated ASPM-NLCs showed that ≈22% of the drug was released in 2h in HCl solution of pH 1.2 (fig. 2). *In vitro* drug release study of NLCs which were enteric coated using organic solution of enteric polymer was not performed because the polymer was precipitated during enteric coating procedure and thus there was no formation of enteric coated NLCs. Hence we moved further for aqueous method of enteric coating for NLCs. EC-NLCs with lipid: coat ratio of 1:2 showed 18.3±3.1% drug release in HCl solution of pH 1.2 in 2h; whereas, EC-NLCs with lipid: coat ratio of 1:3 showed the drug release of 16.1±1.8% of drug. EC-NLCs containing 1:2 lipid: coat, which were lyophilized without cryoprotectant, showed a drug release of 18.8±1.3% in HCl solution.

In ammonium acetate buffer of pH 4.5, ASPM-NLCs showed a drug release of 17.4±1.6% in 1h; whereas EC-NLCs (lipid: coat, 1:3) showed 13.5±0.8% drug release (fig. 3A). In phosphate buffer pH of 6.8, ASPM-NLCs and EC-NLCs (lipid: coat, 1:3) showed 48.1±2.8% and 34.2±2.8% drug release, respectively at the end of 6 h (fig. 3B). In phosphate buffer of pH 7.4, ASPM-NLCs and EC-NLCs (lipid: coat, 1:3) showed a drug release of 75.0±3.4% and 69.2±3.4%, respectively at 24h (fig. 3C).

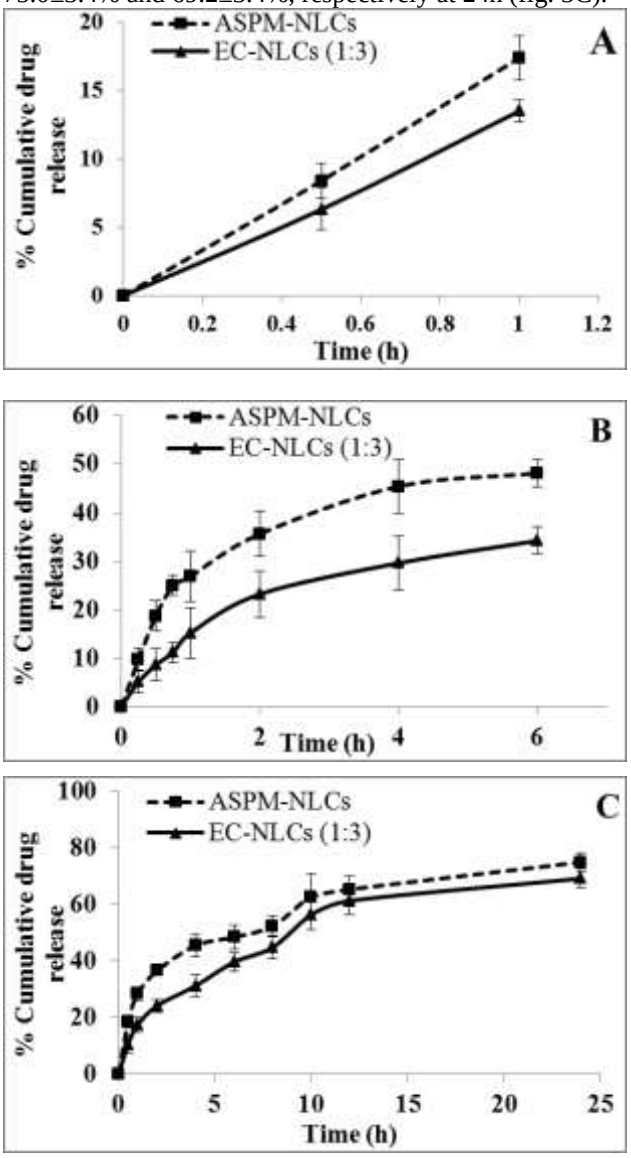


**Fig. 3**: *In vitro* drug release profiles of ASPM-NLCs and EC-NLCs (lipid: coat, 1:3) in A) ammonium acetate

buffer pH 4.5, B) phosphate buffer pH 6.8 and C) phosphate buffer pH 7.4

ASPM-NLCs= Uncoated nanostructured lipid carriers of ASPM; EC-NLCs (1:3) = Enteric coated nanostructured lipid carriers with lipid: Eudragit® polymer 1:3% w/w.

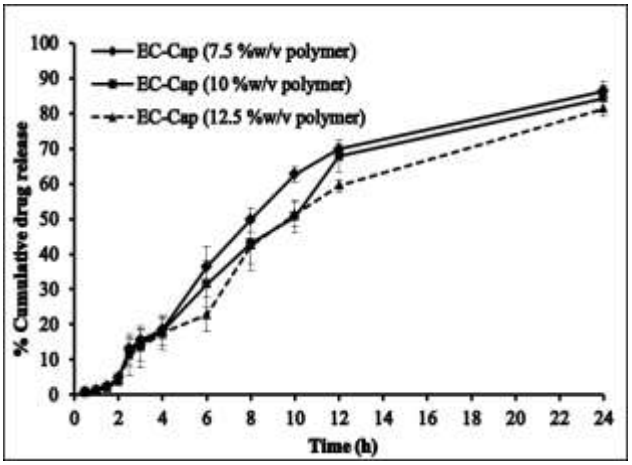


**Fig. 4**: *In vitro* drug release profiles of enteric coated capsules in 0.1N HCl solution of pH 1.2 (up to 2h), ammonium acetate buffer of pH 4.5 (2h to 3h), phosphate buffer of pH 6.8 (3h to 6h) and phosphate buffer of pH 7.4 (6h to 24h)

EC-Cap (7.5% w/v polymer) = Enteric coated capsule using 7.5% w/v polymer; EC-Cap (10% w/v polymer)= Enteric coated capsule using 10% w/v polymer; EC-Cap (12.5% w/v polymer)= Enteric coated capsule using 12.5% w/v polymer.

In pH 1.2 HCl solution, enteric coated capsules of ASPM-NLCs showed the drug release of 5.0±0.4% at 7.5% w/v, 4.7±0.8% at 10% w/v and 4.1±1.6% at 12.5% w/v concentrations of enteric polymers at the end of 2h. At the end of 3h, 15.6±2.7%, 14.4±4.6% and 13.6±5.9% of ASPM was released for 7.5, 10 and 12.5% w/v Eudragit® coat, respectively in ammonium acetate buffer of pH 4.5. At the end of 24 h, 86.3±2.5%, 84.3±3.8% and 81.3±2.1% of ASPM was released for 7.5, 10 and 12.5% w/v Eudragit® coat, respectively in pH 7.4 phosphate buffer (fig. 4).

## DISCUSSION

ASPM-NLCs showed good physico-chemical properties with respect to particle size, PDI and entrapment efficiency. It is clearly observed from the TEM photomicrograph that the particles exhibit narrow size distribution which is in compliance with DLS technique data obtained by Zetasizer instrument. Zeta potential was found to be low (-4.83±0.29 mV) which could be due to the coverage of NLCs with Tween-80. In a previous study, Tween-80 has been reported to decrease the mobility of the particles leading to lower zeta potential values (Martins *et al.*, 2012). Although the zeta potential of the NLCs is at lower side, the physical stability of the NLCs is likely to be unaffected as Tween-80 is able to offer steric stabilization to the NLCs (Kasongo *et al.*, 2011). Enteric coating of NLCs was first attempted by using organic solution of enteric polymer in either acetone or ethanol. However, it was observed that the NLCs or components of NLCs were found to precipitate when organic solution of enteric coating polymers was added to dispersion of NLCs. We initially presumed that the susceptibility of the lipids towards the organic solvent might be due to higher volume of the organic solvents. So we decided to dissolve enteric coating material in little amount of organic solvent and then to incorporate this solution into the dispersion of NLCs. However, immediate evaporation of less volume of organic solvent before it could enteric coat the NLCs led to solidification of enteric polymer. This was evident by the presence of solid polymeric particles. Therefore, we could not coat the NLCs using non-aqueous enteric coating approach under these consitions and hence these enteric coated NLCs were not prepared and characterized in this study. Then we moved to the approach of using aqueous based enteric coating approach for preformed ASPM-NLCs. The increase in particle size and zeta potential of NLCs after enteric coating suggested the deposition of enteric polymer over the surface of NLCs. Our observations with respect to size and zeta potential are in accordance with previous report where the enteric coated citrus pectin nanoparticles showed relatively increased size and zeta potential after coating with Eudragit® S100 (Subudhi *et al.*, 2015).

Both ASPM-NLCs and EC-NLCs were lyophilized in presence of sucrose as cryoprotectant, as explained in Materials and Methods section. During lyophilization process, the properties of the surfactant layer are expected to change due to the removal of water, which further would increase the particulate concentration leading to particle aggregation (Mehnert and Mader, 2001). This may be the reason for increased particle size of NLCs after lyophilization. Similar observation of increase in particle size of lipid nanoparticles after freeze drying was also observed by Cavalli *et al.* (1997).

***In vitro drug release study***

ASPM-NLCs showed ≈22% of the drug release in 2h in HCl solution of pH 1.2 which could be due to the partial degradation of NLCs in HCl solution. For effective delivery of drug in intestine by NLCs, the release of drug in HCl solution of pH 1.2 should be minimal. Hence, we decided to enteric coat the NLCs using Eudragit® polymers.

Eudragit® polymers are practically insoluble in water; however, certain degree of neutralization with bases such as KOH, NaOH, ammonium hydroxide, ammonia, etc is

required to obtain aqueous dispersions of Eudragit® (Skalsky *et al.*, 2011). In case of aqueous enteric coating method, no considerable reduction in drug release was observed with increase in Eudragit® concentration.

Both 1:2 and 1:3 lipid: Coat ratios of EC-NLCs showed a little reduction in amount of drug release in HCl solution in 2h (18.3±3.1% and 16.1±1.8%, respectively) when compared to ASPM-NLCs which released 22.3±3.2% of drug (fig. 2; all these values were not significantly different from each other, $p > 0.05$). This could be because of improper coating of NLCs and partial neutralization of polymer resulting in increased rate of dissolution in media (Skalsky and Petereit, 2008).

We presumed that the cryoprotectant (sucrose) used during lyophilization of NLCs could displace the enteric coating polymer from the surface of NLCs, which further would disrupt the continuity in coating on the NLCs. To assess this, EC-NLCs (1:2 lipid: coat) were lyophilized without cryoprotectant. This formulation showed a drug release profile, similar to EC-NLCs of same lipid: coat ratio, which was lyophilized in the presence of cryoprotectant. This observation revealed the absence of any interference of cryoprotectant in aqueous enteric coating process of NLCs. Based on *in vitro* drug release data, we selected EC-NLCs (lipid: coat, 1:3) for further assessment of drug release in rest of the buffer solutions of different pH as this formulation showed greatest resistance for gastric pH.
In phosphate buffer of pH 6.8, EC-NLCs (lipid: coat, 1:3) showed comparatively low drug release (34.2±2.8%) than ASPM-NLCs (48.1±2.8%), which may be due to alkaline pH specific solubilization property of the enteric coating in EC-NLCs. Eudragit® L100-55 and Eudragit® S100 polymers generally do not dissolve instantly when they come in contact with nearly basic pH; instead a swollen polymeric mass might have formed initially around the NLCs that creates an extra matrix for drug molecules to pass through it.

Either aqueous or non-aqueous methods of enteric coating of NLCs did not show considerably less release of ASPM from NLCs in HCl solution of pH 1.2. Therefore to protect ASPM-NLCs from acidic environment, we attempted conventional gastric protection strategy *i.e*. use of enteric coated capsules filled with lyophilized powder of ASPM-NLCs. Mixture of Eudragit® polymers (Eudragit® L100-55 and Eudragit® S100 in the ratio of 3:2) was utilized for enteric coating of hard gelatin capsules filled with ASPM-NLCs at the polymer concentrations of 7.5, 10 and 12.5% w/v.

Enteric coated capsules of ASPM-NLCs showed very low drug release in HCl solution indicating great gastric resistance of the enteric coating capsules (≈4-5% of drug release in first 2h). Further, less drug release was observed in ammonium acetate buffer of pH 4.5 also (cumulative % of drug release was ≈13-15% in 3h). In phosphate buffer of pH 7.4, good drug release was observed at the end of 24h (cumulative % of drug release was ≈81-86%). Since, ASPM-NLCs showed almost similar drug release patterns in phosphate buffer of pH 6.8 (48.1±2.8, 6h) and phosphate buffer of pH 7.4 (48.4±4.3, 6h), the drug release study for enteric coated capsules was carried out only in phosphate buffer of pH 7.4.

The results of drug release study suggested that enteric coated capsules are promising formulations to provide gastric resistance to ASPM-NLCs for their direct delivery into intestine; on the other hand, low to moderate gastric resistance was offered by ASPM-NLCs and EC-NLCs. Considering the complexity involved in enteric coating procedure for NLCs, uncoated NLCs are generally used for oral delivery of drugs, even though they are susceptible to gastric pH (Luan *et al.*, 2015; Mishra *et al.*, 2016; Shah *et al.*, 2016).

## CONCLUSION

In this study, enteric coated hard gelatin capsules containing ASPM-NLCs exhibited high gastric protection for NLCs against the uncoated ASPM-NLCs and enteric coated NLCs (EC-NLCs), which showed low to moderate level of protection in gastric pH. Aqueous based enteric coating method was found to be suitable for enteric coating of the NLCs in comparison with organic solvent based method. However, due to the complexities involved in enteric coating of nano-carriers, the oral administration of uncoated nano-carriers is the generally followed practice in *in vivo* studies.

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