




Evaluation of carvedilol-loaded Eudragit® nanoparticles

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ABSTRACT: The objective of present study was to prepare positively charged carvedilol-loaded nanoparticles providing a controlled release formulation by spray dryer technique and was to examine the effect of different derivative of Eudragit® polymers on entrapment efficiency (EE%), dissolution profile and release kinetics. Two non-biodegradable positively charged polymers, Eudragit® RS100 and RL100 were used alone or in combination. The prepared formulations were evaluated for their particle size, polydispersity index, zeta potential, *in vitro* dissolution and *in vitro* release kinetics study. Particle sizes of placebo nanoparticles and carvedilol loaded nanoparticles were 418.2±18.5/ 402.3±22.0/ 416.3±12.5 and 535.5±13.8/ 529.4±10.2/ 530.4±10.4 nm, respectively with PDI values of approximately 0.4–0.6 for each. Carvedilol loading was resulted in positive electrical charge on nanoparticles. The drug encapsulation efficiency was 68.45±4.67/ 63.58±2.30/ 65.67±4.21. *In vitro* cumulative release from the nanoparticles was 80-90 % at 48 hour. Morphology and solid state analyzes were performed also in nanoparticles. The results of this research indicate that spray dryer technique is suitable for carvedilol loaded Eudragit® nanoparticles and no burst effect but a prolonged drug release was observed from formulations.

KEYWORDS: Nanoparticles, drug delivery, controlled release system, carvedilol, spray dry.

1. INTRODUCTION

Carvedilol (CVL), a Biopharmaceutical Classification System (BCS) class II drug, is a multiple action drug with nonselective β -adrenoceptor antagonist and α_1 -receptor antagonist activities. CRV is used in clinical practice for the treatment of hypertension and other cardiovascular diseases [1]. Despite its beneficial effects on the cardiovascular system, the oral bioavailability of CVL be low, because of it is slightly soluble in water and undergoes first-pass metabolism in the liver. CVL is a drug which has short half-life, requiring 2-3 times oral administration per a day [1,2]. The short elimination time can result in significant variation plasma drug concentrations during repetitive dosing. Rapid absorption coupled with the short elimination half-life can result in significant fluctuation in plasma drug concentrations during repetitive dosing [1-3].

Controlled drug delivery system can provide therapeutically effective plasma drug concentration for a longer period, which by reducing the dosing frequency and minimizing fluctuations in plasma drug concentration by delivering the drug in a controlled [4]. Nanoparticles (NPs) are one of the multi particulate delivery systems and are prepared to obtain prolonged or controlled drug delivery, to improve bioavailability or stability and to target drug to specific sites. Eudragit® polymers are commonly used for enteric coating and also for preparation of controlled-release dosage forms [5,6]. Poor aqueous solubility and intrinsic dissolution rate are the major factors that affect oral delivery of many drugs [7]. Oral polymeric NPs have attract a considerable attention among novel drug delivery carriers because of its success for enhancing the bioavailability, stability, tolerability and efficacy of incorporated drug [8]. Polymeric NPs are stable in the gastrointestinal tract than other colloidal carriers and can protect encapsulated drugs from gastrointestinal environment. As well as, polymeric materials make possible the modulation of physicochemical characteristics, drug release properties and biological behavior of NPs [9]. NP systems preserve the entrapped drug from gastrointestinal system challenge, prolong the systemic circulation time and control release of drug in blood. All these benefits can contribute to reduction of dose and dosing frequency, thereby reducing the side effects and improve the patient compliance [10]. Poly(methacrylic acid-co-ethylacrylate) copolymers (trade name: Eudragit® s) are commonly used for coating of tablets and preparation of controlled release formulations. Eudragit® s are extensively used for pH-sensitive NPs preparation [11]. Eudragit® RL and

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Eudragit® RS are insoluble in aqueous media but they are permeable and both have a pH-independent release profiles due to the presence of quaternary ammonium groups in their structures [5]. Same time these capable of limited swelling, thus show a good material for the dispersion of drugs [10, 12]. Spray-drying is widely used for polymeric NP of many therapeutic agents, due to its reproducibility, consistency, and control of particle-size distribution and drug release. This technique allows the production of dry powders with specific characteristics such as particle size, shape and narrow polydispersity index [13-15].

In this study, polymeric NP formulation containing CVL for oral controlled delivery, was prepared and characterized. It focused on preparing and comparing the release properties of sustained release formulations of CVL NPs using the spray-drying technique using Eudragit® polymers. The main purpose of this study is to examine the effect of different derivative of Eudragit® polymer on Entrapment efficiency (EE%), dissolution profile, release kinetics and also this study is to examine the effect of different polymer types on *in vitro* evaluation. The physicochemical characteristics of NPs were studied by EE%, Dissolution Study, Release kinetics study with DDSolver, Morphology, Particle size (PS), Polydispersity Index (PDI), Zeta potential (ZP), X-ray diffraction analysis (XRD), Fourier transform infrared (FTIR) spectroscopy.

2. RESULTS AND DISCUSSION

2.1. Preparation of polymeric nanoparticles

The NPs were prepared by spray drying technique. It is reported that polymeric NPs fabricated using spray drying module to control particle size and distribution. The NPs obtained by spray-dryer showed good performance in terms of a high yield and encapsulation efficiency [16, 17]. Polymer types, polymer concentration and amount of drug were optimized. In this study with CVL, mainly pH sensitive polymers; Eudragit® RL 100 and Eudragit® RS 100 were used. Prepared NPs were then characterized for morphology, particle size, PDI, zeta potential, entrapment efficiency, release study and solid state properties.

2.2. High Pressure Liquid Chromatography (HPLC) conditions and Assessment

The HPLC method developed was validated for precision, accuracy, specificity and linearity. Linearity was determined to be at a concentration range of 100-700 µg.mL⁻¹. The method for CVL was decided to be precise due to RSD values of <2 % for repeatability and intermediate precision. Recovery of the method was satisfactory owing to <2 % RSD value. Validation study of HPLC method used for quantification of carvedilol showed a linearity of $y=10.985,6515x+159.386,8571$ ($r^2=0.9996$), accuracy of $100.401\pm 0.403\%$, $100.124\pm 0.145\%$ and $99.376\pm 0.721\%$ for the concentrations of 40 µg.mL⁻¹, 80 µg.mL⁻¹ and 120 µg.mL⁻¹, respectively ($n = 6$). Limit of detection (LOD) was determined to be 0.0001 µg.mL⁻¹ while limit of quantitation (LOQ) was 0.0003 µg.mL⁻¹. Conclusively, procedure proposed in this study can be used for routine, simultaneous and concurrent CVL determination. This stability indicating method can be adaptable for the determination of CVL in similar pharmaceutical dosage forms [3, 18, 19].

2.3. Morphology

Detailed observation of morphological characters of the NPs were performed by Scanning electron microscopy (SEM). SEM images of pure CVL and CVL loaded NPs are given in Figure 1. The drug-loaded NPs were small, spherical, uniform and there was no adhesion between particles seen. Pure CVL's non-uniform shapes were not observed in the formulation images. It is known that to obtain a smooth particle surface spray drying is a good technique [20]. The SEM images confirmed the spherical shape with smooth surface and small structure of the NPs [21-23].

2.4. Measurement of PS, PDI, ZP and EE

The mean PS, PDI and ZP of CVL loaded NPs is shown in Table 1. The particle size distribution of all formulations was analyzed and the formulations showed narrow and uniform particle size distribution and PDI of formulations ranged from 0.44 to 0.62 for placebo NPs and drug loaded NPs. Smaller PDI is desired for stable and better NPs as it indicates homogeneity of dispersion. The acceptable value for PDI range is between 0.05 and 0.7, values greater than 0.7 indicate that the sample has a very wide size distribution and is probably not suitable for the dynamic light scattering technique [21-23]. As shown in Table 1, acceptable values for PDI were obtained for all formulations.

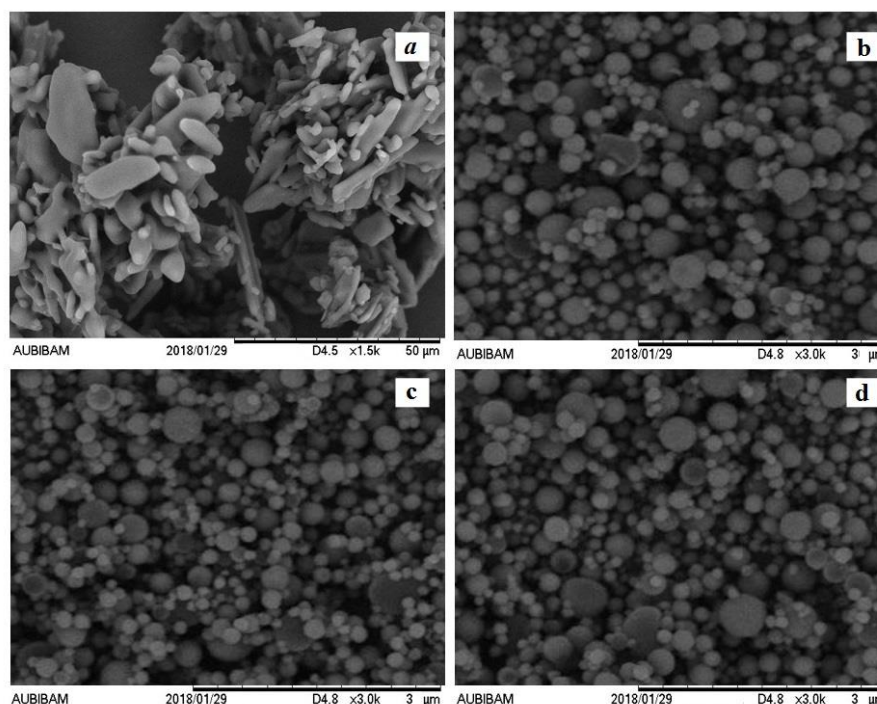


Figure 1. Scanning electron micrographs of formulations, a: CVL b: CL-1 c: CS-1 d: CSL-1.

Table 1. Particle size distribution, PDI, Zeta potential and Encapsulation efficiency of nanoparticle formulations.

Code	Particle size (nm)	PDI	Zeta potential (mV)	EE %
CL-P	418.2±18.5	0.44±0.02	+40.1±1.1	-
CS-P	402.3±22.0	0.52±0.01	+42.3±1.4	-
CSL-P	416.3±12.5	0.53±0.07	+42.4±1.5	-
CL-1	535.5±13.8	0.46±0.07	+41.2±1.3	68.45±4.67
CS-1	529.4±10.2	0.51±0.06	+40.6±2.4	63.58±2.30
CSL-1	530.4±10.4	0.62±0.02	+40.6±2.3	65.67±4.21

Note: CL-P, CS-P and CSL-P: Placebo formulations, CL-1, CS-1 and CSL-1: CVL loaded formulations, EE%: Entrapment efficiency %

NPs mean sizes were 418.2 nm, 402.3 nm, 416.3 nm, 535.5 nm, 529.4 nm, 530.4 nm for CL-P, CS-P, CSL-P, CL-1, CS-1 and CSL-1, respectively. The SEM images of both Eudragit® RS 100 and RL 100 revealed particle sizes in accordance with the size obtained from dynamic light scattering. No difference was found in the size of polymer type and rate. The size and zeta potential of the nanoparticles did not change significantly ($p = 0.13$).

Zeta potential gives data on the surface charge of the prepared NPs. Zeta potential value is an important factor for predicting the stability of the NPs [26]. The Zeta potential of NPs were +40.1 mV, +42.3 mV, +42.4 mV, +41.2 mV, +40.6 mV, and +40.6 mV for CL-P, CS-P, CSL-P, CL-1, CS-1 and CSL-1, respectively. Zeta potential value was found to be around +40 mV, indicating that the NPs have good stability. Higher Zeta potential is expected for the stable colloidal system as it overcomes the particle aggregation due to repulsion forces [24]. Positive surface charge of NPs were attributed to the cationic nature of Eudragit® polymers [23, 24]. *In vitro* release profiles of the drugs can also be influenced by zeta potential of the NPs. Furthermore, the surface charge of the particles is the important parameter that controls the drug loading efficiency [25-27].

A good correlation between particle size and drug entrapment percent was reported as small size particles possessed low entrapment efficiency [28, 29]. The entrapment efficiency, important factors for a drug delivery system, are used to evaluate the usability of nano-based carriers [30]. CVL loading efficiencies on all NPs showed over 60%; CL-1, CS-1 and CSL-1 had loading efficiencies of 68.45±4.67%, 63.58±2.30% and

65.67±4.21 respectively (Table 1). The results showed that the formulation prepared using Eudragit® RL 100 showed higher EE% than Eudragit® RS 100 but this difference is not significant ($p = 0.22$). Several factors can be affect successful entrapment of drug in the NPs; these contain, low drug solubility in the aqueous phase; fast precipitation rate of polymer(s) in the aqueous phase, low viscosity of the internal phase, as well as, drug solubility in the polymer [31-33].

2.5. *In vitro* release of CVL from polymeric nanoparticles

The *in vitro* drug release profile of NPs and pure CVL are shown in Figure 2. The *in vitro* release of CVL from polymeric NPs were performed in phosphate buffer (pH 6.8) containing 30% Polyethylene glycol 400 to simulate the physiological conditions of the human body. All the NPs revealed slower drug release rate in comparison with the pure CVL. 80% of pure CVL was released in the first 3 h. By contrast, only 20 % was released from NPs in the first 3 h. During the observation period (48 h), the total release amount was almost 70 - 80 %. Drug release was slow from Eudragit® RS 100 compared to Eudragit® RL 100 NPs and this may be due to the greater aqueous permeability of Eudragit® RL 100 polymer [34]. In general, all three nanoparticle formulations showed a prolonged release, no burst effect could be observed (see Fig. 2). A slow release pattern was observed for all nanoparticle preparations. The explanation for the low release from Eudragit®-containing particles was a strong ionic interaction between the polycationic Eudragit® and the CVL. Drug release rates from Eudragit® RS particles were very slow, from the same formulations prepared using Eudragit® RL as polymer different drug release profiles were observed. Release was less retarded because of the greater water permeability of the latter, due to the higher quaternary ammonium group content [35-37].

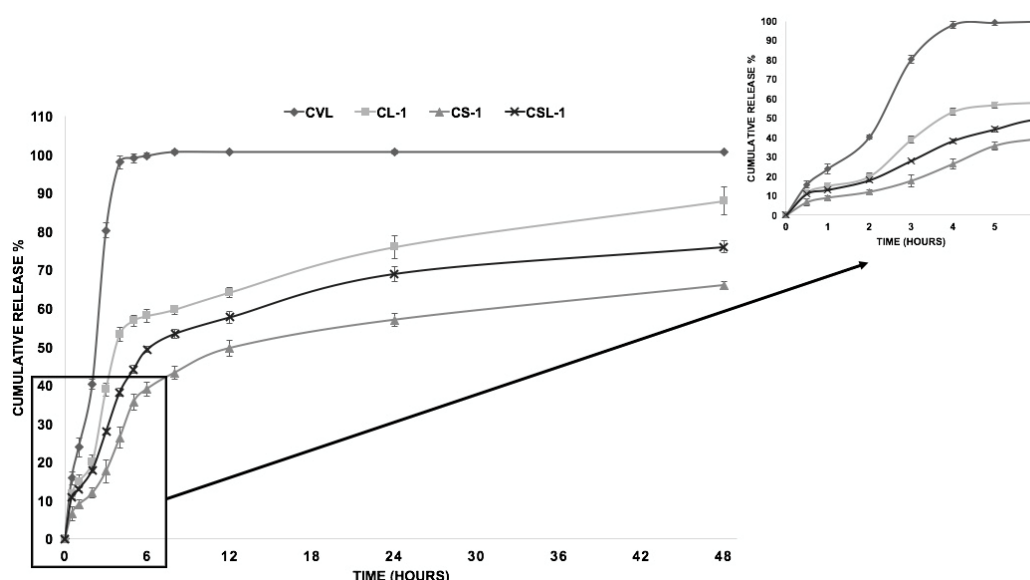


Figure 2. *In vitro* release profiles of pure CVL and CVL from polymeric nanoparticles (Mean ± SE; n = 3).

2.6. Release Kinetics Study

Various release kinetic models (zero order, first order, Higuchi, Hopfenberg, Korsmeyer-Peppas and Peppas-sahlin model) were fitted with the release data of NPs and analyzed for release mechanism. An Excel add-in DDSolver program, which allows modeling of dissolution data, was designed by researchers to reduce computation time and eliminate computational errors [38]. When all the NPs were analyzed for cumulative solubility in time versus time, all formulations appeared to be continuously released for 48 hours. In this study, DDSolver computer program was used to shorten the calculation time, to eliminate calculation errors and to determine the correct release profile. After obtaining the release profiles in this study, data was transferred to the DDSolver program to determine the most important and popular four criteria; coefficient of determination (R^2), adjusted coefficient of determination ($R^2_{adjusted}$), Akaike Information Criterion (AIC) and Model Selection Criterion (MSC). The highest R^2 , $R^2_{adjusted}$ and MSC values and the lowest AIC values were used for evaluating zero order, first order, Higuchi, Hopfenberg, Korsmeyer-Peppas and Peppas-sahlin model [39]. R^2 , $R^2_{adjusted}$, MSC and AIC found are shown in Table 2.

Table 2: *In vitro* release kinetic models of the formulations.

Code	Model	Evaluation Criter			
		R ²	R ² _{adjusted}	AIC	MSC
CL-1	Zero-order model	-0.318	-0.318	113.842	-0.718
CS-1		0.139	0.139	102.585	-0.215
CSL-1		-0.156	-0.156	109.089	-0.565
CL-1	First-order model	0.715	0.715	95.458	0.812
CS-1		0.702	0.702	89.865	0.845
CSL-1		0.686	0.686	93.446	0.739
CL-1	Higuchi model	0.743	0.743	94.239	0.916
CS-1		0.870	0.870	79.859	1.679
CSL-1		0.811	0.811	87.372	1.245
CL-1	Hopfenberg model	0.898	0.552	102.906	0.194
CS-1		0.906	0.611	95.046	0.414
CSL-1		0.908	0.558	99.542	0.231
CL-1	Korsmeyer-Peppas model	0.711	0.679	86.482	0.878
CS-1		0.706	0.674	81.973	0.862
CSL-1		0.747	0.719	82.384	1.010
CL-1	Peppas-Sahlin model	0.969	0.932	82.330	1.908
CS-1		0.969	0.929	76.575	1.953
CSL-1		0.979	0.950	75.361	2.246

The R² and R²_{adjusted} values for the zero order, first order, Higuchi, Hopfenberg, Korsmeyer-Peppas are smaller than Peppas-Sahlin model for all NPs, which are relatively small. This suggests the drug release does not comply with zero order, first order, Higuchi, Hopfenberg, Korsmeyer-Peppas model. The MSC provided by MicroMath Corporation is a statistical criterion for model selection, which is attracting increasing attention in the field of dissolution data modeling. The AIC has been used for selecting optimal models for more than 35 years. Its general simplicity and applicability make it a perfect and popular criterion for various purposes, including drug dissolution analysis [38]. Depending on the R², R²_{adjusted}, MSC and AIC values obtained, Peppas-Sahlin model was found to be more suitable than the other release kinetics. In the literature, this release kinetic model appears to be attributed to polymeric carriers [39].

In an other saying, higher correlation was observed in Peppas-Sahlin model. Peppas-Sahlin model is combined Fickian diffusion and erosion of the nanoparticle matrix. During the first hours of the release process, active agent likely mainly diffused from the nanoparticles and into the aqueous medium by Fickian diffusion. Peppas-Sahlin model was used to quantify the contribution of Fickian diffusion and case-II transport on drug release for this study. For using method, mathematical equation was modified with a term representing the surface free drug fraction and normalized by the nanoparticle size. All the results indicated that the drug release from the formulations occurred by Fickian diffusion mechanism mimicking swellable slab like structures. The contribution of relaxation mechanism was found lesser than the diffusion mechanism indicating porous matrices with irregular surfaces [40,41]. Therefore, results of in this study indicate that release of CVL from NPs is not predominantly driven by a solo mechanism, but a combined mechanism of Fickian and non-Fickian release. When the literature is examined, similar results are found [42].

2.7. Powder X-ray diffraction (XRD)

XRD analysis is an important technique in the pharmaceutical research. It plays an important role in drug delivery system development [40]. XRD analysis is a well-defined analytical method frequently used in research because it reveals the molecular structure of NPs, examines the crystal state, performs polymorphism studies and also provides information about stability [37]. XRD profiles of pure CVL, pure polymers and NPs prepared are shown in Figure 3. Figure 3 clearly shows that CVL exhibits crystal structure while the X-ray diffractogram of polymers are typical of amorphous structure. Characteristic peaks of CVL were not observed in XRD profiles of NPs formulation. This indicates that CVL was molecularly dispersed within NPs and there exists less or no free drug in crystalline form on the surface of NPs [43, 44].

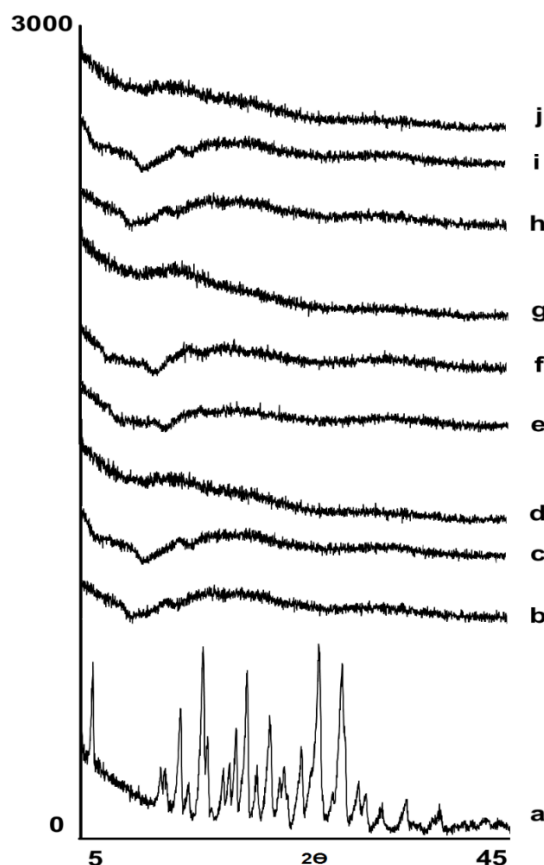


Figure 3. XRD profile of pure CVL, polymers and CVL loaded formulations (a: CVL b: Eudragit® RL 100 c: Eudragit® RS 100 d: Eudragit® RL 100- Eudragit® RS 100 (1:1 Physical Mixture) e: CL-P f: CS-P g: CSL-p h: CL-1 i: CS-1 j: CSL-1).

2.8. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of moisture free powdered samples of CVL, polymers, physical mixture and formulations were obtained using a FTIR instrument. FTIR analysis was used to evaluate the possible intermolecular interaction between CVL, polymers and physical mixture. There was no significant difference in the FTIR spectra of pure CVL and physical mixture of CVL and polymers. CVL have bands at 2924 cm^{-1} , related to C-H stretching, and at 1598 cm^{-1} , related to the bending vibrations of the NH group [43]. It has been used to assess the interaction between carrier and guest molecules in the solid state. Characteristic peaks of drug were also present in FTIR spectra of and physical mixture. These results suggested that there was no interaction between CVL and polymers. The FT-IR spectra is depicted in Figure 4.

3. CONCLUSION

The findings of the present study show that carvedilol loaded polymeric nanoparticles were prepared successfully by spray-dryer process. Formulations were characterized with several parameters. The prepared NPs have a spherical morphology with negative zeta potential and good colloidal stability. It was found that the developed NPs had ideal physical properties and drug release profiles to be used as controlled release. Particle size and zeta potential did not significantly differ between the formulations Eudragit® RL100 and Eudragit® RS100 ($p > 0.05$). Drug release was slow from Eudragit® RS100 compared to Eudragit® RL100 NPs and this may be due to the greater aqueous permeability of Eudragit® RL100 polymer. The formulations were successful in retarding drug release over the test period of 48 h *in vitro* studies. Therefore, it can be concluded that Eudragit® formulations are a potentially useful carrier for oral delivery of CVL. The developed formulation is stable and safe, and represents a promising system for the sustained and controlled delivery of CVL to target cells, tissues and organs.

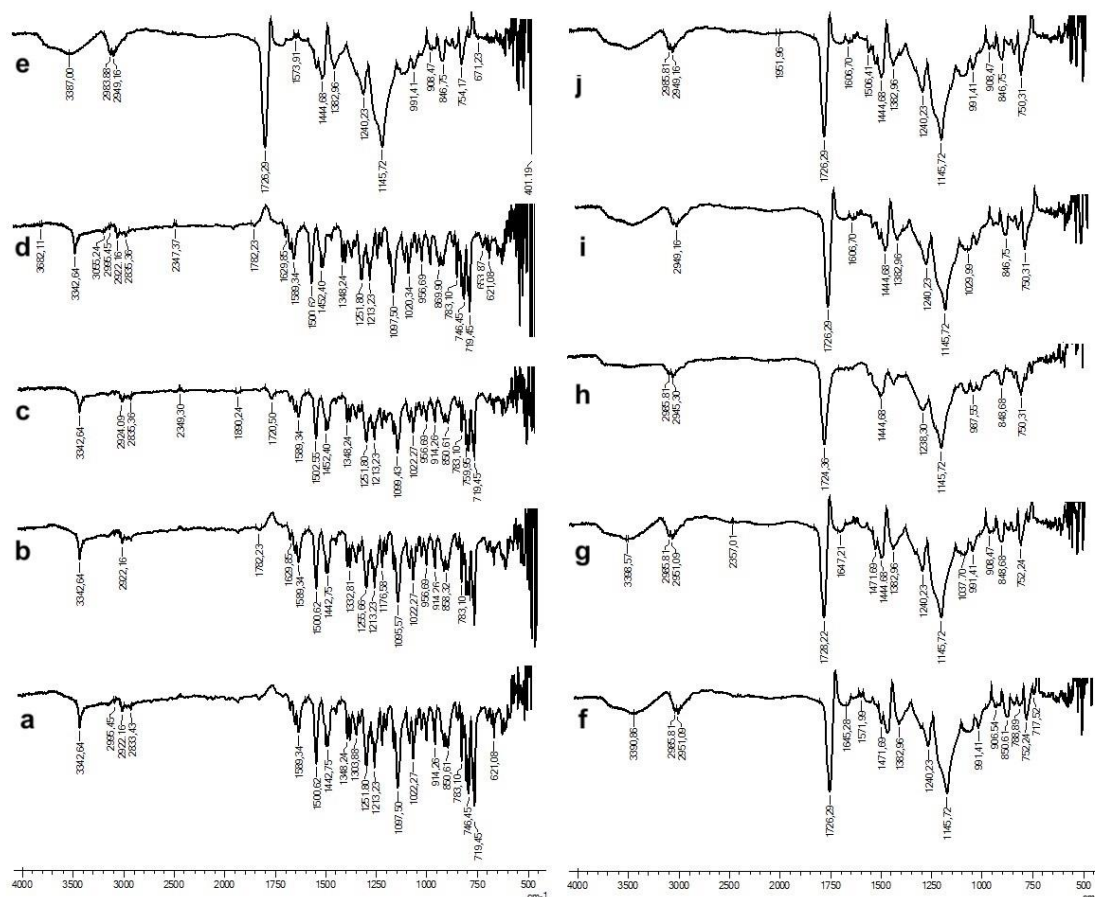


Figure 4. FT-IR profile of pure CVL, physical mixture (PM) and and formulations (a: CVL b: CVL -Eudragit® RL 100 PM c: CVL-Eudragit® RS 100 PM d: CVL- Eudragit® RL 100-Eudragit® RS100 PM e: CL-P f: CS-P g: CLS-P h: CL-1 i: CS-1 j: CLS-1).

4. MATERIALS AND METHODS

4.1. Materials

Carvedilol was a kind gift from Santa-Farma (İstanbul, Turkey). Eudragit® RS100 and Eudragit® RL100 were obtained from Degussa Röhm Pharma Polymers (Germany). Methanol, dimethyl sulfoxide and deuterio chloroform were purchased from Merck (Germany) while acetonitrile, 2-propanol, potassium dihydrogen phosphate and sodium hydroxide were purchased from Sigma-Aldrich (Germany). All other chemicals and reagents used were of pharmaceutical and analytical grade.

4.2. Methods

4.2.1. Preparation of polymeric nanoparticles

For the preparation of the polymeric solution with Eudragit® RL 100, Eudragit® RS 100 and Eudragit® RL 100: Eudragit® RS 100 (1:1 physical mixture) were dissolved in 100 mL methanol under a magnetic stirrer at 500 rpm for 1 hour to obtain a clear solution. For the preparation with active agent, CVL was added to this clear solution and stirred further for another 15 min. Spray-dryer was conditioned 30 min using methanol to obtain the desired levels of spraying.

The spray dryer was connected to the Inert Loop B-295 (Spray-Dryer B-90, BUCHI, Switzerland) because of the organic solvent. Carbondioxide gas was used at a flow rate of 450 mL/min. The inlet temperature was selected as 120°C for having maximum dried NPs [22]. Inlet temperature of 120°C, outlet temperature of 55°C were used during application. Dried NPs were collected in the collecting container. Contents of formulations prepared are summarized in Table 3.

Table 3. Formulation of polymeric nanoparticles.

Code	Eudragit® RL 100 (g)	Eudragit® RS 100 (g)	CVL (mg)	Methanol (mL)
CL-P	2	-	-	100
CS-P	-	2	-	100
CSL-P	1	1	-	100
CL-1	2	-	200	100
CS-1	-	2	200	100
CSL-1	1	1	200	100

4.2.2. HPLC conditions

Analytical experiments were performed with HPLC (Shimadzu Corporation, Kyoto, Japan) with reversed-phase GL Sciences Inc. InertSustaine® column (4.6mmx150 mm, C18 Gravity, 3 µm). The mobile phase was a mixture of 0.03M potassium dihydrogen phosphate (pH 3.0) buffer: acetonitrile: methanol (60:50:10, v:v:v), prepared daily and de-gassed by sonication and filtered through 0.45 µm membrane filter before the experiment. The flow rate was set at 0.5 mL/min resulting in a run time of 10 min per sample. The injection volume was 20 µL. Detection was performed at 242 nm and samples were analyzed at 40 °C.

4.2.3. Morphology

Morphological and structural characteristics of the PLGA NPs prepared were investigated using SEM (Hitachi TM 3030 Plus, Japan). Lyophilized samples were coated with thin layer of gold using a coater (Karaltay Scientific Instruments, China) under 50 mA for 30 seconds before observation under a scanning electron microscope (SEM).

4.2.4. Measurement of particle size, polydispersity index, zeta potential

The mean size, polydispersity Index (PDI) and zeta potential of the CVL NPs was determined using a Zetasizer Nanoseries (Malvern Instruments, England). Samples of all NPs were dispersed in double-distilled water (adjusted to a constant conductivity of 50 µS.cm⁻¹ using 0.9% NaCl) just prior to analyses [28]. Each sample was appropriately diluted with distilled water for analysis. Measurements were repeated in triplicate.

4.2.5. Assessment of entrapment efficiency (EE)

CVL loading to NPs was determined by high performance liquid chromatography (HPLC) method described in the previous section which was previously validated to demonstrate its precision, accuracy and linearity. CVL content of NPs was assessed by direct extraction of CVL from NPs. Spray dried NPs (about 5 mg) were accurately weighed, 2 mL methanol was added and vortexed to dissolve the particles in the organic phase. Complete solution was filtered through 0.22 µm polyamid filter and analyzed using HPLC. Drug content was expressed as EE (%) following Equation 1 [3, 32].

$$EE\% = (\text{Actual amount of CVL loaded in NPs} / \text{Theoretical amount of CVL loaded in NPs}) \times 100 \text{ Eq. (1)}$$

4.2.6. In vitro release of CVL from polymeric nanoparticles

The dialysis bag (cellulose membran, MW of 12-14 kDa, Sigma) method was used to study the *in vitro* release of Eudragit® NPs. The NPs containing 5 mg of CVL and 5 mg pure CVL were placed in the dialysis bags and stirred at 100 rpm using magnetic stirrer at 37 °C. *In vitro* drug release was initiated in a buffer system at pH 6.8 containing 30% Polyethylene glycol 400. One milliliter was withdrawn at different time (30 min, 1, 2, 3, 4, 5, 6, 8, 12, 24, 48 h) intervals at the same time, the same amount of fresh medium was replenished to maintain the sink condition. Samples were then tested using the validated HPLC method. All experiments were performed in triplicate.

4.2.7. Release kinetics study

Data obtained from the *in vitro* drug release studies were further investigated for release kinetics using DDSolver software program [35].

4.2.8. Powder X-ray diffraction (XRD)

The X-ray diffraction patterns of pure CVL and polymeric nanoparticles were obtained using a Rikagu Corporation (D/Max-3C, Japan) within the range of 2-40° at 2θ with 2°/min scanning rate and using 40 kV voltage with 20 mA current intensity level.

4.2.9. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum of the CVL formulations were determined at a wavelength of 4000-500 cm⁻¹ using FTIR (Schimadzu IR Prestige-21, Japan) instrument. Pure polymers, pure CVL, physical mixtures and polymeric nanoparticle formulations were also analyzed and were used as references.

4.2.10. Nuclear magnetic resonance (NMR)

¹H-NMR of the samples was carried out by dissolving in deuterio chloroform (CDCl₃), NMR (Bruker 500 MHz UltraShield NMR, Germany). Pure polymers, pure CVL and polymeric nanoparticle formulations were also analyzed and were used as references.

4.2.11. Statistical analysis

The statistical significance of the differences in particle size and zeta potential values and entrapment efficiency percentages between the different nanoparticle formulations was tested by one-way analysis of variance (ANOVA) and DDSolver were employed for in vitro release kinetics calculations. Differences were considered as statistically significant at a level of $p \leq 0.05$.

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