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Formulation and evaluation of colon targeted pulsincap delivery of Carvedilol for treatment of hypertension

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Abstract: The objective of this work is to ensure the controlled release of carvedilol at a given time and location for treating hypertension as a pulsincap delivery system. The capsule body was made of insoluble hard gelatin and filled with carvedilol microspheres, then a hydrogel plug was used to close it. The microspheres were made by oil/water emulsification followed by a solvent evaporation method in different drug: polymer ratios using Eudragit L100 and S100 as polymers. Direct compression was used to make the hydrogel plugs. Various assessment parameters, FTIR analysis, and in-vitro release experiments were performed on the produced microspheres. The results showed that formulated microspheres had satisfactory results for various micrometric properties. The microsphere formulations showed the highest entrapment efficiency were F9, F14 and F15 (89.18±2.25, 83.91±2.13 and 92.2±2.28% respectively). FTIR results revealed that carvedilol was molecularly dispersed into the polymeric matrix. In-vitro release studies of carvedilol microspheres showed sustained action over 8 hours. The pulsincap system was evaluated for the in-vitro release studies. PF9 had the highest cumulative release after 12 hours (94.161±0.83 %) and reduced drug release during lag time so, PF9 was chosen for the in-vivo study. The in-vivo study was carried out on 16 hypertensive patients using HPLC method to measure carvedilol conc. in the blood and compare PF9 with commercially available tablets of the same strength. The in-vivo study revealed that the prepared pulsincap has shown better pharmacokinetics parameters compared with the marketed formulation with a delayed release pattern for the effective treatment of hypertension.

Keywords: Carvedilol; Colon-targeted; Hypertension; Microspheres; Pulsincap.

1. INTRODUCTION

Hypertension is a long-term condition whose symptoms are most obvious early in the morning and there is a need to control the morning surge[1]. It has been recommended to treat hypertension by using the concept of chronopharmacotherapy to ensure that the maximum concentration of the drug should be present in the bloodstream during the activation of sympathetic tone which results in a rapid increase in blood pressure, known as morning blood pressure surge (MBPS)[2]. Antihypertensive individuals who take their medicine before bedtime will experience less morning discomfort and may have a lower cardiovascular risk[3]. Therefore, we developed a unique pulsincap formulation that releases the medicine according to a predefined schedule. It consists of a water-insoluble capsule body filled with medication content and sealed with a hydrogel plug at the opening end, which is covered by a water-soluble cap. The whole unit was enteric coated to avoid the problem of variable gastric emptying[4]. When the capsule enters the small intestine, the plug is ejected by swelling or erosion, and the medication is released.

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Carvedilol is a nonselective β-adrenergic blocking agent with α1-blocking activity and is indicated for the treatment of hypertension and mild or moderate heart failure of ischemic or cardiomyopathic origin. Because of the carbazole moiety in its structure, carvedilol can also be considered a powerful antioxidant[5]. The drug is rapidly absorbed and undergoes extensive first-pass metabolism in the liver. It reaches a peak concentration 1 to 2 hours post dose and has an elimination half-life of about 4-7 hours[6]. It is also poorly soluble in water resulting in a very low bioavailability (it has a bioavailability of 25-35 %), dose frequency, patient incompliance, and decreased stability. Microspheres provide prolonged and constant therapeutic effect, as well as particle size reduction for improving drug solubility. Microspheres minimise dose frequency, improving patient compliance, provide a better therapeutic effect for medications with a short half-life while also protecting the medication from enzymatic and photolytic degradation. Drug absorption in the stomach is reduced, resulting in less local side effects. In general, microspheres prevent the first pass metabolism, improve the biological half-life and also improve the bioavailability[7]. Microspheres are matrix systems in which the medication is uniformly dispersed, either dissolved or suspended. Its structure is made up of solid or liquid particles that are dispersed or dissolved in a matrix[8]. Emulsion solvent evaporation techniques have proven to be more useful when compared to other methods of preparing microspheres. In this technique, the drug is dissolved in the polymer/solvent system. Then it is added dropwise to the aqueous phase by continuous agitation until the solvent is evaporated. This process results in hardened microsphere which contains the drug[9].

The aim of the present study is to formulate and evaluate pulsincap drug delivery system containing carvedilol microspheres. This system can release the drug at a predetermined time after a lag period, so that the drug will be released from the formulation according to the physiological needs of the body, which offers benefits like controlled administration of therapeutic dose at the desired rate, reduction of side effects, minimization of dosing frequency, and decreasing cardiovascular risk, especially in the early morning.

2. METHODS

2.1. Materials

Carvedilol was kindly donated from Global NAPI pharmaceuticals, Cairo (Egypt). Eudragit L 100, Eudragit S100, Hydroxy Propyl methylcellulose (HPMC K4M), dibutyl phthalate, cellulose acetate phthalate and lactose were kindly supplied by Egyptian International Pharmaceutical Industries Company (EIPICO), Cairo (Egypt). Polyvinyl Alcohol (PVA), Potassium dihydrogen orthophosphate, Di-sodium hydrogen orthophosphate anhydrous, Dichloromethane, Ethanol, hydrochloric acid, formaldehyde solution (37 % formaldehyde solution stabilized by 15% methanol, Merck), acetyl acetone, ammonium acetate, glacial acetic acid, acetone, and potassium permanganate were purchased from El Gomhoureya for Drugs Trade & Medical Equipment, Cairo (Egypt). Acetonitrile, Ethanol, and methanol, HPLC grade; Merck. Darmstadt, Germany. Diethyl ether, ethyl acetate, dichloromethane, butanol, sodium hydroxide, trichloroacetic acid, and chloroform were purchased from El Nasr Chemical Co. Triethanolamine and Ortho-phosphoric acid, analytical grade; Riedel-dehaene, Germany.

2.2. Formulation and in-vitro evaluation of the pulsincap system

2.2.1. Preparation of Carvedilol microspheres

An accurately weighed amount of carvedilol and polymers (Eudragit L100 and Eudragit S100) in various proportions were dissolved in 25 mL of the organic solvent (Ethanol+ Dichloromethane 1:1) at room temperature as shown in table (2). The mixture was stirred with a magnetic stirrer (Wise stir ®MSH-20 D DAIHAN scientific, Co., Ltd., Korea) to make a homogeneous polymer dispersion of the medication. The previous organic phase was added drop-by-drop to 100 mL of distilled water containing 0.5% polyvinyl alcohol as an emulsifying agent. A mechanical lab stirrer (HS-120A, DAIHAN Scientific Co., Ltd., Korea) was used to maintain constant stirring at 1000 rpm until the organic phase was evaporated. The microspheres were filtered, washed with distilled water, and left to dry overnight[10].

2.2.2. Evaluation of Carvedilol Microspheres

2.2.2.1. Determination of percentage yield and entrapment efficiency: The yield of the microspheres was calculated using the following equation[11],

$$\text{Percantage yield} = \frac{\text{Weight of the produced microspheres}}{\text{Total weight of drug and polymer used}} \text{ X } 100$$

For the evaluation of entrapment efficiency, 25 mg microspheres were weighed and dissolved in 10 mL of the organic solvent (Ethanol + Dichloromethane 1:1). Add 20 mL of 0.1 N HCl (pH 1.2) to the beaker containing the organic solvent to precipitate the polymer. The organic solvent was evaporated by heating the beaker on a magnetic stirrer then the solution was filtered through a double-layered filter paper (Whatman 42), in a 100 mL volumetric flask, and the volume was adjusted to 100 mL using (0.1 N HCl). The solution was filtered, and from the filtrate, the absorbance was measured at 240 nm [12].

$$\% \text{ Entrapment Efficiency} = \frac{\text{Actual drug content (mg)}}{\text{Theoretical drug content (mg)}} \text{ X } 100$$

2.2.2.2. Saturation solubility study: The Solubility was investigated using shake-flask method[13] for a blank formula of pure carvedilol and carvedilol microspheres (F9, F14, and F15) as they had the highest entrapment efficiency. In 250 mL conical flasks containing 25 mL of distilled water, an excess quantity of drug and microspheres was introduced. At 37 ± 0.5ºC, the sealed flasks were shaken for 24 hours. Following that, aliquots were filtered using Whatman filter paper. Carvedilol concentration was evaluated using a UV spectrophotometer set at 240 nm. A saturation solubility study was also performed in ethanol, 0.1 N HCl (pH 1.2), and phosphate buffer solutions (pH 6.8 and 7.4).

2.2.2.3. Study of the external morphology (Scanning electron microscopy and microscopic images): Scanning electron microscopy (SEM) (JEOL JSM-6480LV, Japan) was used to study the surface shape and structure of microspheres. Microspheres were placed on a sample holder before being sputter-coated with a conducting metal (platinum). After that, the sample was examined for particle size and surface morphology with a focused fine electron beam[14]. The microscopic images were captured using optical microscope (Labomed 9131040 40x Semi-Plan Achromatic Objective, USA)

2.2.2.4. Fourier transformed infrared specrtroscopy (FTIR): FTIR spectra of pure carvedilol, Eudragit L100, Eudragit S100, and the prepared microspheres were determined using Bruker VERTEX 80 (Germany) combined platinum diamond ATR, comprises a diamond disk as that of an internal reflector in the range 4000-400 cm^{-1} with resolution 4 cm^{-1}, refractive index 2.4 (National research Centre).

2.2.2.5. Particle size analysis and determination of flow properties of microspheres

2.2.2.5.1. Particle size analysis: Optical microscopy equipped with a calibrated ocular micrometer was used to determine the average particle size of the microspheres. A small number of microspheres were put on a glass slide and were determined in each formula by making use of Edmondson's equation: D mean =Σnd/Σn

Where "n" means the number of counted microspheres, and "d" stands for the mean size range[12].

2.2.2.5.2. Angle of repose: The angle of repose was determined by a fixed funnel method. In this method, microspheres were poured through the walls of a funnel which was fixed at a position such that its lower tip was at a height of exactly 2.0 cm above hard surface. The sample was poured till the time when upper tip of the pile surface touched the lower tip of the funnel. Draw a circle around the conical heap and measure its diameter. The height of the pile (h) and the radius were determined. Then, the angle of repose was calculated using the following equation[15]:

$$\text{Tan } \theta = h/r$$

2.2.2.5.3. Bulk Density and Tapped density: Bulk density was measured by gently introducing a known sample mass through a glass funnel into a 10 mL graduated cylinder and leaving the powder without compacting it. The apparent untapped volume is then read to the nearest graduated unit.

Bulk density = (Weight of the powder)/ (Bulk volume)

Tapped density was determined by pouring gently a specified quantity of sample through a glass funnel into a 10 mL graduated cylinder. The initial volume was observed, and then the cylinder was allowed to stroke. The tapping was continued until no further change in volume was noted. Volume occupied by the sample after tapping was recorded and the tapped density was calculated [16].

Tapped density = (Weight of the powder)/(Tapped volume)

2.2.2.5.4. Carr's index (CI)[17]:

Carr's index = (Tapped density-Bulk density)/(Tapped density)

2.2.2.5.5. Hausner's ratio (HR)[18]:

Hausner's ratio = (Tapped density)/(Bulk density)

2.2.2.6. In-vitro release study of microspheres: Carvedilol microspheres were tested for in-vitro release using dialysis membrane method. A dialysis tube (2.5 cm in diameter and 6 cm in length) acted as a donor compartment. A dialysis membrane (semi-permeable cellophane membrane) which had been pre-soaked for 30 minutes in warm water (to open the pores of the membrane) was inserted over the glass tube's lower end and made watertight by a rubber band. The receiver compartment held 100 mL of phosphate buffer solution pH 6.8 (release media). Microspheres equivalent to 12.5 mg of carvedilol were dispersed into 5 mL of pH 6.8 buffer and positioned in the donor compartment. The system was maintained at 37 ± 0.5ºC for 8 hours in a thermostatic shaker water bath at 50 rpm. Samples of 5 mL were taken at intervals of 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 hours. To maintain constant volume, the volume of each sample was replaced with the same amount of new buffer. The samples were analyzed spectrophotometrically at 240 nm[19].

2.2.3. Preparation of cross-linked gelatin capsules with formaldehyde treatment

Hard gelatin capsules of size 1 were taken. The caps were detached from their bodies. To create formalin vapors, 25 mL of formaldehyde solution (37% formaldehyde solution stabilized by 15% methanol) was placed in desiccators and potassium permanganate was added. Unfilled capsule bodies were subjected to formalin fumes. Because the caps were not exposed, they remained water-soluble. The bodies were removed after 6 hours of reaction and dried at 50°C for 30 minutes in a hot air oven. After that, the bodies were dried at an ambient temperature to remove any remaining formaldehyde[20].

2.2.4. Tests for unfilled capsules treated with formaldehyde

2.2.4.1. The capsules' diameter, Length and weight were measured before and after formaldehyde treatment using a caliper.

2.2.4.2. Solubility studies of treated capsules: Both treated and untreated capsules were stirred in a beaker containing 100 mL dissolution medium by using a lab stirrer. The dissolution medium taken were 0.1 N HCL (pH 1.2), phosphate buffer solution (pH 7.4 and 6.8). The capsule bodies were subjected to formaldehyde solution for different hours ranging from 2 -12 hours and checking the effect of exposure time in decreasing the solubility of capsule shell. Then formaldehyde exposed capsule bodies were dried in hot air oven. The time at which the capsule became soluble was noted [21].

2.2.4.3. Qualitative estimation of free formaldehyde: Residual content in treated gelatin capsule bodies (Colorimetric Estimation of Formaldehyde):
Standard formaldehyde solution: 3 mL of formaldehyde solution (37% formaldehyde solution stabilized by 15% methanol, Merk) were put in 50 mL volumetric flask and the volume was made up to 50 mL by distilled water to give a standard reference solution containing 20 µg/mL concentration of formaldehyde (The limit for residual formaldehyde according to FDA is 0.002%).

Sample solution: formaldehyde treated bodies (about 25 in number) were cut into small pieces and taken into a beaker containing 25 mL distilled water. This was stirred for 1 hour with a magnetic stirrer to solubilize the free formaldehyde. The solution was then filtered into a 50 mL volumetric flask and volume was made up to 50 mL with distilled water.

Acetyl acetone Reagent: Prepared by dissolving 15.4 g of ammonium acetate in 50 mL of reagent water in a 100-mL volumetric flask. To this solution, add 0.20 mL of acetyl acetone and 0.30 mL of glacial acetic acid. Mix the solution thoroughly, then dilute to 100 mL with reagent water.

Method

To 1 mL of sample solution, 9 mL of water was added. 1 mL of resulting solution was taken into a test tube and mixed with 4 mL of water and 5 mL of acetyl acetone reagent. The test tube was warmed in a water bath at 40 ºC and allowed to stand for 40 minutes and the color intensity of the sample solution was compared with the standard formaldehyde solution which was prepared and processed in the same procedure as the test solution using 1 mL of standard solution in place of the sample solution[22].

2.2.5. Preparation of the hydrogel plug

Each hydrogel plug was made by compressing an equal amount of HPMC K4M and lactose in a single punch tablet machine (Korsch pressen, EKO-DMS, USA) to make tablets with a flat surface weighing 70-100 mg and compressed with a 7 mm punch. A 2 mg talc powder was added to act as lubricant[23]. Table (1) and figure (1) show the composition of hydrogel plug tablets.

Table 1. Composition of the hydrogel plug tablets.

Ingredients (mg)	Hydrogel plug 1	Hydrogel plug 2	Hydrogel plug 3
HPMC K4M	34	44	49
Lactose	34	44	49
Talc	2	2	2
Total weight (mg)	70±0.34	90±0.25	100±0.33

2.2.6. Evaluation of the prepared hydrogel plugs

2.2.6.1. Thickness and hardness test: Thickness and Hardness were measured using electronic digital tablet hardness tester (pharma test, PTB 311, Germany).

2.2.6.2. Swelling index: Hydrogel plug tablets were sequentially immersed in media of three different pH (pH 1.2, pH 6.8, and pH 7.4). For each formulation, one tablet was weighed (dry weight) and placed in a beaker containing 200 mL of buffer solution. After each hour the hydrogel plug tablet was removed from beaker and weighed again up to 6 hours. The tablets were wiped off to remove excess of surface water by using filter paper. The percentage weight gain by the hydrogel plug tablet was calculated [24].

% swelling = ((Wet weight-Dry weight))/(wet weight) X 100

2.2.6.3. Lag time: The lag time test was calculated indirectly by measuring the time necessary for ejection of hydrogel plug from the impermeable capsule body mouth completely. For the study, an insoluble capsule body containing carvedilol microspheres in the bottom and HPMC K4M hydrogel plug tablet tightly put in the opening of the impermeable capsule body was connected to the paddle of the USP apparatus II by a thread and suspended in phosphate buffer solution (pH 6.8) for 6 hours [25].

2.2.7. Formulation of a pulsatile drug delivery system (modified pulsincap)

Carvedilol microspheres equivalent to 12.5 mg were weighed and manually hand-filled into the previously formaldehyde-treated bodies. The microsphere-containing bodies were subsequently filled using hydrogel plugs. Then, using a tiny amount of the 5 % ethyl cellulose ethanolic solution, seal the capsule body and cap together. The sealed capsules were thoroughly coated with 5% Cellulose Acetate Phthalate (CAP) [26].

2.2.8. Coating of Pulsincap

Cellulose acetate phthalate solution (5% w/v) was prepared by adding 5 gm CAP to 0.75 gm dibutyl phthalate as a plasticizer using acetone: ethanol (8:2) as a solvent. Dip coating method was used. The capsules were alternatively dipped in 5 % CAP solution and dried. Coating was repeated until an expected weight gain of 8-12 % was obtained [27].

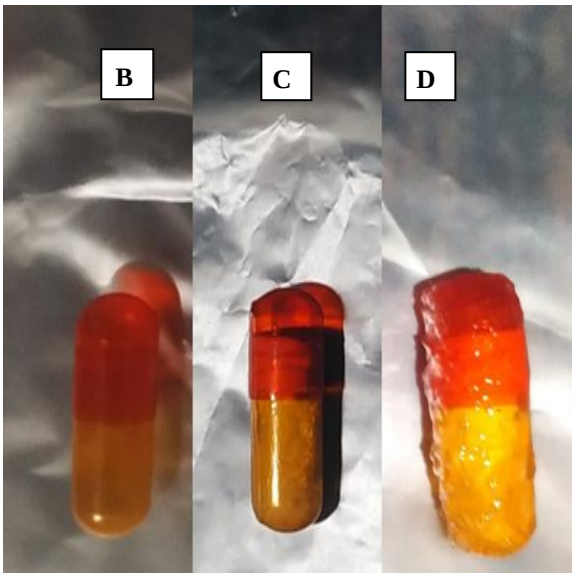

Figure 1. A) Hydrogel plugs with three different thicknesses., B) empty capsule, C) formulated pulsincap before coating, D) pulsincap after coating with 5% cellulose acetate phthalate solution.

2.2.9. Evaluation of the modified Pulsincap

2.2.9.1. Weight variation: 10 capsules were chosen at random from each batch and weighed individually.

2.2.9.2. Coating thickness of cellulose acetate phthalate: Was measured using a screw gauge.

2.2.9.3. Drug content: This test was done to ensure that Equivalent weight of microspheres introduced into the capsule was within the pharmacopoeial limit (95-105%). Microspheres (equivalent to 12.5 mg of carvedilol) were immersed in 50 mL of 0.1 N HCl for 30 minutes before sonication using a probe sonicator (Model 275 T, Crest Ultrasonics Crop, Trenton, USA) for 10 minutes to break the microspheres and facilitate extraction of the drug. The solution was centrifuged using a centrifuge (Phoenix CD-0412-50 GMbH, Germany) and was filtered. The clear supernatant solution was analyzed spectrophotometrically at 240 nm[28].

2.2.10. In-vitro release studies of carvedilol pulsincap

Release studies of carvedilol pulsincap were done using the USP dissolution type II apparatus (Copley, NG 42JY, Nottingham, U.K.) paddle type. A cotton thread was used to attach a capsule to the paddle, ensuring that it was thoroughly submerged in dissolution media making them all in one level. Three dissolving media with pH 1.2, 7.4, and 6.8 were employed consecutively to imitate pH variations along the GI tract, known as the "sequential pH change approach." The pH 1.2 medium was utilised for 2 hours (since the usual gastric emptying duration is 2 hours) before being removed and replaced with a fresh pH 7.4 phosphate buffer solution. The medium was withdrawn after 3 hours (typical small intestine transit time) and replaced with a new pH 6.8 dissolving medium for the subsequent hours. 900 mL of dissolving medium were used, at each time. The temperature was maintained at 37±0.5ºC with a spinning speed of rpm. At specific time intervals, 5 mL of dissolution medium was removed and replaced with new dissolution media. At 240 nm, the withdrawn samples were spectrophotometrically examined, and the total percentage release of the drug was computed[29].

2.2.11. Kinetic modelling of drug release

The mathematical modeling and the in-vitro drug release kinetics of carvedilol pulsincap were calculated according to zero order, first order and Higuchi's diffusion model. The best kinetic order was calculated from the highest values of the obtained correlation coefficients.

2.3. In-vivo therapeutic effect and bioavailability study

The aim of this study is to study the effect of carvedilol on blood pressure especially in the early morning when the cardiovascular risk increases so, we formulate pulsincap to enable the patient to take the medicine at bedtime and the drug will be released from the pulsincap after a lag time in the early morning. The study included 16 hypertensive male adults ranging in age from 33 to 47 years and weight from 72 to 84 kg (non-obese). Each patient signed a written consent form. Each participant underwent a thorough physical and clinical examination. All the patients were supervised by a physician who was responsible for their safety and the collection of samples during the study. Each patient's liver function was assessed by measuring ALT and AST because carvedilol is primarily eliminated through the liver. The patients were instructed not to take any over-the-counter medications for 72 hours before the study. The study was approved by the Research Ethics Committee of Al Azhar University- Faculty of Pharmacy (Girls) in Cairo, Egypt no. 38 on 18-11-2015 and was carried out in the outpatient clinic_internal medicine department_Al Hussein Hospital. The patients were randomly numbered and divided into two dosing groups of equal size, each with eight patients. They were then treated as follows: Group I: They received the best-prepared formulation of Pulsincap (PF9) with a cup of water, and after a 7-day washout period, they received the conventional marketed tablet (Carvipress® 12.5 mg) in the second period. Group II: Orally administered the standard marketed tablet in the first period and the pulsincap (PF9) in the second period.

2.*3.1. Sample collection*

For Group I, blood samples were collected in heparinized tubes using a vein puncture cannula before carvedilol administration (blank), as well as at 4,5, 6, 7, 8, 10, 12, and 14 hours after the dose. Sample withdrawal at 4 hours post dose was to confirm that there was no release of the drug.

For Group II, blood samples were collected using a vein puncture cannula before carvedilol administration (blank), as well as at 0.5, 1, 1.5, 2, 4, 6, and 8 hours after the dose. The sampling time was different because pulsincap is a controlled release dosage form so, the appearance of carvedilol in plasma was after 5 hours and that was confirmed by

withdrawal of blood sample before 5 hours. It was found that no drug release in plasma. However, in conventional tablet the drug appears rapidly after 1-2 hours.

2.3.2. Instrumentation and chromatographic conditions

HPLC (Agilent 1100, Germany) instrument that was equipped with G1313A Autosampler, G1315B DAD Detector, G1316A Column Compartment, G1322A Vaccum Degasser, G1311A quaternary pump, and solvent tubing. The chromatographic analysis was performed on a reversed phase column, Kromacil® 250x4.6mm (i.d), 5µm with a guard column (4×3 mm i.d., Phenomenex) packed with the same material at a flow rate of 1.0 mL/min and the injection volume was 20µL. The mobile phase consisted of water – acetonitrile – methanol – ethanol-1M- triethylamine (83:58:55: 3:1), the pH was adjusted to 2.5 with ortho-phosphoric acid. The mobile phase was filtered using 0.45-micron membrane filter paper and degassed for 15 minutes[30]. Detection was performed at 240 nm.

2.3.3. Pharmacokinetic parameters and statistical analysis

The pharmacokinetic parameters were analysed by the statistical package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). The mean and standard deviation were used to summarise the data. Unpaired t-test was used to compare the two groups. P-values less than 0.05 were considered statistically significant. The calculated pharmacokinetic parameters obtained for pulsincap and conventional tablet were:

C_{max} is the maximum observed plasma concentration.
T_{max} is the time to reach the maximum observed plasma concentration.
K_{el} is the apparent terminal elimination rate constant.
$(t_{1/2})_{el}$ is the apparent terminal half-life.
AUC_{0-t} is the area under the plasma concentration-time curve from time zero to the time of the last measurable concentration.
$AUC_{0\rightarrow\infty}$ is the area under the plasma concentration-time curve from time zero to infinity.
$AUMC_{0\rightarrow\infty}$ is the area under the first moment curve from time zero to infinity.
MRT is the mean residence time.
R.B is the relative bioavailability.

3. RESULTS

3.1. In-vitro evaluation of the pulsincap system

3.1.1. Evaluation of carvedilol microspheres

3.1.1.1. Determination of percentage yield and entrapment efficiency: The prepared microspheres' percentage yield ranged from 75±1.13 % to 94.66±1.46 %. The best value was found to be for F9 (94.66±1.46). The results are revealed in table (2) and showed that the entrapment efficiency increased when the polymer concentration was increased until it reached a particular limit, after which it declined. The formulations had the highest entrapment efficiency were F9, F14 and F15 (89.18±2.25, 83.91±2.13 and 92.2±2.28 % respectively). By increasing carvedilol: Eudragit L100 ratio from 1:1 (F1) to 1:3 (F3), the entrapment efficiency increased from 39.17±1.011 to 68.93±2.22. However, carvedilol: Eudragit L100 ratio of 1:4 (F4) and 1:5 (F5) gave lower values (37.975±1.14 and 28.69±1.17 respectively). By increasing carvedilol: Eudragit S100 ratio from 1:1 (F6) to 1:4 (F9), the entrapment efficiency increased from 50.114±1.13 to 89.18±2.25. However, carvedilol: Eudragit S100 ratio of 1:5 (F10) gave lower value (34.89±0.02). By increasing carvedilol: Eudragit L100: Eudragit S100 ratio from 1:0.5:0.5 (F12) to 1:2:2 (F15), the entrapment efficiency increased from 21.59±1.22 to 92.2±2.28. However, carvedilol: Eudragit L100: Eudragit S100 ratio of 1:2.5:2.5 (F16) and 1:3:4 (F11) gave lower values (78±2.25 and 25.15±1.04 respectively).

3.1.1.2. Saturation solubility study: The results of saturated solubility study are shown in table (3).
3.1.1.3. Study of external morphology (Scanning Electron Microscopy and microscopic images):
Examination of the formulae (F9) by SEM showed that the microspheres were found to be round and resembled aggregates or distinct particles.
Microscopic images and SEM photograph of the carvedilol microspheres are revealed in figure (2).

Table 2. Composition, percentage yield, and entrapment efficiency of different formulations for Carvedilol microspheres.

Formulation	Carvedilol (mg)	Eudragit L100 (mg)	Eudragit S100 (mg)	Ratio (Drug: polymer)	Total weight (mg)	Percentage yield (%)	Entrapment efficiency (%)
F1	300	300	------	1:1	600	83.33±2.74	39.17±0.01
F2	300	600	------	1:2	900	94.44±2.33	54.87±0.08
F3	300	900	------	1:3	1200	91.66±1.19	68.93±0.22
F4	300	1200	------	1:4	1500	80.00±1.23	37.97±0.14
F5	300	1500	------	1:5	1800	91.66±2.45	28.69±0.17
F6	300	------	300	1:1	600	80.00±1.67	50.11±0.13
F7	300	------	600	1:2	900	91.11±1.25	63.93±0.12
F8	300	------	900	1:3	1200	83.33±1.43	73.00±0.05
F9	300	------	1200	1:4	1500	94.66±1.46	89.18±0.25
F10	300	------	1500	1:5	1800	80.55±1.14	34.89±0.02
F11	300	900	1200	1:3:4	2400	87.50±1.20	25.15±0.04
F12	300	150	150	1:0.5:0.5	600	75.00±1.13	21.59±0.22
F13	300	300	300	1:1:1	900	86.66±1.06	66.29±0.19
F14	300	450	450	1:1.5:1.5	1200	83.33±2.33	83.91±0.13
F15	300	600	600	1:2:2	1500	93.33±2.97	92.20±0.28
F16	300	750	750	1:2.5:2.5	1800	83.33±1.30	78.00±0.25

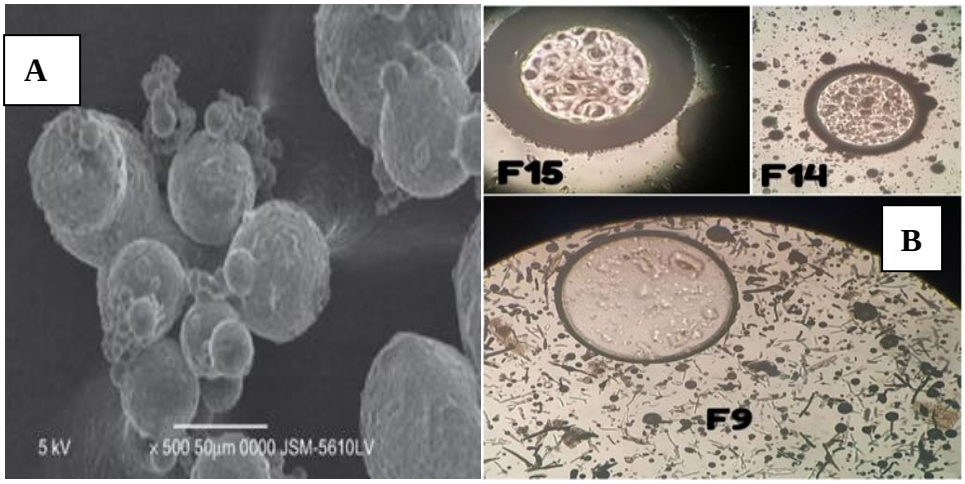

Figure 2. A) SEM photograph of Carvedilol microsphere, B) Microscopic images of carvedilol microspheres (F9, F14, and F15).

3.1.1.4. Fourier transformed infrared spectroscopy (FTIR): The spectrum of pure carvedilol, Eudragit S100, Eudragit L100, and microspheres are shown in figure (3). In the spectra of the produced microspheres, the secondary amine sharp peak present in the pure carvedilol disappeared and a broad peak at the same wavenumber range (around 3366.21 cm^{-1}) was observed.

Table 3. Saturated solubility of blank formula for pure carvedilol and carvedilol microspheres.

Solvent	Saturated solubility (mg/mL)			
	Pure carvedilol	F9	F14	F15
Distilled water	0.0045±0.0	0.0067±0.0003	0.0052±0.0001	0.0031±0.0001
Ethanol	10.9± 1.2	12±0.25	12.7±0.14	11.3±0.15
pH (1.2)	0.271±0.00	0.009±0.006	0.005±0.002	0.002±0.004
pH (6.8)	0.153±0.00	0.8±0.005	0.75±0.001	0.71±0.005
pH (7.4)	0.064±0.00	0.7±0.004	0.68±0.008	0.66±0.003

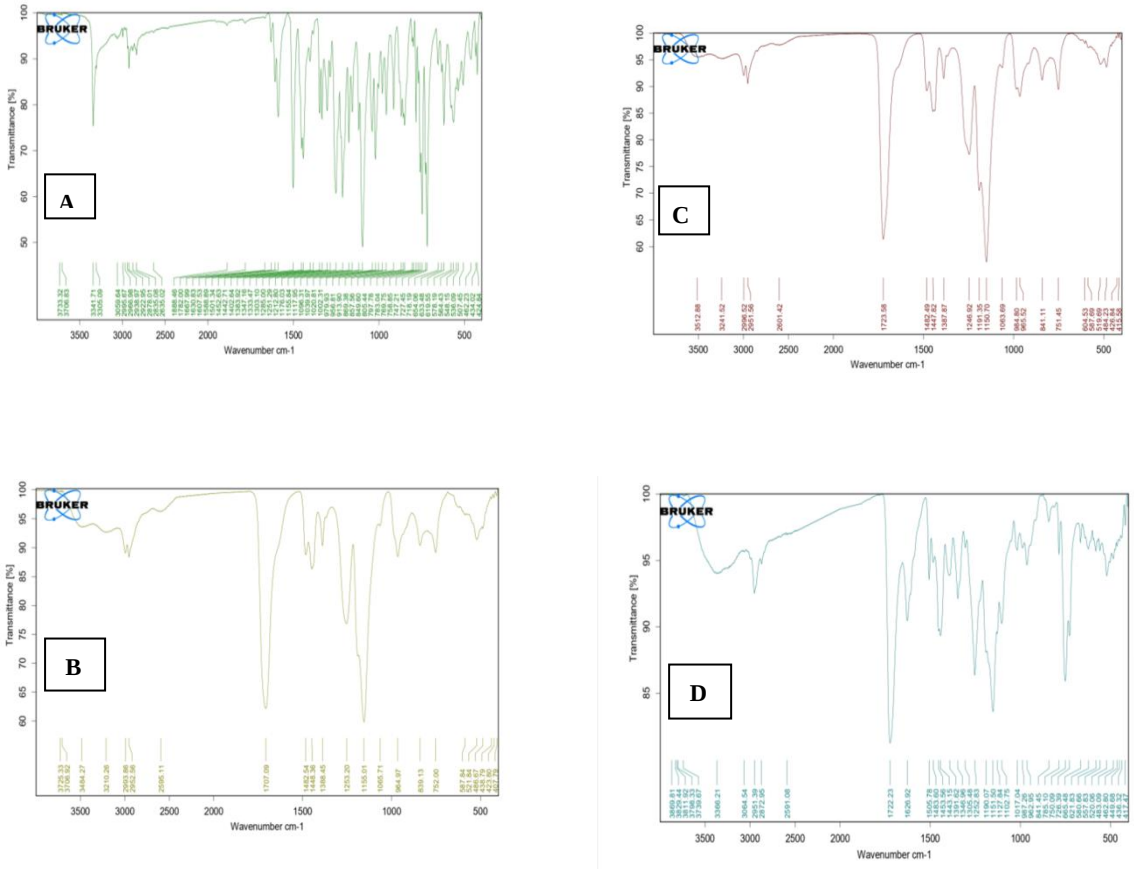

Figure 3. FTIR spectrum of A) pure carvedilol, B) Eudragit L100, C) Eudragit S 100, D) microspheres.

3.1.1.5. Particle size analysis and determination of flow properties of microspheres: The mean particle size of the microspheres increased dramatically as the polymer concentration increased, ranging from 79±1.50 to 249.9±1.91 µm. The evaluation results of prepared microspheres are shown in table (4). The calculated properties were all satisfactory in all formulations and showed good flow properties [31].

Table 4. Flow properties of carvedilol microspheres.

Formulations	Particle size(µm)	Angle of repose	Bulk density (g/cm^3)	Tapped density(g/cm^3)	Hausner's ratio	Carr's index(%)
F1	79.00±1.50	27.64±0.04	0.398±0.034	0.477 ±0.066	1.195±0.035	16.56±0.201
F2	85.00±3.20	26.93±0.03	0.432±0.032	0.526 ±0.051	1.375±0.030	17.87±0.125
F3	89.80±1.28	22.10±0.08	0.474±0.059	0.586 ±0.031	1.370±0.015	19.11±0.059
F4	97.33±1.64	26.75±0.09	0.456±0.055	0.541 ±0.087	1.307±0.020	15.71±0.236
F5	110.00±0.70	20.52±0.02	0.399±0.051	0.449 ± 0.026	1.120±0.010	11.13±0.221
F6	83.54±0.30	26.14±0.03	0.767±0.058	0.898±0.033	1.170±0.013	14.58±0.142
F7	95.36±1.28	25.71±0.07	0.523±0.032	0.604±0.025	1.150±0.045	13.41±0.246
F8	102.50±0.64	26.71±0.06	1.320±0.025	1.530±0.02	1.159±0.017	13.72±0.075
F9	108.66±1.20	25.42±0.03	1.110±0.025	1.250±0.01	1.126±0.054	11.20±0.207
F10	114.25±0.63	26.81±0.01	1.160±0.065	1.600±0.021	1.379±0.024	12.29±0.033
F11	249.90±1.91	26.52±0.01	0.519±0.041	0.606±0.085	1.167±0.012	13.64±0.122
F12	132.55±1.80	26.64±0.02	0.749±0.031	0.884±0.053	1.180±0.029	15.28±0.155
F13	148.90±0.71	23.43±0.02	0.492±0.057	0.578±0.075	1.174±0.018	14.87±0.143
F14	167.80±1.30	26.32±0.02	0.781±0.007	0.903±0.036	1.156±0.048	13.49±0.186
F15	178.46±1.20	23.95± 0.05	0.248±0.048	0.279±0.016	1.125±0.036	11.11±0.136
F16	189.00±0.66	21.52±0.02	0.508±0.064	0.591±0.024	1.163±0.160	13.98±0.131

3.1.1.6. In-vitro release study of carvedilol microspheres: The in-vitro release is shown in figure (4) and it was biphasic, with a quick phase followed by a delayed phase. The initial rapid effect may be desired to guarantee therapeutic medication plasma concentrations at the start of treatment. The drug released from F9 (91.7±0.69%) after 8 hours was higher than F14 and F15 (75.256±0.48 and 73.716±0.03% respectively).

3.1.2. Evaluation of unfilled capsules treated with formaldehyde

3.1.2.1. The diameter, length, and weight of empty capsule bodies were measured before and after formaldehyde treatment. The formaldehyde treated capsules were found to be slightly thicker. It was found that the length of capsules increased from 20±0.3 mm (before formaldehyde treatment) to 20.3±0.1mm (after formaldehyde treatment), also the capsule external diameter was found to increase after formaldehyde treatment from 7±0.1 mm to 7.3±0.2 mm. The weight of empty capsule bodies increased from 74.51 ±0.23 mg to 75.36±0.25 mg after formaldehyde treatment.

3.1.2.2. Solubility studies for the treated capsule bodies

The results are revealed in table (5) and showed that both untreated capsule bodies and caps dissolved within less than 20 minutes while formalin-treated capsules bodies remained intact for longer time.

Table 5. Solubility studies of formaldehyde-treated capsule bodies.

Time of exposure to formaldehyde solution in hours	Time needed for solubility in distilled water	Time needed for solubility in 0.1 N HCL (pH 1.2)	Time needed for solubility in phosphate buffer solution (pH 7.4)	Time needed for solubility in phosphate buffer solution (pH 6.8)
2	4:15 h	50 minutes	1h	1:10 h
4	7:30 h	1:25 h	1:30 h	1:15 h
6	10:20 h	2 h	2:10 h	2:15 h
8	15:10 h	3:20 h	3:25 h	3:30 h
10	17 h	5:15 h	5:30 h	5:20 h
12	22 h	6 h	6 h	6:10 h

3.1.2.3. Qualitative estimation of free formaldehyde residual content in treated gelatin capsule bodies): The test showed that a yellow-colored solution was produced for both sample and standard solutions. The sample solution was not more intensely colored than the standard solution indicating that less than 20µg free formaldehyde is present in 25 capsules.

3.1.3. Physical evaluation of the hydrogel plugs: Hydrogel plug 3 showed the highest %swelling index and the longest lag time of 5.35±0.12 as shown in table (6).

Table 6. The thickness, hardness, lag time and percentage swelling index of the prepared hydrogel plug.

Hydrogel plug No.	Thickness (mm)	Hardness (Kg/cm^2)	% Swelling index after 6 h			Lag time (h)
			In pH 1.2	In pH 6.8	In pH 7.4	
Hydrogel plug 1	2.80±0.042	2.4±0.014	73.28±3.27	73.14±0.16	72.19±2.33	4.30±0.13
Hydrogel plug 2	3.05±0.035	2.7±0.075	78.13±2.41	79.96±0.19	79.08±2.77	5.05±0.18
Hydrogel plug 3	3.60±0.021	2.9±0.023	84.09±1.17	84.13±0.17	83.98±0.22	5.35±0.12

3.1.4. Evaluation of the prepared pulsincap

Dibutyl phthalate was used as a plasticizer. The percentage of the plasticizer is 15% of cellulose acetate phthalate. The addition of plasticizers improves the water resistance of this coating material, and formulations using such plasticizers are more effective than when cellulose acetate phthalate is used alone.

Table 7. Evaluation of the prepared pulsincap.

Formulation No.	Weight of empty capsule (mg)	Weight of microspheres equivalent to 12.5 mg carvedilol	Weight of hydrogel plug tablet (mg)	Total weight of capsule (mg)	Thickness of CAP Coating (mm)	Average weight after coating with 5 % CAP (mg)	% Drug content
PF9	75.36±0.25	70.07±0.15	100±0.11	245.43±0.51	0.63±0.061	269.973±3.58	99.31±1.36
PF14	75.22±0.18	59.591±0.11	100±0.21	234.811±0.5	0.62±0.032	258.292±2.17	98.54±3.74
PF15	75.95±0.31	67.78±0.42	100±0.13	243.73± 0.86	0.65±0.015	268.103±3.11	98.45±2.13

3.1.5. In-vitro release studies of pulsincap

The in-vitro release of pulsincap for selected formulations (PF9, PF14, and PF15) was performed and it was found that PF9 had the highest % cumulative release in pH 6.8after 12 h (94.161±0.83 %) compared to PF14 and PF15 (84.262±1.23 and 80.068±0.67 %) respectively and reduced drug release during lag time. The release in pH 1.2 after 2

hours was zero for the three formulations. While PF14 released 4.964±0.22% of the drug and PF15 released 4.88±0.05% of the drug after 5 hours in pH 7.4, as shown in figure (4).

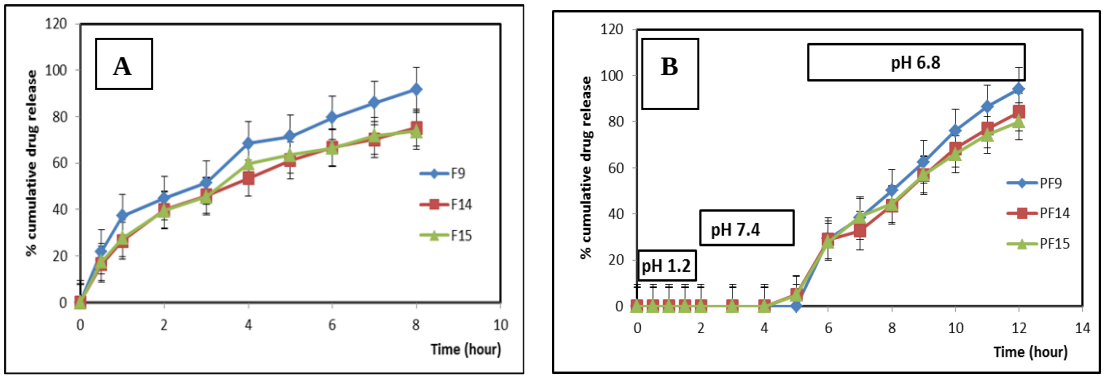

Figure 4. A) Percentage of carvedilol released from loaded microspheres (F9, F14, and F15), B) B In vitro release profile of pulsincap (PF9, PF14, and PF15).

3.1.6. Kinetic modeling of drug release

The kinetic analysis of all release profiles follows zero order models.

3.2. In-vivo therapeutic effect and bioavailability study

3.2.1. Plasma Concentration-Time data

Figure (5) show the mean plasma concentration of carvedilol following oral administration of 12.5 mg conventional tablet and pulsincap by sixteen patients.

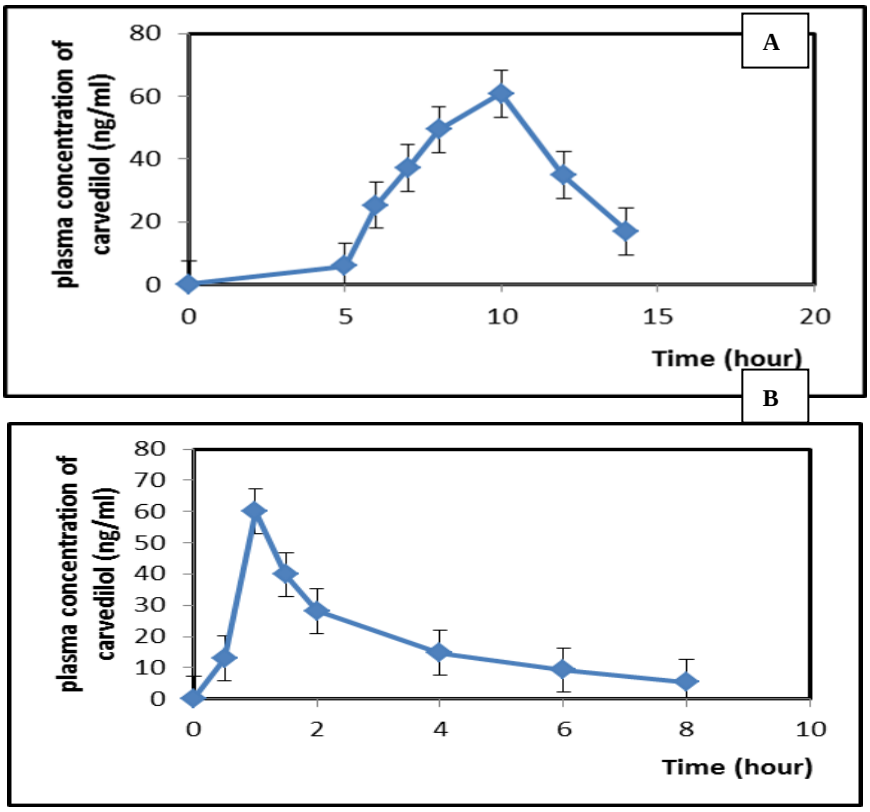

Figure 5. A) The mean plasma concentration of carvedilol (ng/ml) versus time (hours) following administration of 12.5 mg conventional tablet by sixteen patients, B) The mean plasma concentration of carvedilol (ng/ml) versus time (hours) following administration of 12.5 mg pulsincap (PF9) by sixteen patients.

3.2.2. Pharmacokinetic parameters and statistical analysis

Results of pharmacokinetic parameters are presented in table (8).

Table 8. The pharmacokinetic parameters of 12.5 mg carvedilol oral administration for both conventional tablet and pulsincap (F9) were measured and calculated in sixteen patients.

Pharmacokinetic Parameters	Conventional tablet (mean ± S.D)	Pulsincap (PF9) (mean ± S.D)	*p*-Value
C_{max} (ng/mL)	60.00±4.1	60.73±2.84	0.558
T_{max} (hr.)	1	10	<0.001*
K_{el} (hr^{-1})	0.296±0.015	0.090±0.097	<0.001*
$(t1/2)_{el}$ (hr.)	2.343±0.096	7.683±0.042	<0.001*
$AUC_{0\rightarrow\infty}$(ng.hr/mL)	165.445±9.391	559.22±9.01	<0.001*
$AUMC_{0\rightarrow\infty}$($ng.hr^2$/mL)	559.22±9.01	6207.819±98.265	<0.001*
MRT (hr)	3.38±0.084	11.01±0.043	<0.001*
$Cmax/AUC_{0\rightarrow t}$ (hr^{-1})	0.408±0.014	0.178±0.006	<0.001*
Relative bioavailability (R.B)		338%	

4. DISCUSSION

In the preparation of microspheres, surfactant concentration used was 0.5% PVA. The goal in preparing emulsions must be to reduce the interfacial tension to promote a more intimate blending of the two phases. This can be achieved by reducing the viscosity of the internal phase to make a good emulsion form. Low PVA concentration (0.2% w/v) might be insufficient to stabilize the droplets, which led to low encapsulation efficiency. PVA concentration of 0.5% w/v was found to be sufficient to stabilize the droplets leading to high encapsulation. Moreover, high PVA concentration (1% w/v) can affect the encapsulation efficiency of lipophilic drug due to increased viscosity and formation of molecular aggregates or micelles in aqueous phase. The micelles have tendency to solubilize lipophillic drug molecules resulting in increased solubility of lipophillic drugs thereby leading to poor encapsulation of drug[32].

For the entrapment efficiency, as the ratio of drug-to-polymer increased, encapsulation efficiency increased as seen in the formulations (from F1 to F3), (From F6 to F9) and (from F12 to F15); this is due to the fact that higher ratio of drug-to-polymer would produce large size droplets with decreased surface area, such that diffusion of drug from such microsphere will be slow, resulting in higher encapsulation efficiency[33]. While further increase in polymer content leads to a decrease in the encapsulation efficiency as seen in the formulations (F4, F5, F10, F11 and F16). This can be due to the fact that an additional increase in polymer content led to an enhancement of the concentration gradient between the emulsion droplets and the continuous phase; as a result, increasing the amount of drug partitioning into the continuous phase[34].

The results of saturated solubility study revealed that carvedilol exhibits pH-dependent solubility. It is a weak base (pKa = 7.8), and hence it is ionisable only at very low PH values (acidic pH of the stomach). However, in basic pH, carvedilol may precipitate in a crystalline form under digestion condition. Lower solubility of carvedilol in buffers at high salt concentrations might be related to the

higher ionic strength values of the dissolution media, where it has been shown that solubility decreases with an increase in ionic strength. Solubility of microspheres in distilled water and pH 1.2 exhibit very low values. This could be due to the presence of the drug in a relatively more localized way in the core of the microspheres. And neither Eudragit S100 nor Eudragit L100 polymers dissolved in distilled water or the acidic medium (1.2). When the pH of the dissolution medium was increased to the slightly basic buffer medium (pH 6.8 and pH 7.4), the solubility increased. This may be due to the increase of the porosity of the microspheres due to the enhanced solubility of Eudragit S 100 and L100 in this medium and its subsequent gradual removal from the microsphere matrix structure[35].

The results of FTIR analysis showed that in the spectra of the produced microspheres, the secondary amine sharp peak present in the pure carvedilol disappeared and a broad peak at the same wavenumber range (around 3366.21 cm−1) was observed, which is indicative of formation of hydrogen bond (which is a physical bond) between the O- and NH groups of the drug and polymers[36]. This might be evidence indicating a breakdown in the crystalline structure of carvedilol, leading to the formation of the amorphous state within the microspheres. These results could explain that the reduction in crystallinity of drug led to a decrease of the energy required in the dissolving process and also to a highly dispersed state of the drug. This bonding is most probably related to the controlled release of the drug[37].The carbonyl stretching peak of Eudragit L100 and S100 appears as a strong band at 1722.23 cm-1in microspheres with minor shifting which confirm that carboxylic moieties are linked to the carvedilol backbone chain in microspheres. Therefore, the microspheres could envelop carvedilol, and strong chemical interactions between carvedilol and polymers were absent[38]. These movements that occur during the process may indicate that Eudragit S100 and L100 polymers have been changed from a linear chain to a polymeric matrix form [39].

The main aim of formaldehyde treatment was to modify the solubility of hard gelatin capsules. Cross-linking of gelatin molecules was achieved by exposing to formalin vapors. Cross-linking involves the reaction of amino groups in gelatin molecular chain with aldehyde groups of formaldehyde by a "Schiff's base condensation" forming an irreversible complex so that the gelatin becomes water insoluble giving enough time for the polymer to swell and extend the drug release[29].

During estimation of residual formaldehyde content, formaldehyde reacts with acetylacetone in presence of ammonium acetate leading to the formation of 3,5-diacetyl-1,4-dihydrolutidine (DDL) which is characterized by yellow color (Hantzsch reaction) [40].

Swelling behavior of hydrogel plugs was assessed by the weight method. A gradual increase in the swelling indices was achieved with increasing the amount of HPMC K4M and lactose attributable to the ability of HPMC to absorb water due to the presence of hydrophilic groups in its structure leading to increased viscosity, more hydration and gel formation around the surface of the tablet, attributing to high swelling index. Due to high viscosity, matrix integrity is maintained for a longer duration leading to least erosion (stronger diffusional layer that is resistant to diffusion or erosion) [41]. The swelling equilibrium (maximum swelling index) is reached when the osmotic forces of the functional groups are balanced by the restrictive forces of the higher ordering of the polymer chains[42].

The enhanced bioavailability of carvedilol may be due to the preparation of microspheres using Eudragit, which improves the drug's solubility and provides a high level of protection against early drug release in the stomach and small intestine. Carvedilol microspheres formed with Eudragit convey the majority of the drug load to the colon, allowing for medication release at the appropriate spot after adequate transit time[20]. Also, the hydrogel plug creates a lag phase (no release of drug) that delays the drug release. Cross-linking of the capsule body by formaldehyde vapour and coating the entire capsule with cellulose acetate phthalate (5%CAP) modified the solubility of the capsule and was effective in delaying the drug release.

5. CONCLUSIONS

It can be concluded that pulsatile drug delivery systems provide a solution for the distribution of medications with chronopharmacological behavior, significant first-pass metabolism, night-time dosing requirements, or GIT absorption window. The carvedilol pulsincap was successfully administered to the colon region, meeting the chronotherapeutic strategy for better hypertension therapy.

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Conflicts of Interest: All authors declare that they have no conflicts of interest.

Ethical Statement: This study was approved by the Research Ethics Committee of Al Azhar University-Faculty of Pharmacy (Girls) in Cairo, Egypt no. 38 on 18-11-2015 and was carried out in the outpatient clinic_internal medicine department_Al Hussein Hospital. This material is the authors' own original work, which has not been previously published elsewhere. The paper is not currently being considered for publication elsewhere. The paper reflects the authors' own research and analysis in a truthful and complete manner.

Author Contribution: The authors confirm contribution to the paper as follows: Study conception and design: Amira F. Elghamry, Zeinab Alkasaby Zalat , Amal S. Abu El-Enin and Hosny A. Elewa; Data collection: Amira F. Elghamry, Zeinab Alkasaby Zalat , Amal S. Abu El-Enin and Hosny A. Elewa; Analysis and interpretation of results: Amira F. Elghamry carried out the experimental analysis and performed the numerical calculations for the suggested experiment.; Manuscript preparation: Amira F. Elghamry wrote the manuscript with input from all authors. All authors reviewed the results and approved the final version of the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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