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In vitro and *in vivo* Evaluation of Ivabradine Loaded Microsphres Prepared by Multiple Emulsion Solvent Evaporation Method

Firke Sagar N * and Ghiware Nitin B
CRPS, Nanded Pharmacy College Nanded (Affiliated to SRTM University Nanded)
Opposite Kasturba Matruseva Hospital, Shyam Nagar, Nanded- 431602. MS

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***Corresponding Author Email:** sagar1385@yahoo.co.in

Abstract
The Present study is an attempt to avoid the repetitive administration and release of drug in a controlled manner, thereby improve the bioavailability. Ivabradine HCl Floating microspheres were prepared by multiple emulsion solvent evaporation technique using Ethyl Cellulose as polymer and Tween 80 as emulsifying agent. During process optimization various parameters were studied such as: drug: polymer ratio and stirring speed. Selected optimized formulations were studied for SEM, entrapment, floating behavior, drug release and kinetics. Microspheres prepared were spherical shaped with smooth surface. Size of microspheres was in the range of 137.40 µm to 183.23µm. Formulation prepared using 400 mg of Ethyl cellulose gives the highest yield of 89.10±10 %, 91.5±0.10 % of drug loading, and 78.20±0.27 percent of drug release and 92.10±0.26 of Buoyancy**.** *In vivo* floating ability (X-ray) study, *in-vivo* Cardioprotective activity was performed on rats. Formulation remained buoyant for more than 8 h. Optimized formulation treated group shows significant increase in bioavailability. Ivabradine loaded floating microspheres expected to give new choice for safe, economical and increased bioavailable formulation for effective management of Angina Pectoris.

Keywords
Ivabradine, Microspheres, Multiple Emulsion Solvent Evaporation Method

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INTRODUCTION
Cardiovascular diseases have now become the leading cause of mortality in India, attributed to cardiovascular disease (CVD). Ischemic Heart diseases like Angina Pectoris and Stroke are the leading cause of deaths in India and are responsible for >80% of CVD deaths. Ivabradine HCL is a pure heart rate lowering agent, acting by selective and specific inhibition of the cardiac pacemaker I_f current that controls the spontaneous diastolic depolarization in the sinus node and regulates heart rate. The absolute bioavailability is around 40%, due to first-pass effect in the gut and liver; elimination Half half-life of 2 hours[(1)]. In the patient diagnosed with Angina Pectoris needs special attention in the treatment; since the Angina attack may be impulsive in the night or in the early morning hours[(2)]. This can be treated by maintaining therapeutic level of drug in plasma over the period of time, with sustained release formulation of Ivabradine.
Microspheres loaded with Ivabradine HCL, increase the effectiveness and release of drug in control

manner from polymeric membrane and thereby, maintain its concentration for longer duration. The aim of the study was to increase the bioavailability and reduce the mentioned side effects of Ivabradine. Moreover, effect of different concentrations of Ethyl Cellulose on drug loading, particle size and *in-vitro* drug release was examined and optimized formulation was subjected to *in-vivo* floating efficiency (X-ray) study.

MATERIALS AND METHOD

Materials

The Ivabradine Hydrochloride was provided as gift sample by Biocon Limited, Pashamylaram, Medak District, Andhra Pradesh, for this research work. Whereas Ethylcellulose (ETHOCEL Standard 4 Premium grade) was provided as gift sample by Colorcon Asia pvt Limited, Verna Industrial Estate, Verna, Goa. Tween 80 and Dichloromethane were purchased from SD Fine Chemicals. All chemical and reagents used were of analytical grade[3].

Methodology.

Preparation of microspheres

Microspheres were prepared by Multiple Emulsion Technique to obtain the W/O/W type of emulsion. A given quantity of Ivabradine HCl and Ethyl Cellulose (Formulation Table 01) was dissolved in a measured volume (10ML) of Dichloromethane; predefined volume of water as an internal phase was added to above drug-polymer solution in order to produce W/O primary emulsion. Primary emulsion was then emulsified into an aqueous phase containing Tween 80 as an emulsifier to produce a stable multiple W/O/W emulsion. The above mixture was stirred at 1000 RPM using three bladed Propellers for 30 minutes at given temperature to obtained microspheres. The microspheres were isolated by filtration through sintered glass filter and dried in oven at 40^{O}C[4].

Table 1: Factorial design (3^2) Formulation table of Ivabradine loaded floating microsphere

Batch Code	Drug mg	Independent variables		Dependent variables		
		Polymer Ratio (X1)	RPM (X2)	Loading % (Y1)	Particle size (µm) (Y2)	%Drug Release (Y3)
EC1	100	-1	0	76.5±0.45	142.89	91.11±0.09
EC2	100	+1	0	90.5±0.40	178.64	79.01±0.18
EC3	100	+1	-1	91.1±0.15	183.23	78.66±0.00
EC4	100	0	-1	82.1±0.40	172.19	85.55±0.09
EC5	100	+1	+1	91.5±0.10	162.55	**78.20±0.27**
EC6	100	0	+1	86.97±0.36	161.81	84.46±0.09
EC7	100	-1	-1	75.16±0.2	157.76	91.08±0.12
EC8	100	0	0	83.1±0.38	165.96	82.72±0.23
EC9	100	-1	+1	77.16±0.53	137.40	89.11±0.13

Code Value	Actual Values		Variable Levels
	X1	X2	
-1	200	500	**Low**
0	300	1000	**Medium**
+1	400	1500	**High**

*** n = 3, all values ± standard deviation, statistically significant at 0.05 level. X1 is polymer concentration (mg), and X2 is stirring speed (RPM). All batches contained 100 mg Ivabradine HCL.**

CHARACTERIZATION OF MICROSPHERES

The percentage yields

Percentage yield of different formulations were determined by weighing the floating microspheres after drying. The percentage yield of different formulation were calculated as follows[6].

% Yield = (Total Weight of Microspheres/Total Weight of Drug and Polymer) ×100.

Percentage of Drug Content / Drug Loading (%)

Fixed amounts of microspheres loaded with Ivabradine were dissolved in Phosphate Buffer of pH1.2 by Ultrasonication (Remi). The solution was then filtered through a 5µm membrane filter. Finally, drug concentration was determined by the UV Spectrophotometer at 286 λ max. Drug content was calculated according to following equation[7].

Micromeritic properties

Particle Size

Particle size analysis of microspheres was conducted by Optical microscopy (Micron 80) and diameter is expressed as projected diameter (d_p). Slide of sample was prepared by putting small amount of microspheres over the slide. A drop of paraffin oil was added in order to prepare the dispersion. A thin smear of dispersion was prepared over the glass slide. The slide was observed under the microscope at (10x) magnification and sizes of the particles were recorded in terms of eyepiece divisions and converted to diameter[8].

Floating Behavior

Formulated microspheres 100 mg were spread over the surface of 200 ml glass beaker filled with 100 ml of 0.1 N HCL containing 0.02% v/v Tween 80. The mixture was allowed to stay for 12 hours overnight. Floating microspheres were separated by decantation. Sinking Particles were again separated by filtration. Particles of both types were dried in dessicator until constant weight was obtained. Both fractions of the microsphere were weighed and percentage buoyancy was determined by using following formula and the results are recorded in table 02[9].

Table 2: Drug loading, particle size and %buoyancy of Ivabradine Loaded floating Microsphere

Batch Code	% Drug Loading	Particle Size µm	% Buoyancy
EC1	76.5±0.45	142.89	84.37±1.86
EC2	90.5±0.40	178.64	91.37±1.31
EC3	91.1±0.15	183.23	92.03±0.93
EC4	82.1±0.40	172.19	89.33±0.81
EC5	**91.5±0.10***	**162.55***	**92.10±0.26***
EC6	86.97±0.36	161.81	87.13±0.75
EC7	75.16±0.2	157.76	87.83±0.60
EC8	83.1±0.38	165.96	88.10±0.61
EC9	77.16±0.53	137.40	87.90±0.70

*** n = 3, all values ± standard deviation, statistically significant at 0.05 level**

% Buoyancy = {[wf / wf + ws] x 100}

Where,

wf = weight of floating microspheres,

ws = weight of sinking microspheres.

Surface Morphology of Ivabradine Microspheres

The external and internal morphology of the microspheres was studied by scanning electron microscopy (SEM). SEM photographs are shown in Figure 03.

DSC Study of Ivabradine Microspheres

Differential Scanning Calorimetry of Ivabradine loaded Microspheres was conducted to ascertain any significant modification in drug characteristics such melting point. A Study was conducted in Shimadzu DSC6. The endothermic melting peak of Ivabradine microspheres was found at 192.09^{O}C against the reported melting point of 192-196^{O}C. The onset of the melting peak started at 189.06^{O}C and end set at 193.89 ^{O}C. The DSC spectrum is depicted in Figure 01.

FTIR Analysis of Ivabradine Microspheres

An Infrared Spectroscopic study was conducted over the Optimized formulation. The said study was aimed to confirm the absence of any physical and chemical incompatibility between the drug and polymer post formulation[3]. The FTIR spectrum is depicted in figure 02.

***In vitro* drug release study**

The *in-vitro* dissolution studies were performed using Dissolution apparatus I (Basket type). An accurately weighed sample of Ivabradine HCl loaded microspheres was placed into 900 ml of Phosphate buffer of pH 1.2 maintained at a temperature 37.0º±0.5ºC and stirred at a speed of 50 rpm. At different time intervals, 05 ml aliquot of the sample were withdrawn, and the volume was replaced with an equal volume of plain dissolution medium kept at 37ºC. The collected samples were filtered and analyzed at 286-λ max of drug using a UV-Visible spectrophotometer against buffer of pH 1.2 taken as blank[4].

Data obtained from the study was analyzed for Model Fitting. In order to describe the kinetics of drug release from sustained release preparation, various mathematical and pharmacokinetic models have been proposed. Five kinetic models including zero order, first order, Higuchi matrix, Peppas Korsmeyer and Hixson-Crowell were applied to process the *in-vitro* release data of microspheres in order to find out the equation with a best fit model using PCP Disso software.

***In-vivo* floating behavior**

Healthy albino rats, weighing 500–600 g was treated with optimized formulation and monitored through radiological method (Tanwar et al., 2007) with modification. The study was approved by Institutional Animal Ethics Committee, (Protocol No: VIWA/061819/PR/A1203). The *In-vivo* gastro retentive Study of provide sample of microsphere formulation was carried out in healthy male/female Wistar rats (500–600 g) fasted for 18 h with free access to water.

The animals were housed individually in polypropylene cages and maintained under standard conditions (12-h light and 12-h dark cycle; 25–30 ^{O}C). Animals were divided into three groups (4 animals each). Group A Control, Group B Test and Group C Standard. The group A was supplied with plain water, group B was supplied with microsphere loaded with Ivabradine HCL and Barium Sulphate (EC5B) and Group C was administered with Barium Sulphate (BS) Loaded microspheres by intra gastric tubing. At varying time intervals X-ray photographs of gastric region were taken for monitoring the floating behavior of microspheres [(10)]. X-ray photographs are shown in Figure 4-6

Cardioprotective activity of ivabradine loaded floating microspheres

The study was performed by Isoproterenol induced myocardial necrosis. Wistar albino rats (150–200 g) were used for experimental models. They were housed in clean polypropylene cages under standard conditions of humidity (50% RH), temperature (25°C) and light (12 h light/12 h dark cycle) and fed with standard diet and water ad libitum. All animals were handled with humane care. Group I was served as control group and was treated with normal saline. Group II was served as negative control receiving Isoproterenol 85 mg/kg/sc alone on two consecutive days to induce ischemia. Group IIII was served as standard and received standard marketed preparation of Ivabradine Hydrochloride for seven days followed by 85 mg/kg/sc injection of Isoproterenol on sixth and seventh day. Group IV was served as Test and was received optimized Ivabradine Hydrochloride microspheres dispersion in normal saline for seven days and followed by 85 mg/kg/sc injection of Isoproterenol on sixth and seventh day. All the Myocardial necrosis in levels of serum marker enzymes AST, LDH and CPK were determined as per standard protocols. Forty-eight hours after the first Isoproterenol administration; the rats were sacrificed and autopsied. The heart of all the animals were removed and weighed and washed immediately with saline and then fixed in 10% buffered formalin. Frontal sections were embedded for histological examination [(10)].

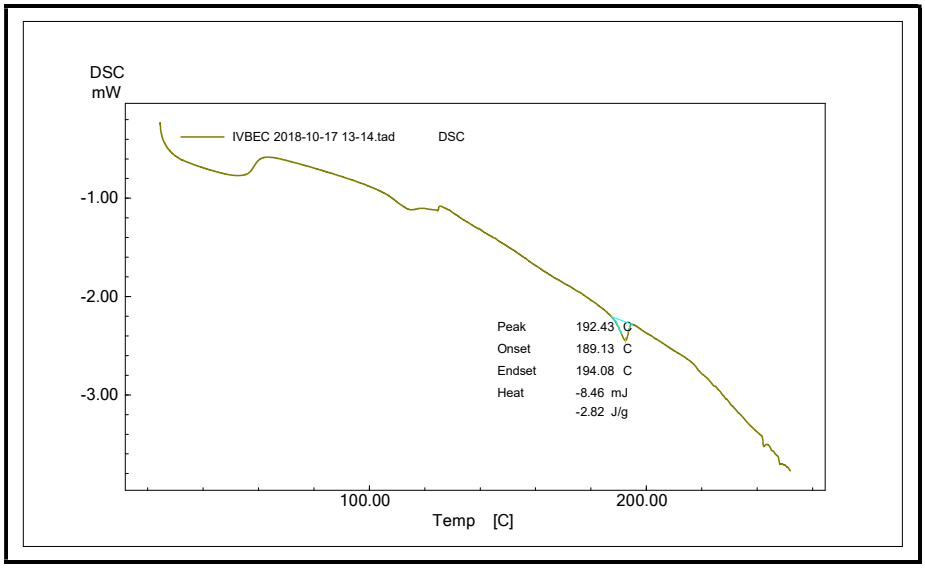

Figure No. 1: FTIR Spectrum of Ivabradine HCl loaded Ethyl cellulose microsphere

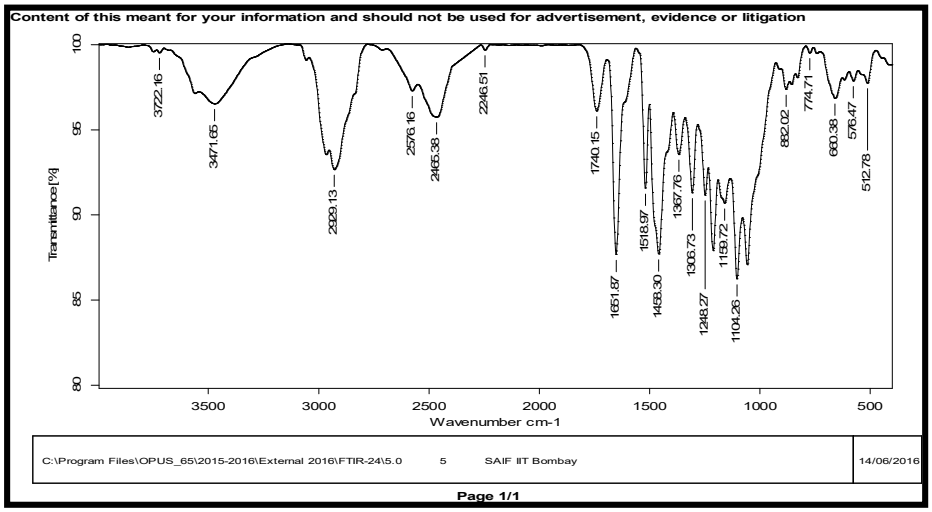

Figure No.2: DSC Thermogram of physical mixture of Ivabradine Hydrochloride and Ethyl Cellulose.

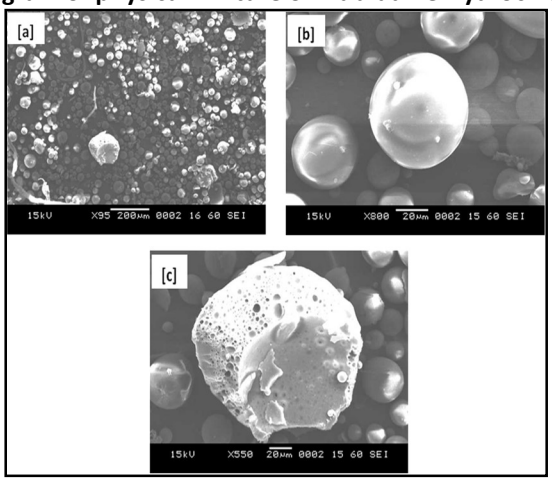

Figure No. 3: Scanning Electron Microscopic (SEM) Images of Microsphere.

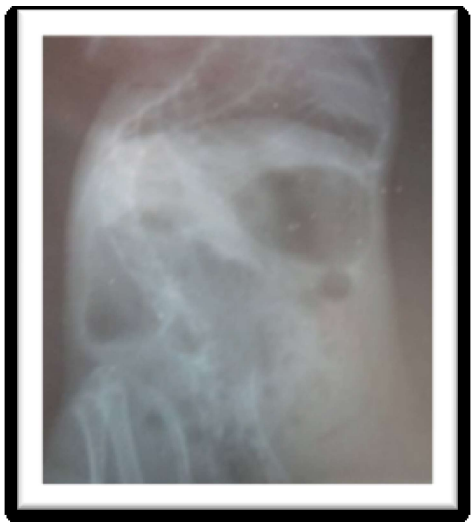

Figure No. 4 Gastric retention at 0 hrs

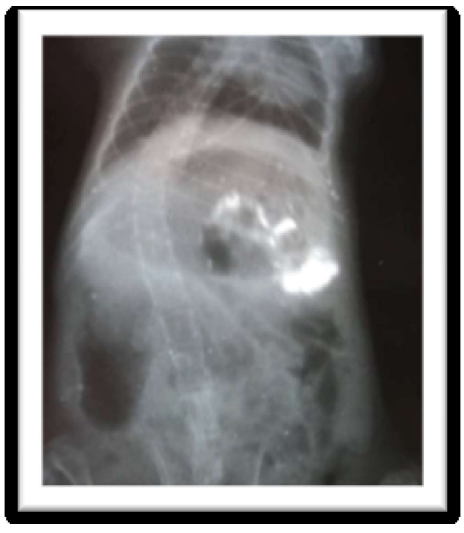

Figure No. 5 Gastric retention at 02 hrs

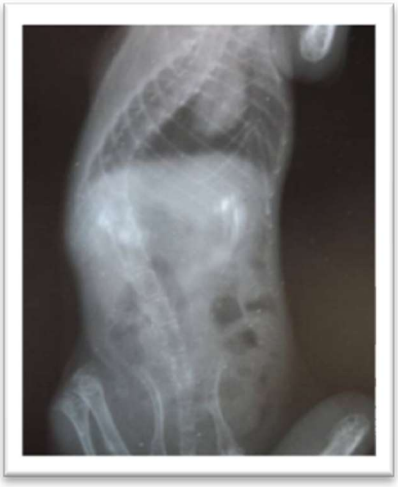

Figure No. 6 Gastric retention at 08 hr

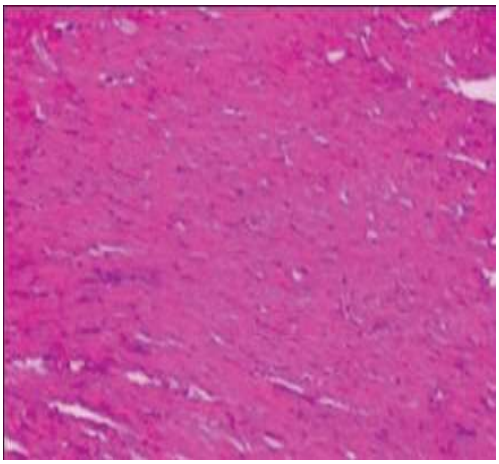

Figure No. 7 Histology of Group-I

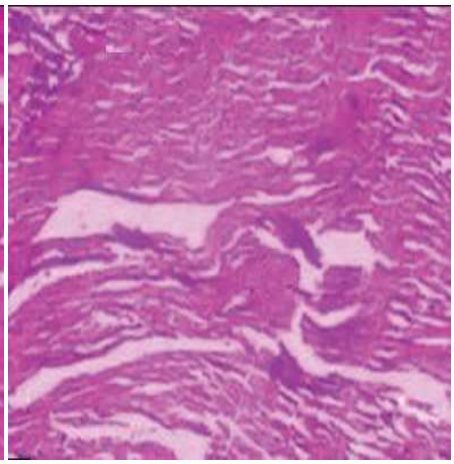

Figure No 8. Histology of Group-II

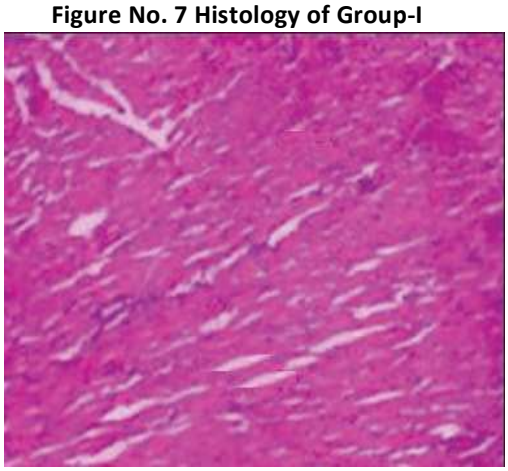

Figure No. 9 Histology of Group-III

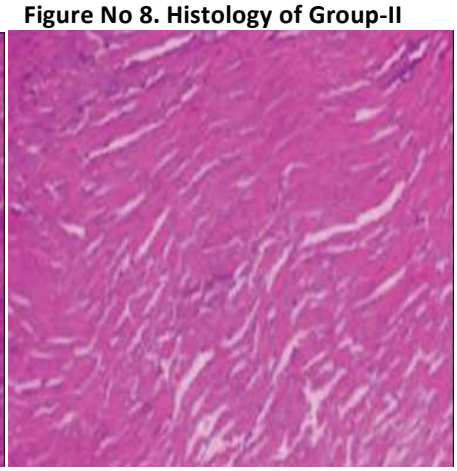

Figure No. 10 Histology of Group-IV

RESULT AND DISCUSSION

Percentage Drug Loading

Drug loading was found in the range of 77.16±0.53 to 91.5±0.10 in all EC1 to EC9 batches respectively. The maximum drug loading was found to be 91.5±0.10 followed by 91.1±0.15 corresponding to EC5 and EC3respectively. The optimum drug loading may be attributed to higher concentration of Ethyl cellulose. Higher concentration of Ethyl cellulose causes sufficient entrapment of drug in polymer matrix. Other formulations also showed drug entrapment, but their entrapment efficiency was found not satisfactory. However, the formulation EC5 showed

relatively smaller particle size related to higher stirring rate.

Ivabradine HCL is water soluble, as a result, it is very difficult to achieve desired loading in microsphere by simple o/w emulsion solvent evaporation method. Therefore, modified w/o/w method was adopted, the presence of the oil phase, i.e., Dichloromethane prevents the diffusion of Ivabradine HCL in aqueous phase and thereby improve its loading.

***In-vitro* buoyancy study**

The % buoyancy of all the preliminary formulations was found to be 84.37±1.86, 91.37±1.31, 92.03±0.93, 89.33±0.81, 92.10±0.26, 87.13±0.75, 87.83±0.60, 88.10±0.61, 87.90±0.70. The buoyancy is the important factor which directly affects the gastric retention of microspheres in the stomach, therefore it is necessary to achieve maximum buoyancy. The buoyancy of the microspheres is principally depending on the density of the microspheres, all the formulations have a bulk density less than the density of the 0.1 N HCL solution. Results are reported in table no 02.

Micromeritic study

Particle Size Analysis

The particle size may affect the flowability and packing arrangement of the microspheres, therefore it is necessary to obtain the optimum particle size. The particle size determination was conducted by the optical microscopic method. The mean geometric diameter (dg) of all the batches EC1 to EC9 was estimated in the range of 142.89µm to 183.23 µm. The maximum particle size, i.e. 183.23 µm (EC3) was attributed to higher concentration of Ethyl cellulose (400 mg) and lowest stirring speed (500 RPM). Lower stirring speed results in insufficient emulsification of organic and aqueous phase, as a result a large sized microsphere may be obtained.

***In vitro* drug Release Study**

The drug releases of Ivabradine HCl from all the batches were tested in a dissolution fluid at pH1.2. Release study was conducted on USP Type-I Basket apparatus at 50RPM and 37°C ±2°C. Cumulative drug release of all EC1, EC2, EC3, EC4, EC5,EC6, EC7, EC8 and EC9 batches at the 8th hour were found to be 91.11±009, 79.01±, 78.66±0.00, 85.55±0.09, 78.20±0.27, 84.46±0.09, 91.08±0.12, 82.72±0.23 and 89.11±0.13 respectively. The optimum release of the drug was found to be 78.20±0.27 at the 8th hour in EC5 batch; corresponding to 400 mg of Ethylcellulose.

In the drug release kinetic study all the batches were found to release the drug by Korsmeyer Peppas model which suggest the release of the drug from microsphere by erosion followed by diffusion mechanism.

***In-vivo* floating behavior**

The optimized floating microspheres (EC5) showed good in-vitro floating ability and enhanced drug release therefore was chosen for studying *in-vivo* floating efficiency by radiological method. The radiographs obtained at 0, 2 and 8 h are shown in Fig 4-6, which indicates the uniform distribution of formulation over the stomach fluid and buoyancy for more than 8 h.

The Cardioprotective activity of ivabradine loaded floating microspheres

As all the values of AST, LDH and CPK are raised in Group II (Negative control) as compared to group I (Control Group) in dose dependence manner, Hence it can be confirmed that the Ischemia and Necrosis is happening with the administration of Isoproterenol (85mg/kg subcutaneously). Therefore, all the groups can be correlated with Group II.

Histopathology

Group-I Histopathological examination of the myocardium of normal animals showed the clear integrity of the myocardial cell membrane. Endocardium and pericardium Fig (7) were seen within normal limits. No inflammatory cell infiltration was seen.

Group-II Group treated with 85mg/kg Isoproterenol showed marked Myocytic necrosis with moderate infiltration of lymphocytes and macrophages. The changes were pronounced against most of the Endocardium and in papillary muscles Fig (8). Considerable inflammatory cell infiltration was also observed.

Group-III Group treated with a standard marketed preparation of Ivabradine Hydrochloride exhibited decreased degree of necrosis (very mild) and negligible infiltration of inflammatory cells. No areas of focal Myonecrosis were observed Fig (9).

Group-IV Group treated with test drug sphere exhibited decreased degree of necrosis (mild-moderate) and less infiltration of inflammatory cells. Occasional areas of focal Myonecrosis were observed and comparable to Group III Fig (10).

Assessments

Isoproterenol, a potent synthetic catecholamine when administered to animals in doses reported above, produces ''infarct-like'' lesions in the heart, which are similar to those found in acute myocardial infarction (AMI) and sudden death in man. In the present experimental study, it has started the dose dependent development of myocardial necrosis which was confirmed biochemically and histologically.

Table 4: Confirmation of Ischemia and Necrosis in Group II in comparison with Group I.

Treatment	AST (IU/l)	LDH (IU/l)	CPK (IU/l)
Group I-Normal Values	159.5±0.21	365.2±1.51	175.5±1.78
Group II-Isoproterenol (85 mg/kg)	159.5±0.21	689.56±0.98	512.67±1.08

Values are mean ±SEM of six animals

Table 5: Weight of Heart of Animals belongs to all groups.

Sr. No	Groups details	Weight of Heart
01	Group I	0.855±2.58
02	Group II	0.917±2.81
03	Group III	0.865±3.02
04	Group IV	0.891±1.98

Values are mean ±SEM of six animals

CONCLUSION

The formulation of Ivabradine loaded EC microspheres was successfully formulated. Different investigations on formulation, characterization, in-vitro release study and *in-vivo* evaluations were carried out and performance of the formulation was evaluated. The proposed optimized formulation depicts an effective way to prolong drug release. The *in-vivo* floating efficiency of optimized formulation was outstanding and microspheres were retained in rat stomach for longer period of time. The histopathological results obtained after administration of microspheres to healthy rats were satisfactory. The developed microspheres are safe and are the need of pharmaceutical industry as an alternate for effective management of Angina Pectoris.

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CONFLICT OF INTEREST Nil

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