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ORIGINAL PAPER

Fabrication and evaluation of pH-dependent polymeric microspheres of ivabradine and their in vitro and in vivo studies

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Abstract

The aim of this work was the development and evaluation of controlled release formulations consisting of methacrylate derivatives and ethyl cellulose synthesized using oil-in-oil solvent evaporation method. Drug release studies were performed in different dissolution media. Maximum drug released was observed at pH 7.4. Fourier transform infrared spectroscopy spectra, SEM and thermal analysis showed compatibility between drug and polymers. Pharmacokinetic parameters were calculated by Phoenix WinNonlin® version 6.3 software. The average AUC_{0-t} was found to be 2483.71 ± 13.173 ng/ml h, 5954.37 ± 12.110 ng/ml h, 6400.82 ± 19.131 ng/ml h and 7427.4 ± 49.322 ng/ml h for group 1–4, respectively. The maximum concentration (C_{max}) of IBH for all groups predicted from pharmacokinetics data was 880.38 ng/ml, 718.43 ng/ml, 721.87 ng/ml and 805.11 ng/ml, respectively. Thus, in vitro and in vivo drug release studies of polymeric microspheres proved their controlled release behavior with preferential delivery for an extended period of time.

Keywords Microspheres · FTIR · Pharmacokinetic models · HPLC · Ivabradine

Introduction

Oral conventional drug administration usually does not deliver rate-controlled release or target specificity. In many cases, conventional drug delivery provides sharp increases of drug concentration at potentially toxic levels. Today new methods of drug delivery are possible: desired drug release can be provided by rate-controlling

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membranes or by implanted biodegradable polymers containing dispersed medication. Over the last 30 years most of research has been focused on biodegradable polymeric microspheres for drug delivery. Administration of medications through such systems has advantageous because microspheres can be injected or ingested; they can be tailored for desired release [1]. Microspheres is a quickly developing technology for achieving controlled release formulations. It is a well-known method that is used to modify and delay drug release from pharmaceutical dosage form. A large number of techniques are available for the formation of sustained and controlled release drug delivery systems [2]. Controlled release polymeric-based system has become the most widespread topics in pharmaceutical technology [3]. Among numerous routes of delivery oral route is the most preferred route to the patient and the clinician alike. However, it presents some problems for a large number of drugs; the enzymes in the gastrointestinal fluid (GIT); pH conditions of GIT; and the enzymes linked to membranes of GIT are the main factors accountable for the bioavailability problems. The blood that drains the GIT transfers the drug directly to the liver leading to first-pass metabolism resulting in poor bioavailability [4–6]. These problems can be solved either by changing routes of administration or by modifying the formulation. Controlled drug delivery is an alternative method of drug administration orally in form of polymeric drug-loaded microspheres.

Ivabradine-HCl (IBH) is 3-[3-[[(7S)-3, 4-dimethoxy-7-bicyclo [4.2.0] octa-1, 3, 5-trienyl] methyl-ethylamino] propyl-7,8-dimethoxy-2,5-dihydro-1H-3-benzazepin-4-onehydrochloride, shown in Fig. 1. Heart rate reduction (HRR) is a significant target for management of patients with stable angina. The drugs available for the management of HRR include calcium channels blockers (CCB) and beta blockers (BB) [7–9]. For the symptomatic management of stable angina pectoris, IBH a novel medication is used [8, 10]. IBH has different modes of action from CCB and BB. IBH is a cardiotonic agent, and it produced anti-anginal effect by reducing the heart rate via specific inhibition of the pacemaker current [11]. The plasma half-life of IBH is about 2 h with 40% bioavailability [12].

The present research focuses on developing polymeric microspheres for enhancing and improving the drug release in a controlled fashion and follows a logical approach in terms of pharmaceutical design using novel drug. Optimized formulations were used by using different methacrylate derivatives and EC. The purpose was to evaluate controlled drug delivery of various polymeric formulations prepared by solvent evaporation technique. Methacrylate derivatives Eudragit® L100-55-EC, Eudragit® FS30D-EC and Kollicoat® MAE 100P-EC were formulated and evaluated

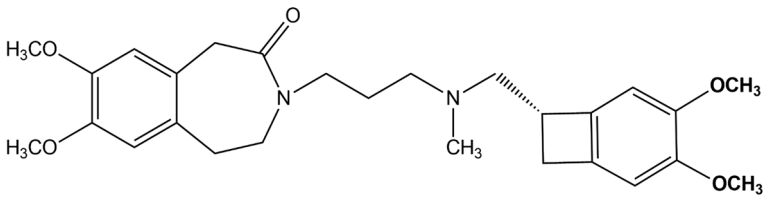

Fig. 1 Structure of ivabradine

for in vitro and in vivo evaluation. In vitro dissolution studies were carried out at pH 1.2, 5.5 and 7.4 at 37 °C to determine the pH-dependent behavior of drug release. FTIR, scanning electron microscopy (SEM), X-ray diffractometry (XRD) and differential scanning calorimetry (DSC) were performed to study to evaluate the polymeric microspheres. On the basis of in vitro results pharmacokinetic analysis of IBH was performed in healthy albino rabbits after oral administration of drug solution and microspheres formulations containing IBH.

Experimental

Materials and methods

Materials

IBH was received as a gift sample from CCL Pharmaceuticals, Lahore. Ethyl cellulose (viscosity 300cP, 5% in toluene/ethanol 80:20, 48% ethoxyl), Span 80, Eudragit® L100-55, Eudragit® FS30D, Kollicoat® MAE100P, light liquid paraffin, *n*-hexane were purchased from Sigma-Aldrich (Evonik Krefeld, Germany). Ethanol, dichloromethane (DCM) and acetone were purchased from BDH, Pakistan. Sodium hydroxide (NaOH) was purchased from Merck, Darmstadt, Germany. Potassium dihydrogen phosphate (KH_2PO_4) was purchased from Merck, Darmstadt, Germany, and Hydrochloric acid was purchased from BDH, Pakistan. Potassium bromide (KBr) of FTIR grade was purchased from Fischer Scientific, Leicestershire, UK. All chemicals and solvents used were of analytical grades.

Development of polymeric microspheres

IBH-loaded Eudragit® L100-55 and EC microspheres were developed by O/O solvent evaporation method. Eudragit® L100-55, Eudragit® FS30D and Kollicoat® MAE100P were dissolved separately in ethanol using magnetic stirrer. EC was separately dissolved in DCM at 25 °C stirred at 300 rpm with a magnetic stirrer. Stirring was continued until a clear solution was obtained. Eudragit® L100-55 and EC are both polymers solution mixed to get a homogeneous solution. Similarly, Eudragit® FS30D-EC and Kollicoat® MAE100P-EC were mixed separately to prepare homogenous solution. Drug was dissolved separately in ethanol at 300 rpm with the help of magnetic stirrer at 25 °C. When clear solution of drug was achieved, it was added dropwise in the polymers solution at 300 rpm. Then, polymers and drug solution were stirred to obtain a homogenous solution. External phase was prepared by dissolving 1% span 80 in liquid paraffin at a stirring speed of 500 rpm. Homogenous solution of drug and polymers was added dropwise by using syringe into liquid paraffin comprising 1% span 80 at 40 °C, whereas stirring was continued at 800 rpm. These mixtures were stirred for 3 h till organic solvents were fully evaporated. After complete evaporation of DCM and ethanol, microspheres were filtered on a Whatman filter paper and collected. Then, developed polymeric microspheres were washed three times with *n*-hexane in order to remove excess of solvents adhering to the surface of microspheres. The washed

microspheres were dried in oven at 45 °C for 24 h. The formulation plan of microspheres is shown in Table 1.

Characterization

Determination of percentage yield, drug loading and entrapment efficiency

Dried microspheres were weighed, and percentage yield w/w was measured by using formula as shown in Eq. 1 [13]. Drug loading was determined by dissolving 50 mg of microspheres in 100 ml of phosphate buffer pH 7.4 for 12 h at 37 °C. After filtration using 0.45-µm syringe filter an analysis of solution was carried out at 287 nm using UV–Vis spectrophotometer (PerkinElmer New York, USA). The absorbance of pure drug (100 mg) was also determined. Drug loading was determined by the following formula as shown in Eq. 2 [14].

$$\text{Percentage yield (\%)} = \frac{\text{weight of microspheres (mg)}}{\text{total weight of drug and polymer (mg)}} \times 100 \quad (1)$$

Table 1 Formulations prepared by varying the ratios of polymers

Formulation code	Polymers used	Polymer ratios	DCM:ethanol	Drug:polymer ratio
F1	EC:Eudragit L100-55	00:10	1:1	1:5
F2	EC:Eudragit L100-55	10:90	1:1	1:5
F3	EC:Eudragit L100-55	20:80	1:1	1:5
F4	EC:Eudragit L100-55	25:75	1:1	1:5
F5	EC:Eudragit L100-55	30:70	1:1	1:5
F6	EC:Eudragit L100-55	40:60	1:1	1:5
F7	EC:Eudragit L100-55	50:50	1:1	1:5
F8	EC:Eudragit FS30D	00:10	1:1	1:5
F9	EC:Eudragit FS30D	10:90	1:1	1:5
F10	EC:Eudragit FS30D	20:80	1:1	1:5
F11	EC:Eudragit FS30D	25:75	1:1	1:5
F12	EC:Eudragit FS30D	30:70	1:1	1:5
F13	EC:Eudragit FS30D	40:60	1:1	1:5
F14	EC:Eudragit FS30D	50:50	1:1	1:5
F15	EC:Kollicoat MAE100P	00:10	1:1	1:5
F16	EC:Kollicoat MAE100P	10:90	1:1	1:5
F17	EC:Kollicoat MAE100P	20:80	1:1	1:5
F18	EC:Kollicoat MAE100P	25:75	1:1	1:5
F19	EC:Kollicoat MAE100P	30:70	1:1	1:5
F20	EC:Kollicoat MAE100P	40:60	1:1	1:5
F21	EC:Kollicoat MAE100P	50:50	1:1	1:5

$$\%\ \text{Drug loading} = \frac{\text{weight of drug in microspeheres}}{\text{weight of microspheres}} \times 100 \tag{2}$$

Entrapment efficiency was calculated using the following formula.

$$\%\ \text{Entrapment efficiency} = \frac{\text{absorbance of microparticles equivalent to 100 mg IBH}}{\text{absorbance of 100 mg IBH}} \times 100 \tag{3}$$

Micromeritic properties of microspheres

The microspheres were characterized by their micromeritic properties such as angle of repose, bulk density, tapped density, compressibility index and Hausner's ratio. These were measured by using the following equations [15].

$$\text{Angle of repose } \tan\theta = 2h/D \tag{4}$$

Bulk density of prepared formulation was measured by using the following formula.

$$\text{Bulk density} = \frac{\text{weight of microspheres}}{\text{volume of microspheres}} \tag{5}$$

Tapped density was determined by the following formula

$$\text{Tapped density} = \frac{\text{mass of microspheres}}{\text{Volume of microspheres after 100 tappings}} \tag{6}$$

Compressibility index is also called as car index (C_i) and was calculated by this formula

$$C_\text{i} = \frac{\text{Initial volume} - \text{Final volume}}{\text{Initial volume}} \times 100 \tag{7}$$

Value of $C_\text{i} < 15\%$ designates good flow properties, while values of $C_\text{i} > 25\%$ characterize poor flow.

Hausner's ratio was measured by the following equation.

$$\text{Hausner's ratio} = \frac{\text{Volume before tapping}}{\text{Volume after 100 tapping}} \tag{8}$$

A value of 1.2 represents free flow, and a ratio near to 1 indicates relatively good flow.

Particle size and morphology of microspheres

The mean particle size of microspheres was measured by optical microscope using pre-calibrated ocular micrometer and stage micrometer. About 100 particles of each

formulation were observed [14]. SEM images were studied by scanning electron microscopy model (SEM, S-3400N, Hitachi, Japan) to determine the surface and morphology of loaded and unloaded microspheres at different magnifications.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were taken to investigate any possible interaction between drug and polymers. The pure drug, polymers and loaded microspheres were subjected to FTIR studies using Shimadzu FTIR spectrophotometer, and sample was scanned at wavelength 4000 and 500 cm^{-1} [16].

Differential scanning calorimetry (DSC)

The nature of drug present in formulations was assessed by performing DSC of pure drug, EC, Eudragit® L100-55, Eudragit® FS30D, Kollicoat® MAE100P and IBH-loaded microspheres carried out simultaneously with STD Q600 DSC/TGA Analyzer, USA. An amount of 4–5 mg of crushed microspheres was placed in aluminum pans and sealed before to carry out test. Each sample was analyzed under stream of nitrogen gas of 100 ml/min and heated from 40 to 350 °C at the rate of 10 °C/min [17].

X-ray diffractometry (XRD)

Crystallinity of IBH, polymers and drug-loaded microspheres was evaluated by using X-ray diffractometer (Bruker D8 Discover, Germany) using Ni-filtered CuK alpha radiation source. The tube voltage of 40 kV, tube current 35 mA and scanning rate 5°/min are over a range of 8°–80° of diffraction angle range [18].

In vitro drug release study

The in vitro drug release study of IBH-loaded microspheres was carried out using USP dissolution paddle apparatus (Pharma test, Germany) at speed of 100 rpm. Temperature was set at 37 ± 0.5 °C. Accurately weighed microspheres (50 mg) were taken in cellulose dialysis membrane and tied it to paddles. Phosphate buffer solution of pH 1.2, 5.5 and 7.4 was used as dissolution medium (500 ml). The samples were collected after fixed intervals of time, i.e., 0.5, 1, 2, 3, 4, 6, 8, 10 and 12 h from dissolution medium. Samples (5 ml) were analyzed via measuring the absorption of IBH at 287 nm by using UV–Vis spectrophotometer (PerkinElmer). Since ivabradine is in its salt form (IBH), there is no need of maintaining sink condition. Measurement of each sample was carried out in triplicate [19]. Drug concentrations were measured by standard calibration curve.

Drug release kinetics

The in vitro drug release data obtained are evaluated by different kinetics models including zero order ($F_t = K_o t$) where F_t is drug release fraction in time t and K_o is rate constant (for zero-order release), first order ($I_n\ (1-F) = -K_1 t$ where F is fraction of drug release in time t and K_1 is release constant (for first order), Higuchi ($F = K_2 t^{1/2}$) where K_2 is Higuchi's constant and F is fraction of drug release at time t and Korsmeyer–Peppas model ($M_t/M = K_3 t^n$) where K_3 is Peppas constant, M_t is quantity of drug released in time t, M is infinity amount of drug release at time infinity, and n is diffusion constant. In microspheres, if n is less than 0.43, it represents Fickian (Case-I) and if value lies among 0.43–0.85, then it is non-Fickian (Case-II) zero-order drug release mechanism [20].

In vivo studies

The pH-dependent polymeric microspheres were successfully developed and characterized for the prevention of plasma drug fluctuation and to control the drug release pattern. For this purpose, methacrylate derivatives and EC-based IBH-loaded carries were synthesized. The major objective was to establish controlled release microsphere having ability to deliver drug at a predetermined rate for an extended period of time. The concentrations were determined using a validated HPLC method. Duffull et al. [21] develop a pharmacokinetic simulation model in healthy male volunteers. IBH pharmacokinetic studies were also reported in animals [22, 23].

Study design

Albino rabbits of weight about 2.0–2.5 kg were obtained from animal house of Pharmacology Laboratory, Faculty of Pharmacy, Bahauddin Zakariya University, Multan, Punjab (Pakistan). The research protocols were studied and approved by the departmental animal ethics committee for use of experimental laboratory animals. All rabbits were housed in well-maintained separate chamber with proper temperature conditions (25 ± 1 °C). These animals are supplied with free access to food and water as a standard diet. Over-night fasted rabbits were used for the experimental studies. Experimental animals were then divided into four groups (1, 2, 3 and 4), 12 rabbits in each group. Animals were fasted at least for 12 h before starting the experiment. Before drug administration animals were allowed a free access to water. Rabbits were appropriately labeled and placed in wooden cages during the process of sampling. At the first stage, drug solution (IBH, 1 mg/kg) was administered to group 1 via feeding tube followed by 10–20 ml of water. This group was tagged as control group. In phase second, group 2 administered formulation F7 (microspheres having IBH equivalent to 1 mg/kg), F14 (microspheres having IBH comparable to 1 mg/kg) was given to group 3, and F21 (microspheres having IBH same to 1 mg/kg) was given to group 4.

Blood sampling protocol

0.5 ml sample were obtained from each experimental animal from jugular vein. The sampling was done at fixed time interval of 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h after oral administration. Blood samples were collected in citrated tubes. These sampling tubes were centrifuged at 5000 revolutions per minutes (rpm) for 10 min. After plasma isolation all samples are stored at − 20 °C till further study. Separation was attained by using a mobile phase (acetonitrile: buffer (pH 6.0), 40:60 V/V), with a flow rate of 1 ml/min at room temperature (22 + 2 °C). The mobile phase was filtered through a 0.45 µm and degassed by sonication before running in HPLC.

Determination of IBH concentration in plasma

Calibration curve was used for the determination of concentration of IBH from pure drug solution of known concentration in plasma. For plasma sample preparation 1 ml of plasma sample was spiked with 50 µl of internal standard (IS) working solution at 2 µg/ml. The plasma mixture was extracted with 4 ml of acetonitrile and mixed by vortex mixer (Seoulin Bioscience, Korea), for 1 min, and then centrifuged at 5000 rpm for 5 min. The organic layer was removed using a micropipette and evaporated under a stream of nitrogen gas in the thermostatically controlled water bath maintained at 40 °C until completely dry. The residue was dissolved in 100 µl mobile phase, vortex mixed for 3 min and centrifuged at 6000 rpm for 20 min. Twenty microliters of this supernatant was injected into column for analysis [24]. Analysis was performed using high-performance liquid chromatography (PerkinElmer, New York, USA) fitted out with column HSC-$_{18}$ (25 cm × 4.6 mm, 5 µm) Supelco (Sigma-Aldrich). A sample of 20 µl was injected with a runtime of 10 min.

Pharmacokinetic parameters

Pharmacokinetic parameters were calculated by Phoenix WinNonlin® version 6.3 software; the linear trapezoidal method was used to calculate AUC from time versus plasma conc. Clearance was calculated by dividing given dose by AUC_{last}. Noncompartmental analysis (NCA) was used to determine each pharmacokinetic profile. Pharmacokinetic parameters such as time of maximum drug conc. in plasma (T_{max}), maximum plasma drug conc. (C_{max}), area under plasma conc. curve (AUC), half-life ($t_{1/2}$), rate of elimination (K_e), area under first moment curve (AUMC), mean residence time (MRT), distribution volume (V_d) and clearance (CL) were determined.

Statistical analysis

Statistical analysis was conducted using analysis of variance (ANOVA) for the purpose of calculating statistical significant as well as nonsignificant analysis at

95% CI, with P value > 0.05 considered to be a significant difference in results. Significance level was fixed at 5%. The value of $P < 0.05$ was considered to be significant, and $P < 0.001$ was measured extremely significant.

Results and discussion

Preparation and percentage yield of microspheres

In the present study, IBH-loaded microspheres were prepared by using oil-in-oil (O/O) solvent evaporation method. This method was selected because drug and polymers were soluble in DCM and ethanol. Microspheres were successfully synthesized and evaluated. The maximum percentage entrapment efficiency and percentage yield for different formulations with various compositions of polymers are shown in Table 2.

Table 2 Size, % entrapment efficiency and % percentage yield of polymeric microspheres

Formulation code	Size (μm)	% Entrapment efficiency	% Yield
F1	10 ± 1.112	63 ± 1.32	67 ± 2.23
F2	12 ± 2.115	69 ± 1.55	69 ± 1.15
F3	20 ± 2.320	71 ± 2.54	71 ± 2.11
F4	22 ± 1.001	73 ± 2.11	70 ± 1.33
F5	27 ± 5.012	77 ± 1.71	75 ± 2.56
F6	34 ± 3.002	79 ± 1.83	82 ± 3.18
F7	39 ± 4.132	81 ± 2.15	88 ± 2.65
F8	20 ± 1.11	28.37 ± 0.005	68.33 ± 2
F9	20 ± 2.221	30.59 ± 0.001	69.16 ± 3
F10	30 ± 2.001	35.85 ± 0.01	68.66 ± 1.5
F11	30 ± 1.234	27.11 ± 0.01	68.16 ± 1.5
F12	30 ± 1.110	30.32 ± 0.001	69.66 ± 2
F13	30 ± 1.234	45.18 ± 0.01	71.66 ± 3
F14	30 ± 1.100	44.06 ± 0.01	70.88 ± 4
F15	55.12 ± 1.2	57.12 ± 1.32	65.15 ± 2.23
F16	61.54 ± 2.3	63.23 ± 1.55	69.34 ± 1.15
F17	66.43 ± 3.2	74.21 ± 2.54	73.23 ± 2.11
F18	73.45 ± 1.7	73.22 ± 2.11	79.11 ± 1.33
F19	76.13 ± 5.4	81.31 ± 1.71	85.34 ± 2.56
F20	80.11 ± 3.7	84.12 ± 1.83	83.22 ± 3.18
F21	82.12 ± 4.3	87.22 ± 2.15	86.22 ± 2.65

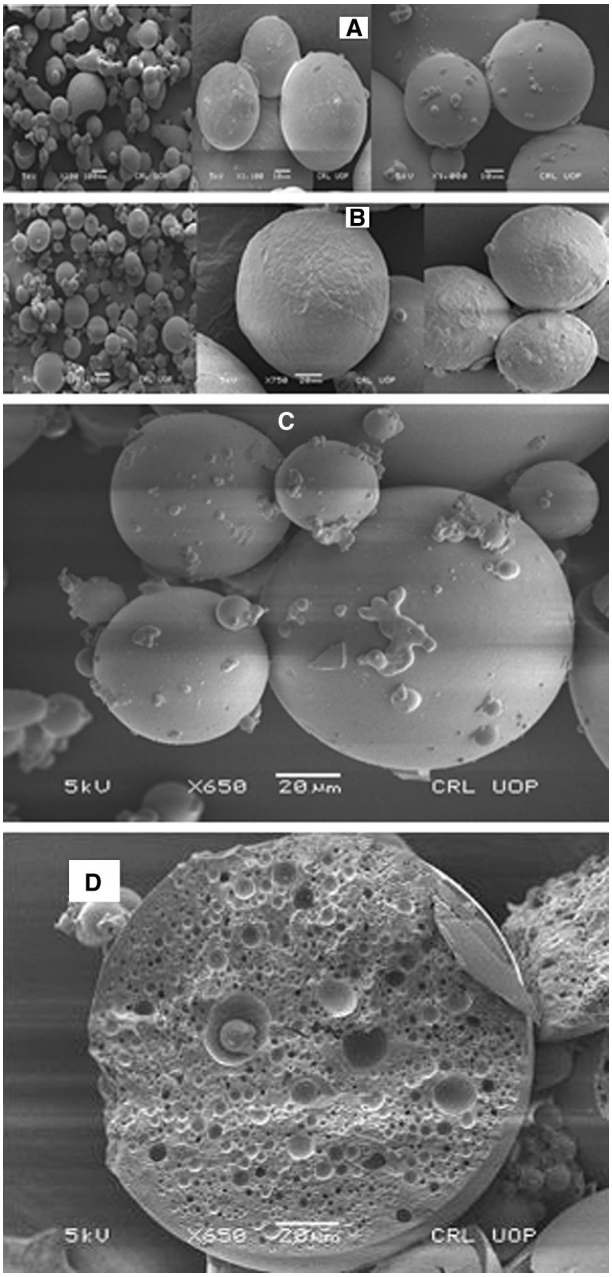

Fig. 2 SEM images of IBH-loaded microspheres of formulations **a** F7, **b** F14 and **c** F21, **d** cross section of microspheres

Particle size and morphology of microspheres

SEM was used to determine the shape and surface morphology of microspheres. SEM images of unloaded microspheres were in spherical form mostly having smooth surfaces, while IBH-loaded microparticles showed comparatively rough surface as shown in Fig. 2. The desired spherical microspheres were obtained at stirring speed of 800 rpm. Internal morphology confirmed the presence of cavity which exhibited matrix responsible for controlled release of drug. Microspheres prepared with Kollicoat® MAE100P were spherical with smooth surfaces while in combination with EC irregular with smooth surface confirmed by SEM. The mean particle size of various formulations of microspheres was 60–80 µm and is given in Table 2. Particle size of microspheres increases gradually as the amount of EC increases, and coating layer around IBH became more and more thick. It was observed in most cases the larger the mean particle size, longer the diffusion path for drug releases, and consequently led to slow release of IBH.

Table 3 Results of micromeritic properties of microspheres

Formulation code	Angle of repose (°)	Hausner's ratio	Compressibility index (%)	Tapped density (g/ml)	Bulk density (g/ml)
F1	18.35 ± 1.35	1.11 ± 0.09	7 ± 1.32	0.41 ± 0.01	0.26 ± 0.01
F2	20.42 ± 1.82	1.15 ± 0.21	10 ± 2.11	0.42 ± 2.01	0.25 ± 0.01
F3	23.70 ± 3.25	1.17 ± 0.04	11 ± 1.52	0.45 ± 0.01	0.29 ± 0.02
F4	24.55 ± 2.45	1.19 ± 0.49	12 ± 2.32	0.47 ± 0.11	0.32 ± 0.01
F5	26.27 ± 1.33	1.20 ± 0.18	14 ± 8.28	0.49 ± 0.02	0.41 ± 0.01
F6	27.66 ± 4.21	1.24 ± 0.40	16 ± 4.62	0.53 ± 0.01	0.47 ± 0.02
F7	28.27 ± 1.33	1.21 ± 0.03	21 ± 1.42	0.57 ± 0.01	0.51 ± 0.01
F8	12.04 ± 1.3	1.15 ± 1.11	13.63 ± 1.01	0.20 ± 0.1	0.18 ± 0.01
F9	11.58 ± 1.2	1.15 ± 1.001	16 ± 1.53	0.19 ± 0.01	0.16 ± 0.001
F10	12.46 ± 1.3	1.15 ± 1.51	13.33 ± 1.22	0.29 ± 0.01	0.22 ± 0.01
F11	11.23 ± 1.3	1.21 ± 1.52	17.54 ± 2.35	0.26 ± 0.11	0.21 ± 0.01
F12	15.6 ± 1.5	1.13 ± 1.21	11.76 ± 1.12	0.27 ± 0.01	0.21 ± 0.01
F13	11.91 ± 1.4	1.14 ± 1.31	12.5 ± 2.18	0.31 ± 0.01	0.27 ± 0.01
F14	12.33 ± 1.19	1.21 ± 1.40	17.94 ± 3.17	0.25 ± 0.01	0.20 ± 0.001
F15	17.35 ± 1.25	1.11 ± 0.09	10.17 ± 1.22	0.41 ± 0.01	0.23 ± 0.01
F16	21.42 ± 1.72	1.13 ± 0.21	11.13 ± 2.11	0.42 ± 2.01	0.22 ± 0.01
F17	22.70 ± 3.25	1.15 ± 0.04	13.36 ± 1.42	0.45 ± 0.01	0.28 ± 0.02
F18	24.55 ± 2.45	1.17 ± 0.49	15.12 ± 2.52	0.47 ± 0.11	0.31 ± 0.01
F19	26.27 ± 1.33	1.21 ± 0.18	16.22 ± 8.58	0.49 ± 0.02	0.47 ± 0.01
F20	27.66 ± 4.21	1.22 ± 0.40	17.26 ± 4.62	0.53 ± 0.01	0.49 ± 0.02
F21	28.27 ± 1.33	1.23 ± 0.03	22.45 ± 1.42	0.57 ± 0.01	0.53 ± 0.01

Many studies have shown that the smaller the size of particle drug release will be more rapid due to increase in particle surface area. So release profile of drug from microspheres is predictable as being dependent on particle size [25].

Micromeritic properties microspheres

Micromeritics included bulk density, tapped density, Carr's index (C_i), Hausner's ratio (H_r) and angle of repose of all formulations were studied as shown in Table 3. C_i values lie within the range 11–21 which indicate an excellent flow of microspheres [26]. H_r values of all formulations were lower than 1.25 demonstrating good flow properties [27]. Values of angle of repose of all formulations were below 30^0 also representing free flow properties of microspheres that indicated that these can be handled easily.

FTIR

Figure 3 depicts the FTIR spectra of IBH, EC, EL100-55, Eudragit® FS30D, Kollicoat® MAE100P and IBH-loaded microspheres. IR spectra of IBH presented characteristics peaks such as C=C stretching bands at 1103 cm^{-1}, C–C stretching bands at 1456 cm^{-1} and aliphatic C–N present stretching bands at 2451 cm^{-1} [28]. EC showed peaks at 2833 cm due to stretching vibration of OH group at carbon numbers 2, 3 and 6 [29]. The spectra of EL100-55 displayed several characteristic bands at 1701 cm^{-1} (CO carboxylic acid groups vibrations), 1736 cm^{-1} (esterified carboxyl groups vibrations), 1157 cm^{-1}, 1184 cm^{-1} and 1261 cm^{-1} (ester vibrations), 1387 cm^{-1}, 1479 cm^{-1} and 2979 cm^{-1} (CHX vibrations) and 3234 cm^{-1} (OH groups vibrations) [30]. Eudragit® FS30D showed band at 1732 cm^{-1} and 1603 cm^{-1} due to the MAA carboxylic acids C=O vibration [31]. The FTIR spectrum of Kollicoat® MAE100P showed a characteristic broad band at 3421 cm^{-1}, which is assigned for OH stretching and a stretching vibration band of C=O at 1637 cm^{-1} as well as a C-H stretching vibration peak at 2915 cm^{-1} [32]. The peaks at 1723 and 1698 cm^{-1} represented the carbonyl group in Kollicoat® MAE100P [33]. The FTIR spectra of IBH-loaded microspheres indicated compatibility between IBH, EC and methacrylate derivatives. Therefore, the drug was chemically steady in microspheres.

DSC

DSC was conducted to explore the melting characteristics of drug and polymers. DSC showed endothermic peak near 193°C which is the indication of melting point of IBH, while drug-loaded microspheres showed no such peaks shown in Fig. 4. Similarly, endothermic peaks of EC were observed at glass transition temperature 132 °C and EL100-55 showed peaks at 97 °C. It suggested that the drug particles were uniformly distributed in polymer matrix. Figure 4 presents DSC pattern of IBH, EC, EL100-55, Eudragit® FS30D, Kollicoat® MAE100P and IBH-loaded microspheres.

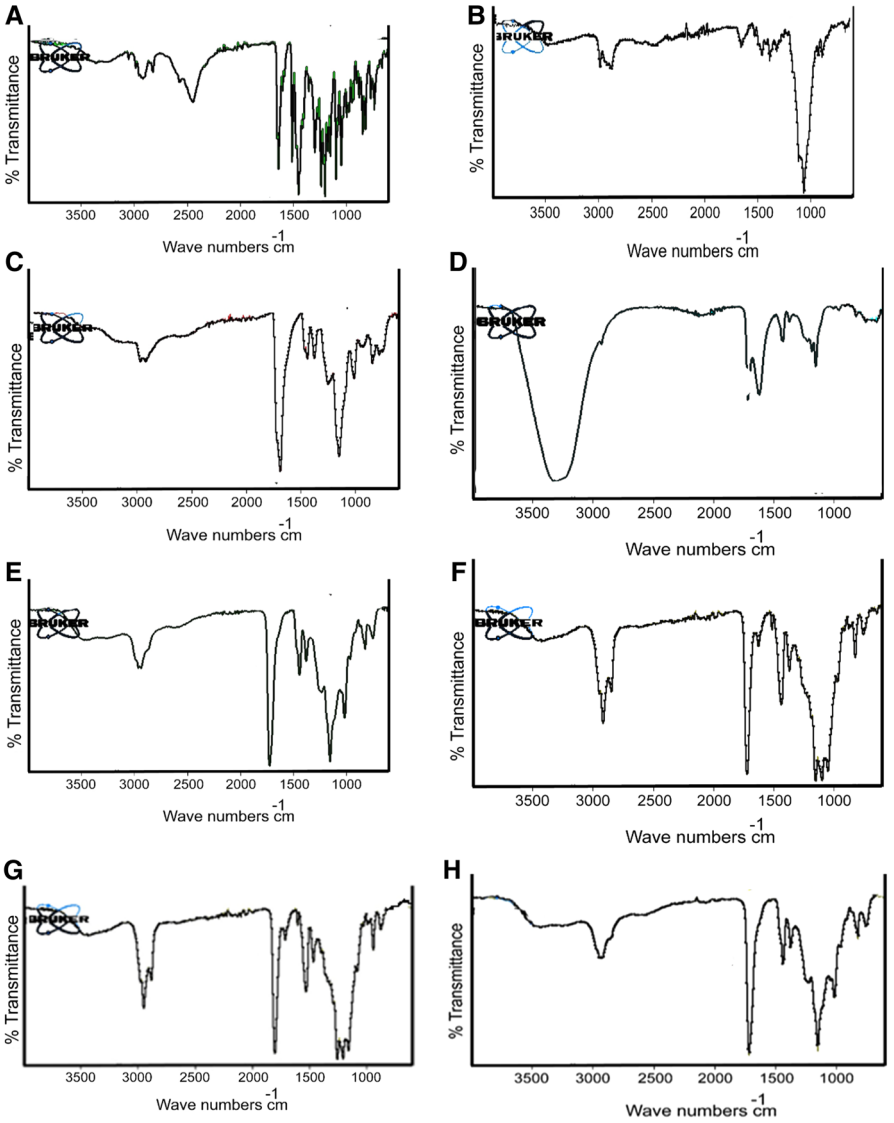

Fig. 3 FTIR spectra of **a** IBH, **b** EC, **c** Eudragit L100-55, **d** Eudragit FS30D, **e** Kollicoat MAE100P, **f** F7-loaded microspheres, **g** F14-loaded microspheres and **h** F21-loaded microspheres

XRD

XRD analysis was used to investigate the crystallinity of drug in prepared microspheres. Ivabradine was shown the characteristics intense peaks at 2θ of 11°, 15°, 20° and 25° due to its crystalline nature. The size of the crystals was not

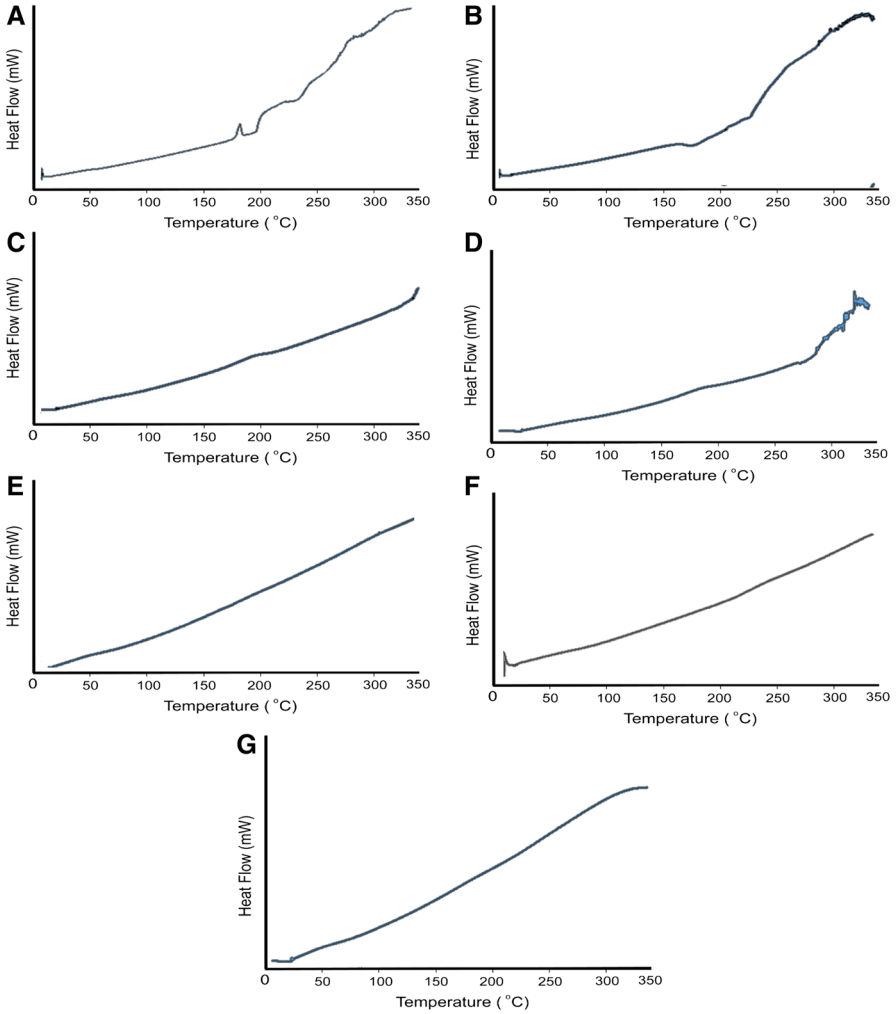

Fig. 4 DSC pattern of **a** IBH, **b** EC, **c** Eudragit L100-55, **d** Kollicoat MAE100P, **e** F7-loaded microspheres, **f** F14-loaded microspheres and **g** F21-loaded microspheres

measured as we have only determined the nature of the particles that whether they are amorphous or crystalline. Furthermore, in formulation form the drug was completely captured by the polymeric matrix and now drug may be in the form of solid solution loss of crystallinity as XRD graphs have shown no identifiable or sharp peaks describing the conversion of drug into amorphous form. XRD patterns of pure drug (a) IBH, (b) EC, (c) EL 100-55, (d) Eudragit® FS30D, (e) Kollicoat® MAE100P, (f) IBH-loaded microspheres F7, (g) IBH-loaded F14 formulation, (h) IBH-loaded F21 formulation shown in Fig. 5 clearly designate that drug particles were distributed at molecular level in the polymeric matrix [34].

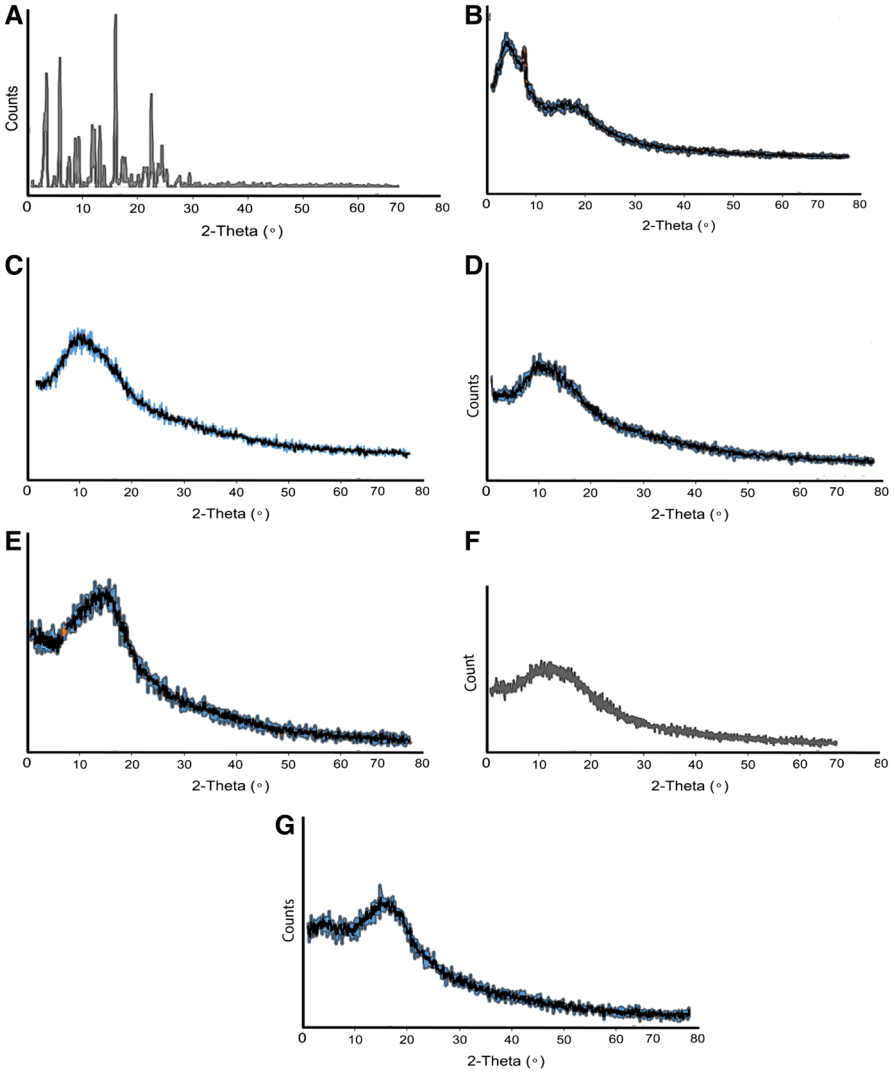

Fig. 5 XRD pattern of **a** IBH, **b** EC, **c** Eudragit L100-55, d Kollicoat MAE100P, **e** F7-loaded microspheres, **f** F14-loaded microspheres and **g** F21-loaded microspheres

In vitro studies

The in vitro drug release of microspheres depends on polymer network characteristics including the chemical structure, network structure and release conditions of polymers. IBH was selected as model drug to study the release kinetics in prepared microspheres having a varying amount of both polymers. % Cumulative drug release from all formulations pH 7.4 is shown in Fig. 6a–c. Figure shows the cumulative release % at buffer solution of pH 7.4 indicating that drug release was higher than

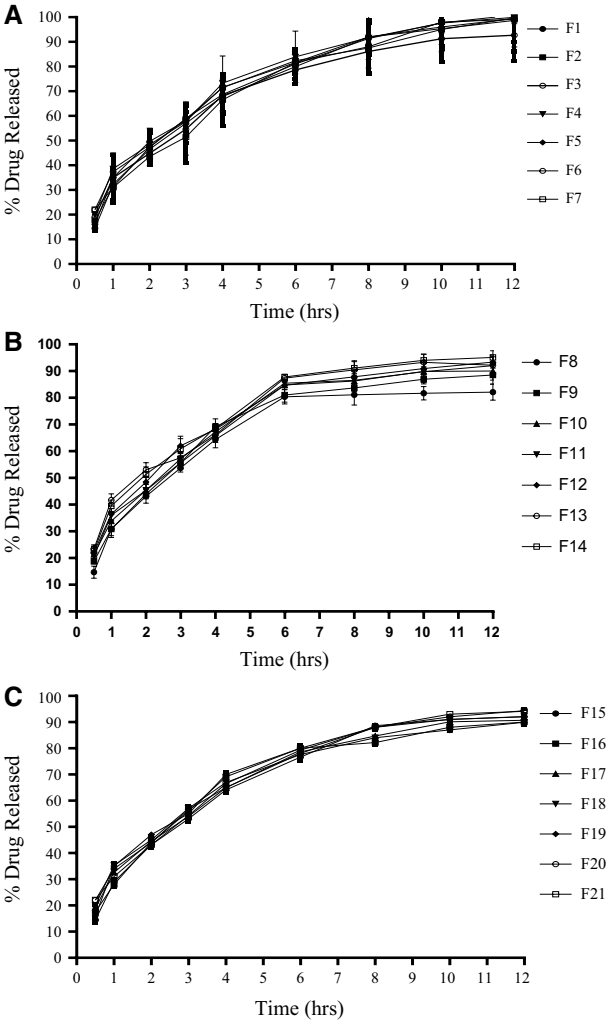

Fig. 6 In vitro drug release of **a** F1-7 formulations, **b** F8-14 formulations and **c** F15-21 formulations at pH 7.4

other pH 5.5 and 1.2. The in vitro drug release study indicated that the combination of polymers and their changed ratio changed the release rate of drug from microspheres [35]. Drug release of formulations was very slow at pH 1.2 because Eudragit derivatives are insoluble in an acid environment, and it should prevent dissolution of IBH as shown in Fig. 7a–c. At pH 5.5 FI showed sustained release rate because Eudragit® EL 100-55, Eudragit® FS30D and Kollicoat® MAE100P are soluble at pH 5.5, and dissolution process takes relatively longer because it involves the process of absorption, swelling and disentanglement, before drug release. Figure 8a–c represents the drug release pattern of all polymeric microspheres. All formulations

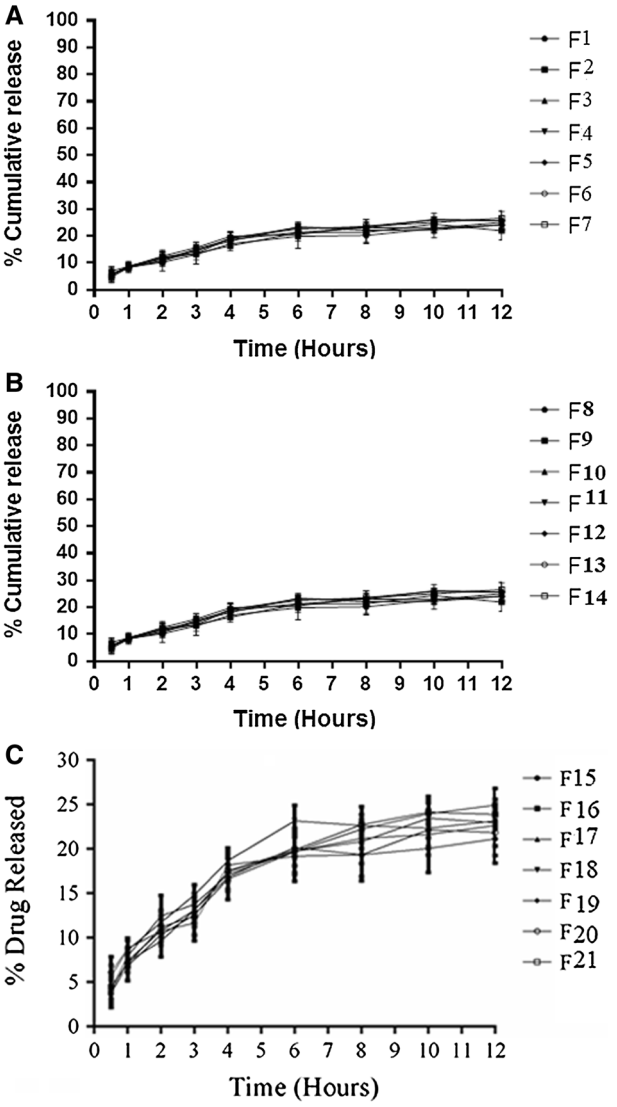

Fig. 7 In vitro drug release of **a** F1-7 formulations, **b** F8-14 formulations and **c** F15-21 formulations at pH 1.2

showed maximum sustained release at pH 7.4 containing equal amount of methacrylate derivatives and EC. Due to EC the microparticles become impermeable to water and give very slow release of drug as observed at pH 7.4. An increase in polymer solution viscosity has produced microspheres with decreased porosity due to thickening of polymer wall. It is clearly known that higher concentration of EC results in a longer diffusional path length, so drug release is extended. The thick polymeric barrier slowed the entry of surrounding dissolution medium into the

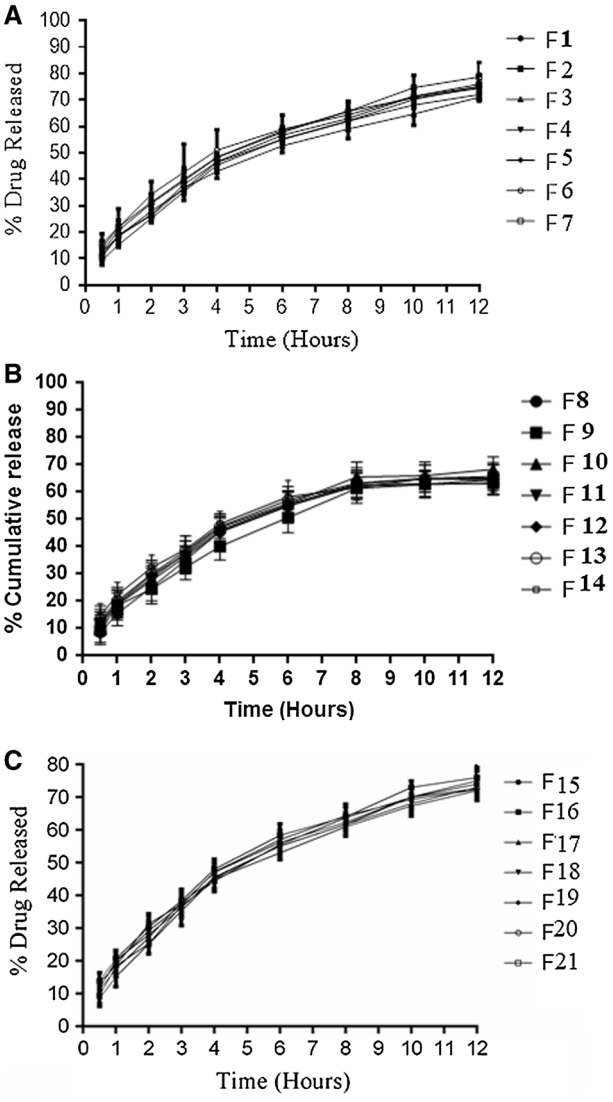

Fig. 8 In vitro drug release of **a** F1-7 formulations, **b** F8-14 formulations and **c** F15-21 formulations at pH 5.5

microspheres and hence less quantity of drug release out from the polymer matrices of the microspheres exhibiting sustained release [36]. It indicates that drug release mechanism gradually transfers from diffusion mechanism to erosion at dissolution medium of pH 7.4. Maximum drug release from all formulations showed almost more than 90% at pH 7.4. Results showed the cumulative release % at pH 7.4 indicating that drug release was higher at pH 7.4 demonstrating pH-dependent behavior. IBH was found to be soluble in pH 1.2, 5.5 and 7.4 phosphate buffer, and

no significant effect of pH on solubility was observed [37]. Similar solubility was observed in different pH-responsive systems. Dissolution studies were repeated three times ($n = 3$).

Drug release kinetics

In vitro drug release mechanism was analyzed by means of applying various kinetic models including zero-order, first-order, Higuchi and Korsmeyer–Peppas models. However, on the basis of regression coefficient (R^2), model best fitted to the release data was selected. The values of R^2 indicated that drug release follows first-order model. The result proposed that first order was best fitted to the data and followed by drug release. By applying Korsmeyer–Peppas model the value of (n) for release of drug was calculated. The value of (n) was found to be between 0.408 and 0.585 which indicates that diffusion mechanism was non-Fickian [38]. Drug release from above formulation was likely controlled by a combination of diffusion and erosion mechanisms.

In vivo studies

On the basis of preliminary investigations, these three formulations (FA7, FA14 and F21) with maximum in vitro cumulative drug release were selected for in vivo evaluation. Single pharmacokinetic study was conducted in animals for in vivo assessment. Mean ± SD conc. of IBH determined in plasma of rabbits after oral administration of drug solution (group 1) and controlled release IBH-loaded microspheres (groups 2, 3 and 4) is shown in Table 4. The mean ± SD of plasma conc. versus time profile of IBH in groups 1, 2, 3 and 4 is illustrated in Fig. 9. Drug was detected quickly in rabbit plasma after oral provision of IBH solution, while from controlled release polymeric microspheres formulations it acquired nearly 2 h to detect measureable extent of drug in rabbit plasma. Lag time of nearby 2 h showed the extended release of experimental drug from microspheres. It revealed that drug might remain intact in stomach and most possibly released at higher intestinal pH. Various pharmacokinetics parameters of all groups (1–4) were assessed and are presented in Tables 5 and 6, respectively. ANOVA test was applied for statistics evaluation of pk. parameters at 5% significance level as given in Table 6. Results indicated that all parameters showed P values were < 0.0001 representing a significant difference among all assessed factors.

The mean plasma concentration (C_{max}) of IBH for all groups (1–4) predicted from pk. data were 880.38 ng/ml, 718.43 ng/ml, 721.87 ng/ml and 805.11 ng/ml, respectively, achieved at T_{max} of 2.01, 4.01, 4.0 and 4.56 h, respectively. As compared to other groups C_{max} was significantly higher for group 1, while T_{max} of groups 2–4 was significantly higher than control group. This prolonged T_{max} revealed controlled release microspheres formulations that represent slow release of drug that resulted controlled released in vivo drug delivery. Our results showed good similarity to drug-loaded hydrogels presented with prolonged T_{max} and lower

Table 4 Plasma conc. (ng/ml) (mean ± SD) of oral drug solution and optimized formulations (F7, F14 and F21)

Oral drug solution (IBH 1 mg/kg)		F7 formulation	F14 formulation	F21 formulation
Time (h)	Plasma conc. (mean ± SD)	Plasma conc. (mean ± SD)	Plasma conc. (mean ± SD)	Plasma conc. (mean ± SD)
00	00	0 ± 00	0 ± 00	0 ± 00
0.5	56.776 ± 20.865	0 ± 00	0 ± 00	0 ± 00
1	285.735 ± 46.257	61.013 ± 16.243	57.341 ± 11.123	24.311 ± 9.112
2	880.381 ± 131.229	190.627 ± 51.617	167.953 ± 90.286	207.943 ± 70.276
3	636.092 ± 74.388	364.99 1 ± 72.170	403.235 ± 115.078	503.235 ± 105.078
4	430.293 ± 97.478	718.432 ± 71.289	721.866 ± 95.506	805.110 ± 117.011
6	131.051 ± 22.884	573.626 ± 88.719	608.478 ± 102.371	711.457 ± 118.305
8	34.0 ± 10.864	412.979 ± 106.400	498.808 ± 92.463	518.691 ± 107.557
10	–	313.733 ± 90.591	352.842 ± 87.083	457.533 ± 87.874
12	–	189.240 ± 64.784	218.613 ± 48.385	238.613 ± 48.385
24	–	58.331 ± 74.774	42.212 ± 64.784	74.112 ± 75.554

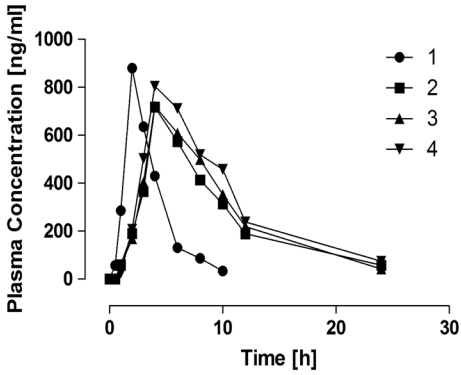

Fig. 9 Comparative plasma conc. versus time of drug solution of groups 1–4

C_{max} as compared to conventional dosage form [10], reported similar finding of pk. parameters after oral administration of IBH with coadministration of puerarin.

AUC is very important tool for the assessment of bioavailability. In the current research AUC_{0-t} for oral IBH solution (group 1); F7 (group 2); F14 (group 3); and F21 microspheres (group 4) was found to be 2483.71 ± 13.173 ng/ml h, 5954.37 ± 12.110 ng/ml h, 6400.82 ± 19.131 ng/ml h and 7427.4 ± 49.322 ng/ml h, respectively. $AUC_{0-\infty}$ for oral IBH solution (group 1); F7 (group 2); F214 (group 3); and F21 microspheres (group 4) was found to be 2558.95 ± 62.112 ng/ml h, 6422.72 ± 253.06 ng/ml h, 6680.98 ± 123.01 ng/ml h and 8020.44 ± 134.12 ng/ml h, respectively.

Results indicated clearly that AUC_{0-t} and $AUC_{0-\infty}$ for the microspheres were higher significantly than oral IBH soln., thus signifying enhanced bioavailability in rabbits. Increased AUC could be correlated to increase the bioavailability [39]. Similar results were reported for improving bioavailability of IBH solid lipid microparticles [40]. Lodhi et al. [41] developed controlled release buccal film of IBH. AUC of pH-sensitive hydrogel formulations was greater comparable to conventional dosage form [42–45].

The controlled drug release features of polymeric microspheres also revealed in MRT values. The MRT values were significantly greater for the microspheres than for the IBH solution. Furthermore, microspheres have meaningfully prolonged elimination $t_{1/2}$. This specifies IBH containing microspheres has effectively controlled release drug delivery.

Conclusion

The concepts for fabricating copolymeric microspheres of IBH offer an appropriate, sensible approach to accomplish a lingering therapeutic outcome by continuously releasing the drug over extended period of time. The results indicated that the present HPLC method is very simple and applicable to pharmacokinetic and bioavailability studies of IBH. Collectively, these in vivo results manifested that pH-dependent microspheres had a reasonable controlled release, with better drug delivery at

Table 5 Pharmacokinetic parameters (mean ± SD) of rabbits of groups 1–4

S. no.	Pharmacokinetic parameters	Group 1	Group 2	Group 3	Group 4
1	C_{max} (ng/ml)	880.38 ± 11.03	718.43 ± 0.002	721.87 ± 0.001	805.11 ± 0.004
2	T_{max} (h)	2.01 ± 0.001	4.0 ± 0.001	4.1 ± 0.003	4.56 ± 0.002
3	AUC_{0-t} (ng/ml h)	2483.71 ± 13.173	5954.37 ± 12.110	6400.82 ± 19.131	7427.4 ± 49.322
4	$AUC_{0-\infty}$ (ng/ml h)	2558.95 ± 62.112	6422.72 ± 253.06	6680.98 ± 123.01	8020.44 ± 134.12
5	$AUMC_{0-t}$ (ng h^2/ml)	7321.18 ± 23.121	49,894.11 ± 57.557	52,701.34 ± 146.561	63,477.3 ± 167.110
6	$AUMC_{0-\infty}$ (ng h^2/ml)	8089.59 ± 34.111	64,894.72 ± 254.221	61,284.59 ± 231.10	82,455.77 ± 231.22
7	$t_{1/2}$ (h)	1.53 ± 0.11	5.57 ± 1.12	4.6 ± 1.34	5.55 ± 2.11
8	K_e (h^{-1})	0.45 ± 0.23	0.12 ± 0.11	0.15 ± 0.21	0.12 ± 0.16
9	MRT_{last} (h)	2.95 ± 1.23	8.38 ± 1.88	8.38 ± 2.34	8.55 ± 2.11
10	$MRT_{0-\infty}$ (h)	3.16 ± 0.338	10.1 ± 0.331	9.17 ± 0.220	10.28 ± 0.123
11	CL (L/h/kg)	0.39 ± 0.251	0.16 ± 0.112	0.15 ± 0.341	0.12 ± 0.341
12	V_d (L/kg)	0.86 ± 0.128	1.26 ± 1.112	0.99 ± 0.982	1.13 ± 0.212

Table 6 Summary of ANOVA applied to various pharmacokinetics parameters of all groups

Pk. parameter	ANOVA						
	Df	SS	MS	F	*P* value	*F* crit.	Statistical results
C_{max}	11	3.2804	3.107317	3.244797	0.0009151	2.2073082	Highly significant
T_{max}	11	1.271607	0.11561	5.340234	0.00096	2.216409	Highly significant
AUC_{0-t}	11	740.8187	66.5279	0.000499	1	2.206309	Highly insignificant
$t_{½}$	11	246.117	314.005	3.574782	0.0009	2.12	Highly significant

SS sum of square; *Df* degree of freedom; *MS* mean sum of square

higher pH environment. Results indicated that polymeric network protecting drug from acidic pH of stomach hence improved in vivo retention and decreased plasma drug concentration variation. It would be faster and more cost-effective in modifying imperative properties of the existing drugs than developing new drug entities; hence, this formulation will be windfall to novel drug dosage forms.

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Compliance with ethical standards

Conflict of interest Authors have no conflict of interest to declare.

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