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Preparation and In Vivo Efficacy Study of Pancreatin Microparticles as an Enzyme Replacement Therapy for Pancreatitis

Sonali Raosaheb Naikwade, Rupali Namdeo Meshram, and Amrita Narayan Bajaj

C.U. Shah College of Pharmacy, S.N.D.T. Women's University, Santacruz (W), Mumbai, Maharashtra, India

Deficiency manifestations because of pancreatic insufficiency are treated by oral administration of pancreatic enzymes. As pancreatic enzymes get denatured in hostile acidic conditions of stomach, this investigation was aimed at formulating multiparticulates of pancreatic enzymes coated with enteric polymers such as eudragit L100, cellulose acetate phthalate, and hydroxyl propyl methyl cellulose phthalate, which will circumvent gastric inactivation in addition to providing optimal mixing with chyme. Pancreatin microspheres were prepared by emulsification phase separation by nonsolvent addition and solvent evaporation techniques. This process was optimized for core : coat ratio (1:0.5), stirrer speed (350–400 rpm), dispersant concentration, and amount of nonsolvent added to precipitate microspheres. Optimized formulations were assessed for % enzyme content, acid resistance, flow properties, particle size, particle morphology (by standard electron microscopy), compatibility of drug and polymer in formulation (by differential scanning calorimetry), in vitro release kinetics, and in vivo efficacy study in pancreatitis-induced animal model. Capsules containing enteric-coated pancreatin microspheres offered adequate protection to enzymes and prevented their denaturation in acidic environment. Developed multiparticulate dosage forms promoted effective mixing, instant and complete in vitro release compared with marketed tablets.

Keywords pancreatic enzymes; pancreatitis; microspheres; enteric polymers; in vivo efficacy

INTRODUCTION

Exocrine pancreatic insufficiency is most commonly caused by cystic fibrosis, chronic pancreatitis, or pancreatic resection, which is a chronic and frequently fatal genetic disease of glands that produce or secrete sweat and mucus. This primarily affects the respiratory, digestive, and reproductive tract in children and young adults. The thick mucus seen in lungs has its counterpart in thickening secretions from pancreas, an organ responsible for providing digestive juices that help to break down food (Gilljam et al., 2003; Jain, Subramony, Blanchard, Petro, & Minocha, 2005). These secretions block the movement of digestive enzymes into the duodenum and result in irreversible damage to pancreas, often with painful inflammation (pancreatitis) (Layer & Holtmann, 1994; Opekun, Sutton, & Graham, 1997). The lack of digestive enzymes leads to difficulty in absorbing nutrients with their subsequent excretion in the feces, a disorder known as malabsorption. Malabsorption leads to malnutrition and poor growth and development because of calorie loss. Digestive problems caused by cystic fibrosis (CF) are managed with medications, namely, pancreatic enzymes that help digestion and enemas and mucolytic agents to treat intestinal obstructions. When secretion of pancreatic enzymes falls below 10% of normal, fat and protein maldigestion occurs, which can lead to steatorrhea (Pasquali et al., 1996), azotorrhea, vitamin malabsorption, and weight loss (Kalivianakis et al., 1999; Lankisch, Lembcke, Goke, & Creutzfeldt, 1986; Snook, 1965). Pancreatic enzyme supplements, which contain a mixture of amylase, lipase, and proteases, are the mainstay of treatment for pancreatic enzyme insufficiency (Brayer, Luo, & Withers, 1995).

Deficiency manifestations because of pancreatic insufficiency are treated by oral administration of pancreatic enzymes containing lipase, amylase, and protease activity (Dobrilla, 1989; Layer & Holtmann, 1994; Poustie, Russell, Watling, Ashby, & Smyth, 2006). These enzymes get denatured in hostile acidic conditions of the stomach; thus to prevent this deleterious effect, it becomes imperative to coat pancreatin with pH-sensitive polymers which would release the enzymes above a safe pH threshold (pH 5.0–5.5) (Sax et al., 1987).

Currently available pancreatic enzyme supplements in Indian market are in the form of single-unit dosage forms, such

Address correspondence to Amrita Narayan Bajaj, Professor of Pharmaceutics, C.U. Shah College of Pharmacy, S.N.D.T. Women's University, Juhu-Tara Road, Santacruz (W), Mumbai 400049, Maharashtra, India. E-mail: bajajamrita@rediffmail.com

as tablets, which are enteric coated and show variations in response and drug release pattern (Bansi, Price, Russell, & Sarner, 2000; Fitzsimmons et al., 1997). Large tablets do not pass as quickly as smaller food particles into the small intestine (Dobrilla, 1989). Failure to release the enteric coat will lead to the tablet traversing to distal locations—colon where pH is alkaline and release of enzymes there can cause adverse effects such as colonic fibrosis (Ferrone, Raimondo, & Scolapio, 2007; Genta et al., 2001).

The key to success of proteins as pharmaceuticals is to have in place an efficient drug delivery system that allows the protein drugs to gain access to their target sites at the right time and for proper duration. Four factors that must be considered to fulfill this goal are route of administration, pattern of drug release, method of delivery, and fabrication of formulation. The development of delivery systems for therapeutic proteins and their evaluation depend on biophysical, biochemical, and physiological characteristics of protein molecules including their molecular size, biological half life, immunogenicity, dose requirement, site and rate of administration, pharmacokinetics, and pharmacodynamics. Bruno et al. (1998) reported that enteric microspheres preparations with sphere sizes larger than 1.7 mm pass through the stomach at a slower rate than a meal and therefore may be less efficacious in restoring pancreatic enzyme activity than preparations with smaller sphere sizes.

The objective of this research work was to formulate a multiparticulate delivery system of pancreatin enzymes by encapsulating it with enteric polymer cellulose acetate phthalate (CAP), eudragit L100, and hydroxyl propyl methyl cellulose phthalate (HPMCP), which will circumvent the gastric inactivation of enzymes (Genta et al., 2001; Stevens, Maguiness, Hollingsworth, Heilman, & Chong, 1998; Taylor, 2002; Taylor, Mee, Misiewicz, Barnardo, & Polanska, 1982). The rationale for preparing various formulations was to protect the pancreatin enzymes from degradation in acidic environment after administering orally. To achieve this, we attempted various approaches using different polymers. Capsules containing microspheres of pancreatin enzymes would provide optimal mixing with chyme, a prerequisite for effective enzyme replacement therapy. On arrival at the site of action, that is, duodenum, alkaline conditions prevailing therein will dissolve the polymer, releasing enzyme.

MATERIALS AND METHODS

Materials

Pancreatin 8NF was obtained as a gift sample from Biocon India Ltd. (Bangalore, India) and was standardized as per IP 1996 and USP NF XIV 2000. Pancreatin 8NF contains 200 USP units of amylase/mg, 200 USP units of protease/mg, and 16 USP units of lipase/mg. Eudragit L100 and aerosil were obtained as gift samples from Ideal Cures Pvt. Ltd. (Mumbai, India) and Signet Chemical Corporation (Mumbai, India), respectively. All other polymers and excipients used in the formulation development and analysis were procured from S.D. Fine Chemicals (Mumbai, India).

Analytical Method for the Determination of Enzyme Activities

Protease Content

Protease hydrolyses casein at pH 7.5 and temperature 35°C; this reaction is stopped with the addition of trichloroacetic acid and nondecomposed casein is filtered off. Quantity of the remaining peptides is determined spectrophotometrically. Test tubes were labeled as S1, S2, and S3 for standard and 1.0, 1.5, 2.0 mL standard solution (25 mg standard in 100-mL volumetric flask; volume was made with buffer solution [6.8 g monobasic potassium phosphate and 1.8 g of NaOH were dissolved in 1,000 mL water and the pH was adjusted to 7.5 ± 0.2 using 0.2 N NaOH] and mixed by shaking at room temperature for 25 min; 5 mL of the above solution was taken and the volume was made to 100 mL with buffer) was added, respectively. One test tube was labeled as U for test sample to which 1.5 mL test solution (25 mg test sample was dissolved in 100 mL buffer solution and mixed by shaking at room temperature for 25 min; 5 mL of this solution was taken and the volume was made to 100 mL with buffer) was added. To all test tubes, 5 mL of trichloroacetic acid solution (50 g of trichloroacetic acid in 1,000 mL of water) was added, mixed well, and absorbance was noted at 280 nm. Blank was prepared by mixing 3 mL of buffer solution and 5 mL of trichloroacetic acid. All test tubes were placed in a water bath shaker at 40 ± 2°C. At zero time, 2.0 mL of casein substrate was added to each test tube and shaking was continued for 60 min. After 60 min, reaction was stopped by adding 5 mL trichloroacetic acid; test tubes were maintained at room temperature for complete protein precipitation and filtered off. Absorbance of filtrate was determined against blank at 280 nm with UV Spectrophotometer-Shimadzu RF 5000. Corrected absorbance values were obtained by subtracting values before addition of casein substrate from values after complete reaction. Corrected absorbance values were plotted against corresponding volume of standard solution, and the following equation was obtained to find out unknown concentration, $y = 0.1167x + 0.011$, $R^2 = .9835$.

Amylase Activity

Activity was determined by iodimetric titration based on hydrolysis of soluble starch at pH 6.8 and temperature 25°C in the presence of 0.1 N NaOH solution. Resulting reducing groups were determined iodimetrically. Four stoppered 250-mL conical flasks were taken and marked as S, U, BS, and BU. In each flask, 25 mL of substrate solution (2 g of soluble starch was stirred with 10 mL of water and added to 160 mL of boiling water; it was heated to boiling with continuous mixing and cooled to room temperature; and the volume was made to 200 mL with water), 10 mL of pH 6.8 phosphate buffer, and 1 mL

of NaOH solution were added. Contents of flasks were mixed and flasks were placed in water bath at 25 ± 0.1°C. To flasks BU and BS, 2 mL of 1 N HCl solution was added, mixed, and kept in water bath. To flasks U and BU, 1 mL of assay solution (amount necessary to obtain amylase activity/mL corresponding to the standard solution that is 40 mg was dissolved in 30 mL of phosphate buffer and triturated for 5–10 min and the volume was made to 100 mL with buffer) and to S and BS, 1 mL of standard solution (20 mg of pancreatin enzyme was dissolved in 30 mL of pH 6.8 phosphate buffer, triturated for 5–10 min, and the volume was made to 50 mL with buffer) was added, mixed, and kept in water bath. After 10 min, 2 mL of 1 N HCl was added to S and U and mixed. To each flask, 10 mL of 0.1 N iodine solution was added with continuous stirring followed by 45 mL of 0.1 N sodium hydroxide solution and flasks were kept in dark at temperature between 15 and 25°C for 15 min. To each flask, 4 mL of 2 N sulfuric acid was added and titrated with 0.1 N sodium thiosulfate to colorless end point. Amylase activity was calculated (in USP units/mg of pancreatin) by the following formula:

$$\text{Amylase activity} = \frac{(C_S / W_U)(V_{BU} - V_U)}{V_{BS} - V_S},$$

C_S = amylase activity of standard preparation in USP units/mL
W_U = amount in mg of test sample, pancreatin
V_{BU} = volume in mL of 0.1 N sodium thiosulfate consumed by BU
V_{BS} = volume in mL of 0.1 N sodium thiosulfate consumed by BU
V_U = volume in mL of 0.1 N sodium thiosulfate consumed by U
V_S = volume in mL of 0.1 N sodium thiosulfate consumed by S.

Lipase Content

Analysis was based on potentiometric titration in which hydrolysis of triglycerides of olive oil was determined by means of titration of released carbonic acid at pH 9. Ten milliliters of olive oil substrate (165 mL acacia solution [centrifuged solution of acacia 1 in 10 until clear solution was obtained], 20 mL of olive oil, and 15 g crushed ice were mixed and cooled to 5°C and homogenized at high speed for 15 min, intermittently cooling in an ice bath to prevent temperature rise beyond 30°C), 8 mL buffer solution (60 mg of tris buffer and 234 mg of NaOH in water to make 100 mL), 2 mL bile salt solution (2 g of bile salt in water to make 25 mL), and 9 mL water were mixed in a 100-mL. This beaker was placed in a 250-mL beaker containing water, and this assembly was kept on a magnetic stirrer maintained at 37 ± 1°C. To the beaker, 0.01 N NaOH solution was added drop wise to adjust the pH to 9.2. After adjusting the pH, 1 mL of assay/standard solution (20 mg of standard/test pancreatin was suspended in 3 mL of cold water in a mortar, triturated for 10 min, volume was made to 100 mL and suspension was maintained at 4°C) was added and then 0.01 N NaOH was added continuously for 5 min to maintain pH 9.0. Volume of NaOH added after each minute was noted. Potency was calculated from plotting volume of 0.01 N NaOH titrated against time. Lipase activity was calculated by the following formula:

$$\text{Lipase activity} = \frac{\text{weight of test}}{\text{mL of 0.01 N NaOH for test}} \times \frac{\text{mL of 0.01 N NaOH for standard}}{\text{weight of standard}} \times \text{standard activity}$$

Acid Resistance

Acid resistance characteristics of formulations were studied using USP II dissolution test apparatus for 2 h in acid followed by exposing formulations to pH 6.8 phosphate buffer for 45 min at 75 rpm. Formulation equivalent to 500 mg of pancreatin was weighed and dispersed in acid medium—750 mL of 0.1 N HCl. At the end of 2 h, 250 mL of 0.2 M tribasic sodium phosphate was added to 750 mL of 0.1 N HCl to make the pH 6.8. Stirring was maintained at 75 rpm, and at the end of 45 min, aliquots were withdrawn and subjected to analysis for protease content.

Formulation Development

Solid Dispersions with Conventional Methods

Solid dispersions of pancreatin enzymes were prepared by physical mixing, coevaporation, and kneading methods (Schneider, Knoll Ruzicka, Domschke, Heptner, & Domschke, 1985). Enteric polymers investigated as carriers for preparing solid dispersions of pancreatin enzymes were CAP, eudragit L100, and HPMCP (Sinha & Trehan, 2003; Stead, Skypala, & Hodson, 1988).

Solid Dispersions with Cellulose Acetate Phthalate

Solid dispersions were prepared in ratios 1:1 and 1:2 of pancreatin 8NF and CAP as follows:

- *Physical mixing*: Pancreatin 8NF and CAP were mixed in geometric proportion to prepare formulations P1 (drug : CAP, 1:1) and P2 (drug : CAP, 1:2) as shown in Table 1.
- *Kneading*: Pancreatin 8NF and CAP (P3, 1:1 and P4, 1:2) were mixed in geometric proportions in a glass mortar and kneaded for 30 min to get pasty consistency using 6 and 10 mL of 1:1 acetone : water for P3 and P4 batches, respectively, and then dried in vacuum oven at 35°C for 1 h. Dry kneaded product was scraped and sieved through 60 mesh sieve.
- *Coevaporation*: Pancreatin 8NF (500 mg) was dispersed in 5 mL of water in an evaporating dish, and the contents were stirred at room temperature for

TABLE 1
Formulation Development of Solid Dispersions of Pancreatin

		Batch	Appearance	Flow Properties	Bulk Density (g/cm^3)	Particle Size (μm)	% Protease Content	% Protection Against Acid
Solid dispersions with conventional methods	Cellulose acetate phthalate	P1	Fine powder		0.3	200–250	90.234 ± 2.62	4.183 ± 0.198
		P2	Fine powder		0.44	220–250	86.422 ± 1.381	4.194 ± 0.705
		P3	Fine powder		0.30	250–290	94.027 ± 3.083	20.117 ± 1.372
		P4	Fine powder		0.40	260–280	100.91 ± 0.164	31.740 ± 1.608
		P5	Hard flakes		0.48	450–490	85.269 ± 0.218	23.938 ± 0.939
		P6	Hard flakes		0.45	420–475	96.387 ± 0.602	37.400 ± 2.373
	Eudragit L100	P7	Fine powder	Free flowing	0.45	170–260	97.006 ± 3.052	8.176 ± 0.473
		P8	Fine powder		0.45	230–300	93.002 ± 8.319	34.555 ± 2.101
		P9	Hard flakes		0.45	350–460	95.579 ± 2.304	33.457 ± 3.545
	HPMCP	P10	Fine powder		0.32	140–270	93.819 ± 1.745	7.083 ± 0.417
		P11	Fine powder		0.35	210–260	95.840 ± 3.471	29.310 ± 1.764
		P12	Hard flakes		0.30	290–440	94.256 ± 0.561	24.820 ± 0.581
Solid fluidized system using bed	Cellulose acetate phthalate	P13	Pale yellow colored		0.71	710–800	14.287 ± 0.509	21.899 ± 5.78
		P14	spherical particles		0.68	700–850	27.145 ± 6.255	94.663 ± 5.934
	Eudragit L100	P15	with uniform surface		0.75	720–860	22.336 ± 6.616	10.761 ± 1.636
		P16			0.64	700–840	28.732 ± 0.606	98.915 ± 2.003

5 min using magnetic stirrer. CAP (500 mg for 1:1 ratio) was dissolved in 10 mL acetone and added drop wise to the pancreatin solution with constant stirring. On complete addition, the dish was covered with glass plate, and the contents were stirred at room temperature for 45 min, then the dish was uncovered and the solvent was evaporated at 35 ± 2°C. Dry solid product was scraped and passed through 60 mesh sieve. This batch was coded as P5. Another batch with 1:2 pancreatin and CAP was also prepared in a similar manner and coded as P6 (Table 1).

Solid Dispersions with Eudragit L100

Solid dispersions with eudragit L100 were prepared in 1:2 ratio by techniques and procedures as mentioned above (Stead, Skypala, Hodson, & Batten, 1987). These batches were coded as P7, P8, and P9 (Table 1).

Solid Dispersions with HPMCP

Solid dispersions with HPMCP were prepared in 1:2 ratio by methods as mentioned above. These batches were coded as P10, P11, and P12 (Table 1).

Preparation of Solid Dispersions on Nonpareil Seeds (Sugar Spheres) Using Fluidized Bed System

Solvent system used in the preparation of spraying solution was dichloromethane : isopropyl alcohol : diethyl phthalate (65:35:20% of polymer weight). The equipment used was Labcoater®; spray gun position was tangential with nozzle diameter of 1.8 mm. Atomizing pressure was 350 kPa and inlet temperature was 37°C. Process parameters were optimized to get maximum yield and reproducible results by monitoring conditions throughout the process of coating, namely, spray rate (6–7 mL), rotor speed (200 rpm), and amount of core added (200 g). Sugar spheres (200 g) were loaded into the chamber of a fluidized bed granulator, coater, and fluidized by opening the inlet air flap. As outlet temperature reached 30°C, the spraying solution (enzyme–polymer mixture in organic solvents) was tangentially sprayed onto the fluidized sugar spheres from an atomizing nozzle (10 mm) attached to the peristaltic pump. During the process, spraying rate and inlet temperature were adjusted to maintain outlet temperature between 28 and 31°C. When spraying was finished, the pellets were dried at 30°C for another 5 min and removed.

Solid dispersions thus prepared by spraying enzyme–polymer solution on the surface of nonpareil seeds were coded as P13 and P14 for 1:1 and 1:2 ratios of pancreatin and CAP, respectively. Batches P15 and P16 represented 1:1 and 1:2 ratios of pancreatin and eudragit L100, respectively (Table 1).

Microencapsulation by Solvent Evaporation Technique

Enteric polymers such as CAP, eudragit L100, and HPMCP were used to prepare microspheres in the particle size range of 700–800 μm (Chen & Subirade, 2006; Santini et al., 2000; Stead et al., 1987). Polymers CAP and HPMCP were dissolved in acetone, and eudragit L100 was dissolved in acetone : IPA (1:1). Pancreatin 8NF in particulate form was dispersed in polymer solution; this enzyme–polymer solution was added to liquid paraffin containing surfactants/dispersants to form a stable emulsion. Stirring was continued to evaporate the organic volatile solvent, where upon microspheres obtained were filtered, washed with hexane, and dried. Polymer–drug dispersion to vehicle ratio was varied to get optimum yield. Microspheres were prepared in core : coat ratio of 1:0.25 and 1:0.5 as shown in Tables 2 and 3. Dispersants/surfactants such as aerosil, span 80, and magnesium stearate at varying concentrations were used to prevent coalescence of microspheres at different stirrer speeds.

Microencapsulation by Nonsolvent Addition Technique

Polymer was dissolved in solvent, and pancreatin 8NF in particulate form was dispersed in this solution. This enzyme–polymer mixture was added to liquid paraffin containing surfactant/dispersant to form an emulsion. On stabilization of emulsion, nonsolvent was added drop wise to precipitate out microspheres. Microspheres thus obtained were filtered, washed with hexane till free of oil, and dried. Core : coat ratio used in the preparation of microspheres was 1:0.5 and dispersion : vehicle ratio was 1:5, vol/vol (Table 4).

Evaluation of Developed Formulations

Developed formulations were evaluated for % enzyme content as per the procedure mentioned above. These formulations also tested for flow properties, bulk density, particle size, and % protection against acid. Differential scanning calorimetry (DSC) studies were also conducted using V4 1C Dupont Analyzer and formulations were scanned at a rate of 10°C/min between 20 and 300°C to confirm that there is no interaction between the drug and polymers used. Surface morphology of developed microspheres was analyzed using SEM Studies. In vitro release studies were carried out using USP II dissolution apparatus for 2 h in 0.1 N HCl acid containing pepsin, followed by exposing them to various pH buffers—5.0, 5.5, 6.5, and 7.2—to simulate conditions of duodenal pH. This gastrointestinal pH varies extensively in patients of exocrine pancreatin insufficiency. Acid resistance and in vitro release studies were determined by measuring enzyme activities by methods as mentioned above. Optimized formulations were subjected to stability studies for 6 months as per International Conference on Harmonization (ICH) guidelines. Data were evaluated using two-way analysis of variance (ANOVA) to check the impact of different storage conditions on enzyme activities and acid resistance characteristics. Optimized formulations were filled in capsules and subjected to stability studies for 6 months as per ICH guidelines. Data were evaluated using two-way ANOVA to check the impact of different storage conditions on

TABLE 2
Formulation Development of Pancreatin Microspheres with Solvent Evaporation Technique

	Batch	Core : Coat	Vehicle	Dispersion: Vehicle (vol/vol)	Dispersant/ Surfactant (wt/vol)		Stirrer Speed (rpm)	Observation	% Protease Content	% Protection Against Acid	Mean Particle Size (μm)
Cellulose acetate phthalate	P17	1:0.25	Liquid paraffin	1:3	Span 80	0.25	400	Emulsion not formed	—	—	—
	P18	1:0.25		1:5		0.25	400	Microspheres formed but agglomerated and gave sticky mass	—	—	—
	P19	1:0.25				0.5	400	Microspheres obtained	102.203 ± 2.77	38.113 ± 1.05	—
	P20	**1:0.5**				**0.5**	**400**	**Microspheres obtained**	**108.228 ± 11.54**	**96.776 ± 1.89**	**664**
	P21	1:0.5	Silicone oil			0.5	400	Microspheres obtained	102.46 ± 0.35	97.713 ± 1.46	650
	P22	1:0.5	Liquid paraffin		Talc	0.5	400	Coalescence of microspheres	—	—	—
	P23	1:0.5				1.0	400	Coalescence of microspheres	—	—	—
	P24	1:0.5			Magnesium stearate	0.5	400	Microspheres obtained	103.804 ± 1.05	97.076 ± 1.58	350
	P25	**1:0.5**				**0.25**	**400**	**Microspheres obtained**	**103.781 ± 1.06**	**99.858 ± 4.79**	**655**
	P26	1:0.5				0.25	450	Microspheres obtained	104.178 ± 1.54	99.486 ± 2.28	110
	P27	1:0.5				0.5	350	Coalescence of microspheres	—	—	—
	P28	1:0.5			Aerosil	0.25	400	Microspheres obtained	104.25 ± 1.45	100.619 ± 0.12	85

	P29	1:0.5			0.1	400	Coalescence of microspheres	—	—	—
	P30	1:0.5			0.25	350	Coalescence of microspheres	—	—	—
	P31	**1:0.5**			**0.4**	**350**	**Microspheres obtained**	**103.953 ± 1.64**	**99.116 ± 1.38**	**650**
Eudragit L100	P32	1:0.25	Liquid paraffin	1:5	Aerosil	0.1	350	Microspheres obtained	—	20.275 ± 0.12	2800
	P33	1:05				0.1	350		—	50.375 ± 0.049	2500
	P34	1:0.25				0.25	350		—	21.92 ± 2.404	2000
	P35	1:0.25				0.1	400		—	15.015 ± 0.12	1600
	P36	1:05				0.25	350		—	61.45 ± 1.23	2200
	P37	1:05				0.1	400		—	35.475 ± 0.021	1800
	P38	1:0.25				0.25	400		—	16.35 ± 1.245	700
	P39	1:05				0.25	400		—	97.725 ± 1.676	720
	P40	1:0.5			Span 80	0.3	350		—	—	1820
	P41					0.5	350		—	—	540
	P42					0.3	400		—	—	420
	P43					**0.5**	**400**		**101.228 ± 11.54**	**99.776 ± 1.89**	**810**
	P44				Magnesium stearate	0.15	350		—	—	2340
	P45					0.25	350		—	—	1540
	P46					0.15	400		—	—	1230
	P47					**0.25**	**400**		**94.705 ± 1.07**	**97.265 ± 1.21**	**690**

Bold face characters signify optimized formulations selected for stability testing.

TABLE 3
Formulation Development of Pancreatin Microspheres Using hpmcp as Polymer with Solvent Evaporation Technique

	Batch	Core : Coat	Vehicle	Dispersion : Vehicle (vol/vol)	Dispersant/ Surfactant (wt/vol)		Stirrer Speed (rpm)	Observation	% Protease Content	% Protection Against Acid	Mean Particle Size (μm)
HPMCP	P48	1:0.5	Liquid paraffin	1:5	Aerosil	0.1	350	Microspheres obtained	—	—	2010
	P49					0.2	350		—	—	1320
	P50					0.1	400		—	—	1190
	P51					**0.2**	**400**		**102.25 ± 1.45**	**101.619 ± 0.12**	**680**
	P52				Span 80	0.2	350		—	—	2410
	P53					0.25	350		—	—	950
	P54					0.2	400		—	—	890
	P55					0.25	400		104.178 ± 1.54	101.486 ± 2.28	670
	P56				Magnesium stearate	0.2	350		—	—	2310
	P57					0.25	350		—	—	1230
	P58					0.2	400		—	—	1340
	P59					0.25	400		99.25 ± 1.45	99.619 ± 0.12	710

Bold face characters signify optimized formulations selected for stability testing.

enzyme activities and acid resistance characteristics. The optimum batch was selected based on stability study data, and it was compared for release kinetics with existing marketed tablets of pancreatin enzymes. Pancreatin microspheres, batch P43, were compared for in vitro release profile with three marketed products of pancreatin enzyme tablets, products A, B, and C. In vitro release profile was determined in acid medium for 2 h followed by exposing formulations to pH 6.8 phosphate buffer for 45 min. At the end of 45 min, aliquots were withdrawn from the dissolution media and analyzed for protease content.

In Vivo Efficacy of Developed Formulations

To test the in vivo efficacy of developed pancreatic enzyme formulations, it is necessary to develop an animal model with pancreatic enzyme deficiency (Bruno, Haverkort, Tijssen, Tytgat, & Leeuwena, 1998; Bruno et al., 1998; Ferrone et al., 2007). Rats induced with alcoholic chronic pancreatitis develop lesions in pancreas which are not very different from human chronic pancreatitis. Other symptoms observed in animals, induced with chronic pancreatitis, are steatorrhea, increase in serum levels of triglycerides, bilirubin, and amylase. Animal model for alcoholic chronic pancreatitis was developed using Wistar rats, and developed pancreatin microspheres were administered. The study was conducted as per the protocol sanctioned by Institutional Animal Ethical Committee. Wistar rats comprising both males and females in the weight range of 150–180 g were selected for the study. As alcohol cannot be administered directly, branded alcohol, officer's Choice Whisky, containing 44% (wt/vol) of alcohol was used. Alcohol (2.93 g/kg) and olive oil (0.5 mL) were administered orally through a feeding needle. During the induction period, rats were monitored for weight gain. Composite fecal fat analysis was done at regular intervals of 2, 4, and 5 weeks. Enzyme levels in serum were examined for changes in pancreatic amylase, triglycerides, and bilirubin contents (Table 5). Blood withdrawal for serum analysis was done from retro-orbital cavity. Pathological changes in pancreas were evaluated by sacrificing an animal from group 1 at an interval of 2 and 5 weeks. After induction period, alcohol and olive oil administration was terminated. Rats were kept under observation for a period of 5 days and then treatment regimen was initiated. Dosage of formulations was calculated (group 1, sodium carboxyl methyl cellulose (CMC) 2 mL suspension; group 2, 3 mg of plain pancreatin in 2 mL sodium CMC solution; and group 3, 9 mg of microspheres [formulation P43] dispensed in 2 mL sodium CMC suspension) and administered orally with the help of a feeding needle.

Toxicity studies were performed to assess the safety of the developed formulation. Acute toxicity study indicates morbidity and mortality after a single administration and is probably the simplest method for assessing toxic effects. On the other hand, repeat dose (chronic) toxicity study investigates the effects caused by a long-term (90 days) administration of a

TABLE 4
Formulation Development of Pancreatin Microspheres with Nonsolvent Addition Technique

	Batch	Nonsolvent	Volume of Nonsolvent	Dispersant/Surfactant (wt/vol)		Stirrer Speed (rpm)	Observation	% Protease Content	% Protection Against Acid	Mean Particle Size (μm)
Cellulose acetate phthalate	P60	Water	30	Magnesium stearate	0.25	400	No precipitation	—	—	—
	P61		**40**			**400**	**Microspheres obtained**	**71.698 ± 1.44**	**98.169 ± 2.78**	**659**
	P62					450	Microspheres obtained	88.314 ± 5.58	96.929 ± 10.37	482
	P63					350	Coalescence of microspheres	—	—	—
	P64	Acidic water	30			400	No precipitation	—	—	—
	P65		35			400	Microspheres obtained	83.665 ± 0.49	97.023 ± 1.62	625
	P66	Water: acetone (4:1)	60			400	Microspheres obtained	81.336 ± 1.38	94.978 ± 2.11	442
	P67	Water: acetone (3:1)				400	No precipitation	—	—	—
	P68	**Water**	**40**	**Aerosil**		**400**	**Microspheres obtained**	**79.705 ± 2.72**	**91.791 ± 0.97**	**746**
	P69			Talc	0.2–0.5	400	Coalescence of microspheres	—	—	—
	P70			Span 80	0.25	400	Coalescence of microspheres	—	—	—
	P71				**0.5**	**400**	**Microspheres obtained**	**75.30 ± 1.0**	**93.55 ± 0.49**	**659**
Eudragit L100	P72	Water	30	Magnesium stearate	0.3	400	No precipitation	—	—	—
	P73		40				No precipitation	—	—	—
	P74		**45**				**Microspheres obtained**	**84.761 ± 0.38**	**92.13 ± 5.29**	**754**
	P75	Acidic water	30				No precipitation	—	—	—
	P76		35				Microspheres obtained	83.83 ± 4.28	91.60 ± 7.16	654
	P77	**Water**	**45**	**Aerosil**	**0.25**		**Microspheres obtained**	**78.69 ± 0.47**	**92.33 ± 4.43**	**650**
	P78		45	Talc	0.2–0.5		Coalescence of microspheres	—	—	—
	P79		**45**	**Span 80**	**0.55**		**Microspheres obtained**	**76.827 ± 1.47**	**89.83 ± 0.80**	**754**
HPMCP	**P80**	**Water**	**35**	**Magnesium stearate**	**0.25**	**400**	**Microspheres obtained**	**74.942 ± 1.29**	**91.942 ± 1.51**	**624**
	P81			**Aerosil**	**0.15**			**73.333 ± 0.38**	**95.562 ± 1.71**	**652**
	P82			Talc	0.2–0.5		Coalescence of microspheres	—	—	—
	P83			**Span 80**	**0.45**		**Microspheres obtained**	**71.316 ± 0.45**	**93.468 ± 1.15**	**680**

Bold face characters signify formulations with required physicochemical characteristics.

TABLE 5
Development of Chronic Pancreatitis After 5 Weeks of Induction Period in Wistar Rats

Group (Six Animals/ Group)	Description	Average Weight Gain (g)	% Fecal Fat	Serum Triglycerides (mg/dL)	Serum Pancreatic Amylase (IU)	Serum Billirubin (mg/dL)
Control	Negative control	16.75 ± 3.862	0.86	98.75 ± 2.217	810.25 ± 76.62	0.3 ± 0.082
Group 1	No treatment (induction of pancreatitis but no treatment)	20.2 ± 2.218	4.97	113.2 ± 9.038	795.6 ± 125.1	0.317 ± 0.098
Group 2	Plain pancreatin was administered	26.16 ± 2.639	6.75	124.5 ± 13.487	824.0 ± 60.98	0.35 ± 0.105
Group 3	Pancreatin microspheres were administered	21.167 ± 3.869	5.68	120.667 ± 5.645	767.33 ± 76.47	0.333 ± 0.103

compound. At the end of 90 days, animals were killed; organs were separated and subjected to histopathological studies. The serum sample was analyzed for albumin, alkaline phosphatase, alanine aminotransferase (SGPT), aspartate aminotransferase (SGOT), total bilirubin, and triglycerides (Table 6).

Acute toxicity studies were conducted in such a way that signs of acute toxicity were revealed and the mode of death can be determined. Animals were observed at regular intervals and all signs of toxicity and time of their first occurrence, severity, duration, and progression were recorded. Observations were recorded for 14 days.

RESULTS AND DISCUSSION

Formulation Development

Solid Dispersions with Conventional Methods

Initially, an attempt was made to generate powder dispersions using conventional method in ratios of 1:1 and 1:2 pancreatin 8NF and CAP (Table 1; batches P1–P6) by physical mixing, kneading, and coevaporation that yielded formulations in the particle size range of 200–475 μm with a protease content of 85–96% (wt/wt) and 4–37% protection against acid. These batches were not considered for further evaluation as pancreatin enzyme was found to degrade in acid environment. This approach did not provide sufficient acid protection to enzymes. Solid dispersions (Table 1; P7, P8, and P9) prepared with eudragit L100 and HPMCP (Table 1; P10, P11, and P12) in 1:2 ratio were also not considered for further evaluation as % acid protection was considerably very less (i.e., 7–33%). The next approach was to prepare solid dispersions by coating pancreatin enzymes on sugar spheres.

Preparation of Solid Dispersions on Nonpareil Seeds (Sugar Spheres) Using Fluidized Bed System

In case of solid dispersions prepared by spraying enzyme–polymer solution in ratio of 1:1 and 1:2 of pancreatin and CAP

TABLE 6
Repeat Dose Toxicity Studies: Serum Chemistry Parameters

Serum Chemistry Parameters	Control	Placebo	Test
Albumin (g/dL)	4.60 ± 2.35	4.77 ± 1.23	4.58 ± 5.24
Alkaline phosphatase (IU/L)	37.20 ± 8.56	35.46 ± 6.35	38.52 ± 2.38
Alanine aminotransferase (SGPT) (IU/L)	30.85 ± 5.2	25.68 ± 7.49	28.49 ± 3.24
Aspartate aminotransferase (SGOT) (IU/L)	75.26 ± 10.36	74.22 ± 2.35	78.95 ± 9.87
Total bilirubin (mg/dL)	0.37 ± 8.22	0.29 ± 5.67	0.35 ± 7.52
Triglycerides (mg/dL)	130.5 ± 12.35	125.0 ± 5.47	138.2 ± 4.85

on the surface of nonpareil seeds, batch P13 was omitted from further evaluation parameters and in case of batch P14 though % acid protection was 94.663%, there was loss of enzyme content as shown in Table 1. Same observation was noted in case of batches P15 and P16, respectively, prepared with 1:1 and 1:2 ratio of pancreatin and eudragit L100 (Table 1). This approach provided sufficient acid protection, but it was observed that there was loss of enzyme activity and therefore not considered for further evaluation. To overcome the above mentioned problems, it was decided to coat enzymes using microencapsulation technique. This was attempted by microencapsulation by solvent evaporation and by nonsolvent addition method.

Microencapsulation by Solvent Evaporation Technique

Aerosil, span 80, and magnesium stearate at varying concentrations were evaluated (Table 2; batches P17–P31) as dispersants to prevent coalescence of microspheres at different stirrer speeds. It was observed that greater the dispersants/surfactant concentration and stirrer speed, smaller was the particle size obtained (Table 2; P20, P21, and P24–P26). Dispersants/surfactants act by getting adsorbed on emulsified droplets of microspheres at the surface thereof and thus prevent adhesion or cohesion between microspheres. Core : coat ratio of 1:0.5 positively contributed to acid resistance (batches P20, P21, P24–26, P28, and P31), whereas, other two variables, speed of stirrer and dispersant concentration did not have a significant effect on acid resistance. Core : coat ratio was found to have no effect on particle size. Variables such as stirrer speed and dispersant concentration showed a negative effect (batches P28 and P31), indicating that with increase in stirrer speed and aerosil concentration, there was decrease in particle size of microspheres. As core : coat ratio did not affect particle size, this variable was obliterated from the factorial design for further experiments with magnesium stearate and span 80. Core : coat ratio parameter was fixed to 1:0.5 for further experiments (Tables 2 and 3; batches P40–P59). The optimum particle size was 650–750 μm for effective mixing with gastric chyme. Thus, batches P20, P25, P31, P43, P47, and P51 were selected for stability testing as % enzyme activities for all the above batches was in the range of 92–99% (wt/wt) with % acid protection of 96–101%, and particle size of microspheres was in desired size range that is 650–750 μm (Table 7).

Microencapsulation by Nonsolvent Addition Technique

Water and acidic water were used as nonsolvents to precipitate out microspheres prepared using CAP, eudragit L100, and HPMCP as given in Table 4. Formulations prepared by nonsolvent addition technique are depicted in Table 4. When water as nonsolvent and magnesium stearate (0.25–0.3%, wt/vol) as dispersant were used, 30 mL of water did not precipitate out microspheres (batches P60 and P72). On increasing the volume of water to 45 mL, microspheres with particle size between 650 and 750 μm were obtained (batches P61, P68, and P80); but in all these batches, % protease content was found to be low (71–84%, wt/wt) with % acid protection of 89–98%. Acidic water, 30 mL, did not precipitate microspheres (batches P64 and P75). On increasing the volume to 35 mL, microspheres were obtained with mean particle size of 654.76 μm (batches P65 and P76). In further experiments, 45 mL of water was used as nonsolvent, and the effect of aerosil, span 80, and talc was observed. In batch P77, aerosil (0.25%, wt/vol) gave microspheres with mean particle size of 650 μm. Talc in the concentration range 0.2–0.5% (wt/vol) resulted in coalescence of microspheres (batches P69, P78, and P82). Span 80 when used (0.55%, wt/vol) gave microspheres with mean particle size of 754 μm (batch P79). Though in all batches prepared with nonsolvent addition method, % acid protection was achieved, the % protease content was found to be decreased significantly. However, compared to microspheres prepared by solvent evaporation method, the enzyme activity was retained less in case of microspheres prepared with nonsolvent addition method, indicating loss of activity in the process. The loss in enzyme activity could be due to incomplete precipitation of microspheres with addition of the nonsolvent. Also pancreatin is soluble in water, which might further be a cause of reduction in enzyme content.

TABLE 7
Optimized Stability Batches

	Batch	Enzyme Activities (%)			Acid Resistance (%)	Bulk Density (g/cm^3)	Flow Properties	Mean Particle Size (μm)
		Protease	Amylase	Lipase				
Solvent evaporation technique	P20	96.395	98.692	97.490	96.718	0.712	Free flowing	654
	P25	97.472	97.287	96.445	97.236	0.754		670
	P31	97.772	96.990	97.487	98.925	0.698		658
	P43	94.655	96.792	92.365	99.776	0.758		715
	P47	95.585	93.111	95.437	97.265	0.824		692
	P51	93.437	92.705	93.087	101.619	0.811		682

Evaluation of Developed Formulations

Developed selected formulations (Table 7) were evaluated for bulk density, flow properties, particle size analysis, % enzyme content (protease content, amylase content, and lipase content), and % acid protection as per procedures mentioned above. All formulations were free flowing in nature with bulk density of 0.69–0.82 g/cm^3 and % enzyme content was in the acceptable range of 92–99% (wt/wt). Figure 1 shows SEM photomicrograph of pancreatin microspheres (batch P43) that showed uniform spherical microspheres with no clear demarcation between polymer and pancreatin. Figure 2 shows DSC thermogram of pancreatin microspheres prepared with solvent evaporation technique and other excipients used. Thermogram of microspheres (P43 and P47) showed a shallow endotherm at 59.41°C which was comparable with a deep and sharp endotherm for eudragit L100 at 55.76°C and for pancreatin at 45.87°C. However, endotherm for both pancreatin (208°C) and eudragit L100 (214.23°C) was also seen in endotherms for microspheres at 215.22 and 214.23°C. A differentiating endotherm for pancreatin at 148.95°C was also observed in thermographs of formulations P43 and P47 at 128.57 and 138.54°C, respectively. Thus, from these observations, it was clear that there was no interaction of pancreatin enzyme with enteric polymer and also confirmed microencapsulation of pancreatin enzymes in enteric polymer. Figure 3 showed in vitro release of pancreatin microspheres (P43) upto 92% in phosphate buffer. Thus, the polymer eudragit L100 could protect pancreatin enzymes from degradation in acidic pH of the stomach and allow intact passage of enzymes in the duodenal region.

There was no significant change in physicochemical parameters and release behavior of enzymes at the end of 6 months at 25°C/60% RH. Table 8 shows physicochemical characteristics of microspheres after storage at 40°C/75% RH at the end of 3 months. Enzyme activities and acid resistance of pancreatin microspheres with eudragit L100 as enteric polymer at 40°C/75% RH at the end of 3 months were retained. At 5% significance level, formulations did not differ in their enzyme activities (protease, amylase, and lipase contents) and acid resistance characteristics as tested by two-way ANOVA. Also, there was no significant change in enzyme contents and acid resistance characteristics of formulations on storage at different conditions of temperature and humidity. Amongst all, microspheres of batch P43 were found to retain maximum enzyme activities after storage at 40°C/75% RH, 3 months, and 25°C/60% RH, 6 months. Microspheres did not show any color change and were found to be better in terms of physicochemical characteristics compared with other batches and hence were selected for further studies.

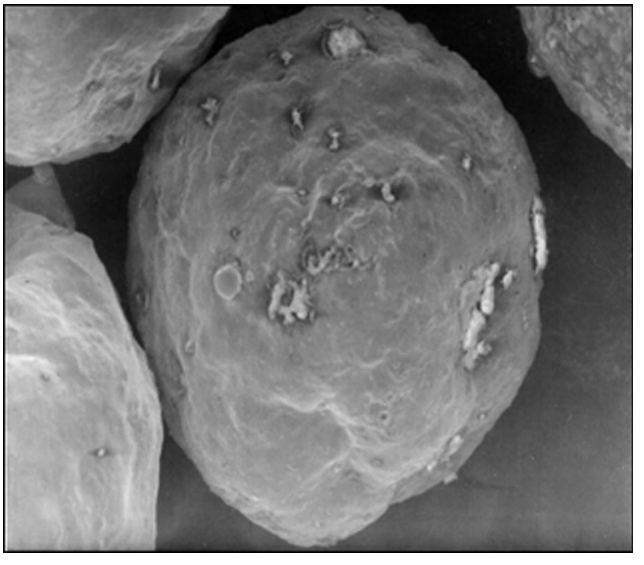

FIGURE 1. SEM photograph of eudragit L100 pancreatin microspheres prepared by solvent evaporation technique.

Microspheres of batch P43 were compared with marketed tablets of pancreatin for release characteristics (Figure 3). As compared with marketed tablets, pancreatin microspheres dispersed uniformly in acid medium, and on addition of phosphate buffer at the end of 2 h, it immediately dissolved within 5 min. Whereas marketed tablets were found to slowly release their enzyme contents by erosion of the matrix. This was revealed from release profiles in which pancreatin microspheres distributed evenly throughout the dissolution media, as compared with marketed tablets which sink to the bottom of the dissolution medium and even in alkaline conditions, released the enzyme contents slowly by eroding the polymer. It was observed that for tablets of product A, two tablets out of six did not disintegrate in alkaline medium even after exposing them to alkaline conditions for a period of 2 h. This could be because of hardening of the enteric coat due to some polymerization. However, the other four tablets disintegrated in alkaline conditions.

In Vivo Efficacy of Developed Formulations

To test the in vivo efficacy of developed pancreatic enzyme formulations, animal model with pancreatic enzyme deficiency was developed by administering alcohol and olive oil, and symptoms like weight gain, % fecal fat, serum triglycerides, serum amylase, serum bilirubin, and histopathological changes (Figure 4) were monitored till 5 weeks of induction period. All symptoms are listed in Table 5. It was observed that % fecal fat and serum triglyceride level were found to be increased significantly in animals induced with pancreatitis. The final dose of pancreatin enzyme to be administered in humans is 500 mg containing amylase 100,000 U, protease 100,000 U, and lipase 8,000 U. After administration of developed pancreatin microspheres to these pancreatitis-induced model, pathological evaluation was performed as shown in Table 9. In case of the group treated with pancreatin microspheres, % reduction in fecal fat was 38.028%, which was significantly high (2.5 times) as compared with the group treated with plain pancreatin.

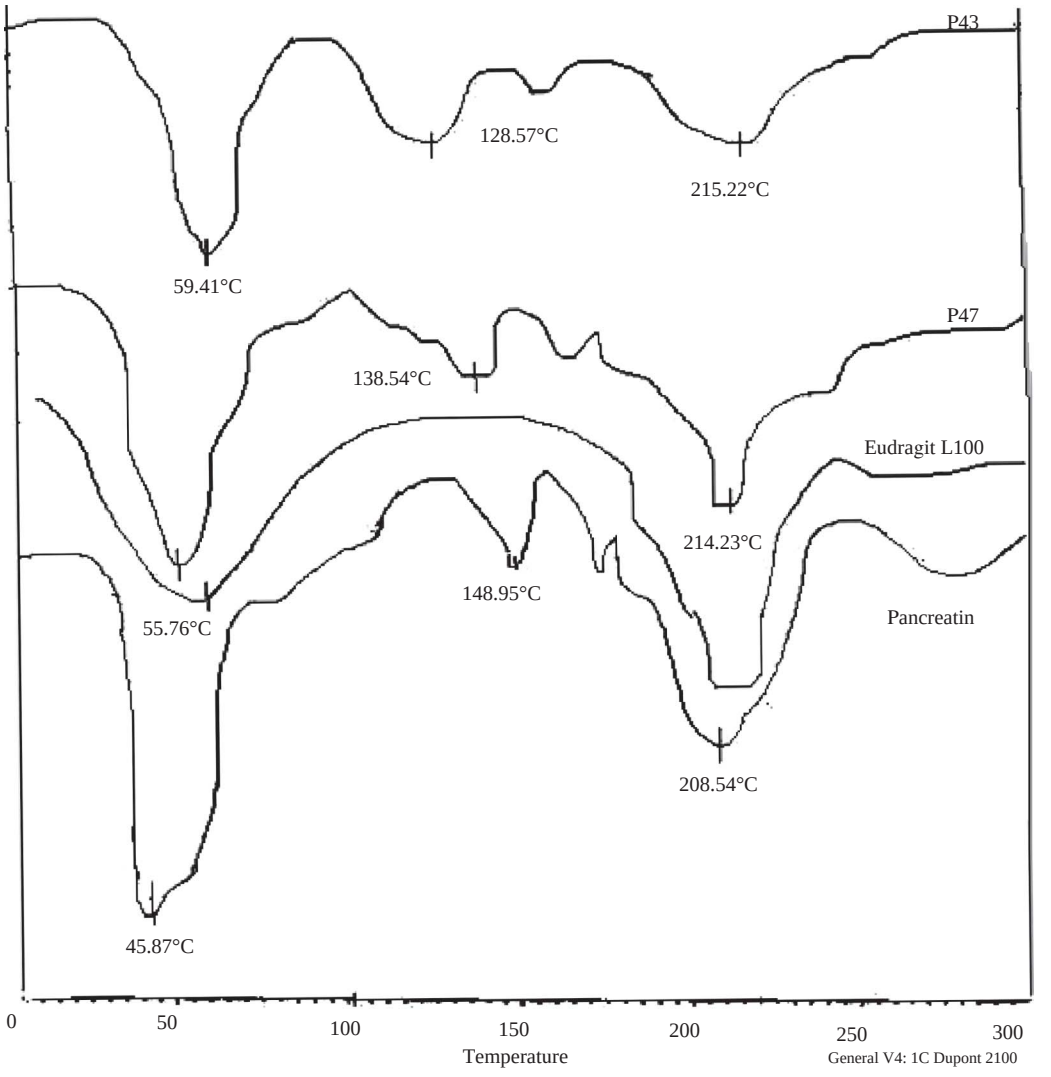

FIGURE 2. DSC thermogram of pancreatin microspheres prepared with eudragit L100 by solvent evaporation technique.

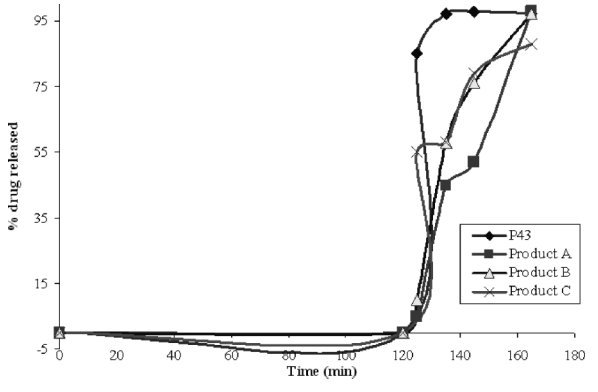

FIGURE 3. Comparison of in vitro release profiles of pancreatin microspheres with marketed tablets.

Repeated dose toxicity studies were performed to assess the safety of developed formulation. At the end of 90 days, animals were killed; organs were separated and subjected to histopathological studies. Result of serum sample analysis for albumin, alkaline phosphatase, alanine aminotransferase (SGPT), aspartate aminotransferase (SGOT), total bilirubin, and triglycerides (Table 6) indicated no significant difference in the values. Histopathological slides (Figure 5) indicated no structural changes in heart, brain, adrenals, lungs, esophagus, stomach, duodenum, small intestine, large intestine, and kidney.

CONCLUSION

Encapsulating pancreatin enzymes with eudragit L100 gave sufficient gastric resistance and released enzymes rapidly in alkaline pH. Microspheres prepared by solvent evaporation method with core : coat ratio 1:0.5 gave good protection against acid. By optimizing concentration of

TABLE 8
Stability Data of Developed Pancreatin Microspheres Tested for Enzyme Activities and Acid Resistance

Formulations	P20	P25	P31	P43	P47	P51
Protease Activity (%)						
Control	94.705	94.655	95.585	85.037	85.302	85.272
25°C/60% RH 6M	88.472	90.275	92.207	84.065	84.422	83.942
40°C/75% RH 3M	84.18	87.852	87.222	83.075	81.13	81.427
Amylase Activity (%)						
Control	95.437	94.542	96.792	84.077	85.867	85.367
25°C/60% RH 6M	87.672	89.7	93.11	83.415	85.35	84.162
40°C/75% RH 3M	77.482	84.527	82.417	83.027	82.007	83.93
Lipase Activity (%)						
Control	95.08	95.58	95.675	85.222	84.12	85.815
25°C/60% RH 6M	86.76	92.365	93.087	84.51	83.657	84.305
40°C/75% RH 3M	78.132	89.03	88.66	83.632	80.192	82.262
Acid Resistance (% release)						
Control	97.265	98.018	97.275	92.863	91.182	91.89
25°C/60% RH 6M	88.957	94.427	93.868	91.828	90.597	90.847
40°C/75% RH 3M	85.193	90.226	92.638	88.48	86.198	87.943

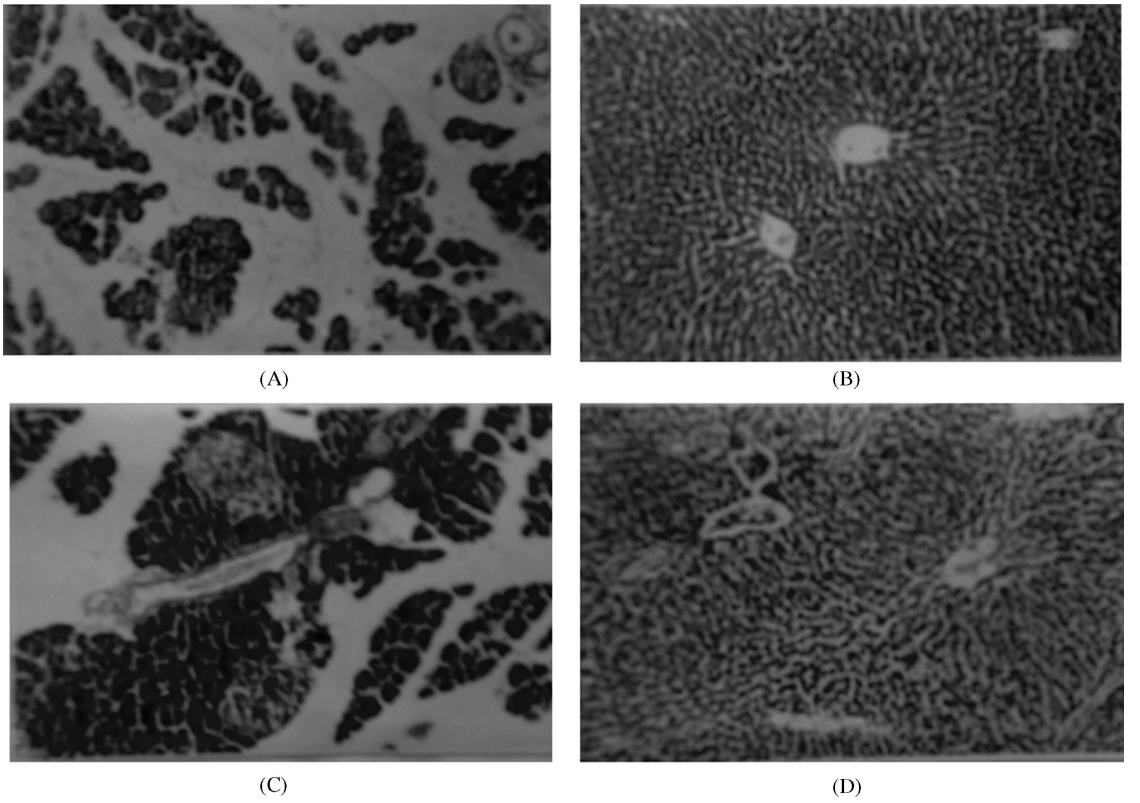

FIGURE 4. Histopathology of organs in induction of pancreatitis. (A) Pancreas, control group; (B) liver, control group; (C) pancreas, induced with chronic pancreatitis (5th week); (D) liver, induced with chronic pancreatitis (5th week).

TABLE 9
Results of Pathological Evaluation After Treatment

Group (Six Animals/Group)	Observations (Weeks)	Average Weight Gain (g)	% Fecal Fat (% Reduction)	Serum Triglycerides (mg/dL)	Serum Pancreatic Amylase (IU)	Serum Bilirubin (mg/dL)
Control	0	—	0.87	—	—	—
	2		1.24			
	4		0.85			
Group 1	0	12.5 ± 1.732	5.1	112.75 ± 7.932	824.25 ± 112.52	0.35 ± 0.129
	2		5.23	110.5 ± 8.426	818.5 ± 112.28	0.375 ± 0.141
	4		4.91 (1.207)	109.25 ± 7.974	809.0 ± 113.66	0.35 ± 0.058
Group 2	0	14.33 ± 6.713	5.87	119.67 ± 9.585	816.0 ± 58.968	0.35 ± 0.105
	2		5.91	116.5 ± 10.07	808.667 ± 53.571	0.4 ± 0.082
	4		5.74 (14.963)	112.83 ± 8.976	803.67 ± 51.875	0.35 ± 0.105
Group 3	0	20.166 ± 3.656	5.41	117.67 ± 3.83	761.67 ± 74.164	0.367 ± 0.127
	2		3.65	113.5 ± 3.937	758.0 ± 73.811	0.283 ± 0.0117
	4		3.52 (38.028)	108.83 ± 4.491	749.67 ± 77.405	0.367 ± 0.121

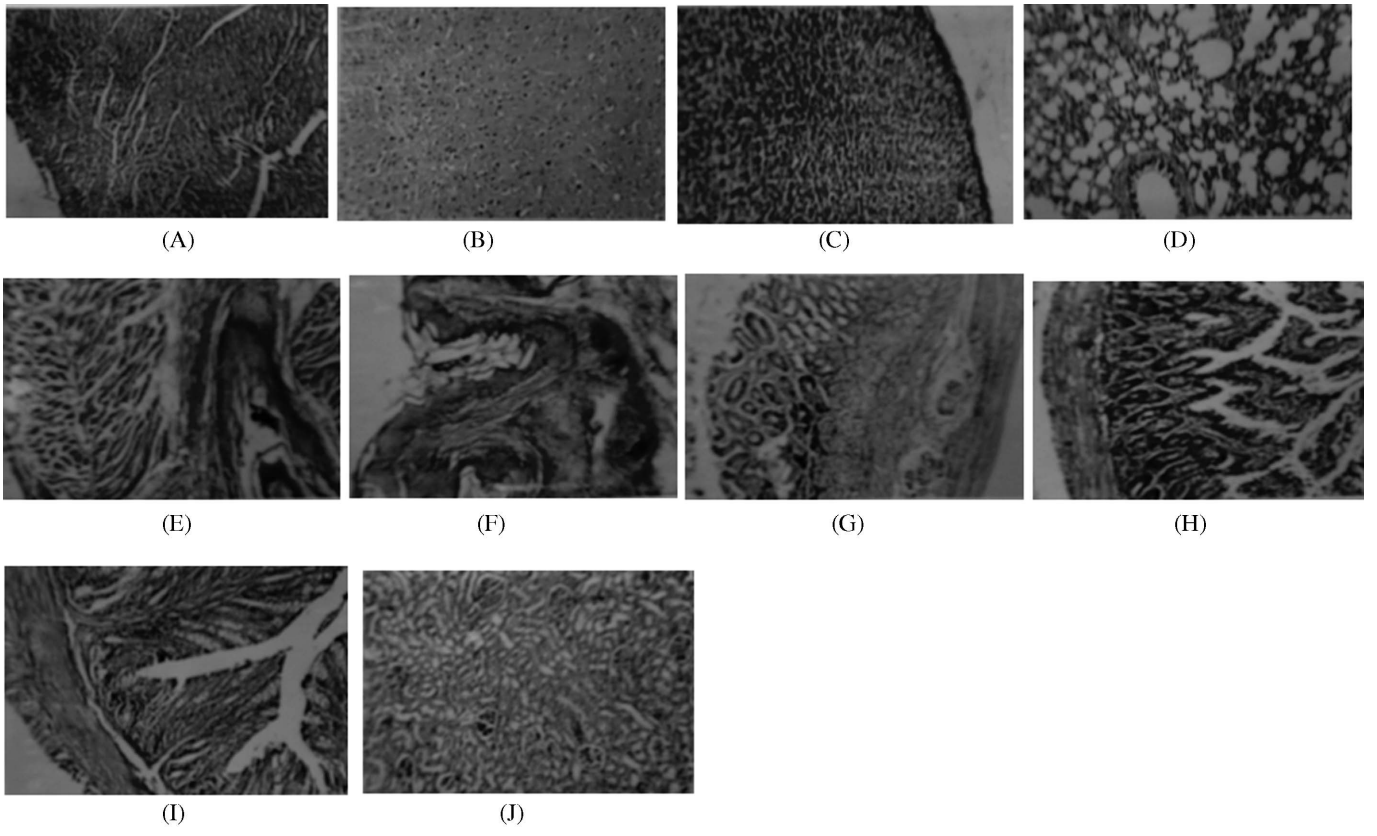

FIGURE 5. Histopathology of organs in repeat dose toxicity studies. (A) Heart, (B) brain, (C) adrenals, (D) lungs, (E) oesophagus, (F) stomach, (G) duodenum, (H) small intestine, (I) large intestine, and (J) kidney.

surfactant and dispersing powders used and stirrer speed, particle size was obtained in the range of 650–750 μm, which is a prerequisite for effective mixing of formulation with food contents.

Comparison of release profiles of pancreatin microspheres with marketed formulations indicated that prepared dosage forms certainly have an edge over the single-unit dosage forms, that is, tablets with respect to mixing efficacy. Thus, the

developed multiparticulate formulations of pancreatin enzyme microspheres have a potential for marketing and patenting.

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