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International Journal of Pharmaceutics 225 (2001) 31–39

international journal of pharmaceutics

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Polymethyacrylate based microparticulates of insulin for oral delivery: Preparation and in vitro dissolution stability in the presence of enzyme inhibitors

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Received 29 September 2000; received in revised form 24 January 2001; accepted 17 May 2001

Abstract

The purpose of this investigation was to (a) evaluate the coprecipitation technique for preparing microparticulates of insulin, (b) study the effect of variables such as addition of salts in the precipitating medium and ratio of polymeric solution to volume of precipitating medium on the dissolution and encapsulation efficiency of insulin microparticulates, and (c) evaluate the in-vitro enzymatic dissolution stability of insulin microparticulates in the presence of chicken ovomucoid (CkOVM) and duck ovomucoid (DkOVM) as inhibitors. Insulin dissolved in 0.01 N HCl was mixed with alcohol USP to get a final concentration of 32% v/v. Eudragit L100, a representative polymethyacrylate polymer, was then dissolved in this solution which was transferred to a beaker containing cold water with homogenization to obtain microparticulates. Dissolution studies were carried out in pH 6.8 phosphate buffer using a 100-ml conversion kit in a standard dissolution assembly. Dissolution stability of microparticulates was evaluated in the presence of 0.5 μM trypsin and 0.1 μM chymotrypsin at various ratios of CkOVM and DkOVM. The results indicated that insulin microparticulates could be prepared using the coprecipitation technique with high encapsulation efficiency by proper selection of experimental conditions and amount of polymer. Presence of salts in the precipitating medium decreased the dissolution of insulin from the microparticulates. As the ratio of precipitating medium with respect to the polymeric solution was increased, the encapsulation efficiency increased. In dissolution stability experiments, insulin was not detected in the presence of enzymes alone. When CkOVM and DkOVM were incorporated, the stability of insulin increased significantly in a concentration dependent fashion. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Insulin; Microparticulates; Coprecipitates; Inhibitors; Chicken ovomucoid; Duck ovomucoid; Dissolution stability

1. Introduction

Oral delivery of therapeutic proteins is limited due to barriers such as enzymatic degradation in the gastrointestinal tract, low epithelial permeability and instability under formulation conditions

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PII: S0378-5173(01)00740-2

(Lee et al., 2000). Extensive research literature is available that addresses approaches used to overcome these barriers. Among them, the use of enzyme inhibitors, permeation enhancers and enteric dosage forms are popular. A synergistic approach to overcome limited oral bioavailability of proteins has also shown promise (Carino and Mathiowitz, 1999).

Among the proteins, insulin has been most extensively investigated for delivery by the oral route. Oral delivery of insulin has been attempted with the use of sustained release polymeric systems with enzyme inhibitors and/or permeation enhancers. Polymeric systems attempted for insulin include enteric coated dosage forms and microencapsulation. Microencapsulation is preferred over coated dosage forms due to their ability to yield consistent microparticulates with limited amount of drug. Microparticulates/microspheres of insulin have been prepared with micro-cystalline cellulose (Trenktrog et al., 1996), polyvinyl alcohol (Kimura et al., 1996), and polymethylacrylates (Morishita et al., 1992a). Some of the disadvantages with these approaches include exposure of the protein to harsh processing conditions such as extrusion spheronization, long processing times to prepare the microparticulates (more than a day), rapid release (within 2 h), and lower encapsulation efficiencies (as low as 40%). The coprecipitation technique involves the precipitation of freely soluble polymer and drug mixture from a solution by addition of a non-solvent (Simonelli et al., 1969; Kislalioglu et al., 1991). Coprecipitates have been formulated as suspensions (Dalal and Narurkar, 1991), hard gelatin capsules (Walker et al., 1980) and directly compressible tablets (Fassihi et al., 1985). Microparticulate formation of proteins by coprecipitation has not been addressed adequately. It was of interest to see if insulin microparticulates can be prepared by this technique.

The role of ovomucoids in the oral delivery of insulin is being investigated in our laboratory. Ovomucoids represent a new class of enzyme inhibitors derived from the egg white of avian species. Extensive reviews detailing their source, active domains and mechanism of inhibitory action can be found elsewhere (Laskowski et al., 1960). Briefly, avian ovomucoids are present in egg white of avian species and account for roughly 10% of egg white of proteins. Their inhibitory activity is species dependent and mediated by binding to the corresponding enzyme through their reactive site. Since they inhibit digestive enzymes such as bovine trypsin and bovine α-chymotrypsin, they might be useful as absorption modifiers for oral delivery of insulin. The stability of insulin in the presence of CkOVM and DkOVM has been studied in our laboratory (Agarwal et al., 2000).

At the present time there is no defined method of selecting the amount of inhibitor in a protein dosage form. This is compounded by the fact that the stability of proteins is dependent on it susceptibility to different enzymes in the gastrointestinal tract. Alternatively, use of enzyme inhibitors need to be tailored to arrest protein degradation against specific degrading enzymes. If the dissolution stability of the protein from the enzyme and inhibitor systems is evaluated, proper selection of type and amount of inhibitor may be made for incorporation in the bioavailability studies.

The objectives of the present investigation were to: (1) evaluate the coprecipitation technique for the preparation of microparticulates of insulin, (2) study the effect of presence of salts in the precipitating medium and ratio of precipitating medium to the polymeric solution on formation of microparticulates, and (3) evaluate the enzymatic dissolution stability of microparticulates in the presence of inhibitors CkOVM and DkOVM.

2. Materials

Insulin was purchased from Intergen Company, Purchase, NY. Duck ovomucoid was provided by Dr Laskowski Jr., Department of Chemistry, Purdue University, IN. Eudragits, lactose, magnesium stearate were samples from Rohm Pharma, CHR Hansen, Mahawah, NJ, Whittaker Clarke and Daniels, South Plainfield, NJ. Talc was bought from Spectrum Chemical Company, Gardena, CA. Trypsin (Type VII, TPCK treated), chicken ovomucoid (Type II-O) and α-chymotrypsin (TLCK treated) were purchased from

Sigma Chemical Company, St. Louis, MO. Alcohol (USP grade) was purchased from Aaper Co., Shelbyville, KY. Solvents used were high-performance liquid chromatography (HPLC) grade. All other chemicals were of reagent grade and were used as received. Deionized water filtered using 0.2 μM filter under vacuum was used for all experiments.

3. Methods

3.1. Microencapsulation by coprecipitation

A representative figure of the experimental set-up is shown in Fig. 1. Insulin was dissolved in 0.01 N HCl to obtain a concentration of 100 IU/ml. Eight milliliter of this solution was added to a 17 ml of alcohol USP contained in a beaker under stirring by a magnetic stirrer rotating at 400 rpm. To this solution, 2 gm of Eudragit L100 was added over a period of 10 min. The polymeric solution was allowed to stir for additional 5 min to allow the polymer to dissolve completely. The solution was then transferred by a peristaltic pump from a fixed height to a beaker containing 100 ml cold water (4 °C) with homogenization (Pro 250, ProScientific, MD) at 10000 rpm. Homogenization was continued for an additional minute after the polymeric solution containing the drug was completely transferred. The suspended microparticulates were separated from the liquid by filtration under vacuum using a Whatman # 4 filter paper. The microparticulates were trans-

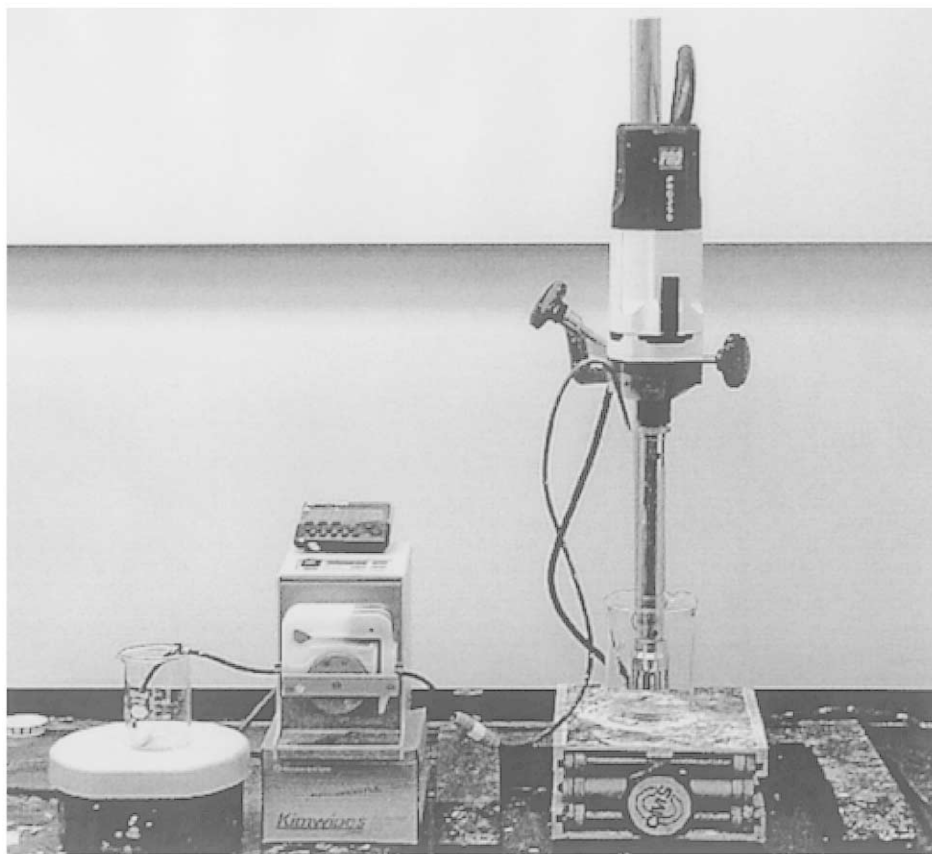

Fig. 1. Apparatus setup for preparation of microparticulates.

ferred to a porcelain dish and allowed to dry overnight in an oven set at 40 °C. They were then passed through sieve #40. Aggregates of microparticulates were milled in a mortar and pestle before passing through the sieves. The microparticulates retained were then weighed and transferred to a screw capped scintillation vial for further use.

To determine the enteric nature of the microparticulates, 25 mg of microparticulates were soaked in 10 ml of 0.1 N HCl that was equilibrated at 37 °C in a water bath. Following the immersion of the microparticulates for 2 h a sample was taken and analyzed by the HPLC method.

The particle size was determined by laser diffraction using a particle sizer (LS230, Beckman Coulter, Miami, FL). The sizing of the microparticulates was determined in a Micro Volume Model in mineral spirit. The sample was placed directly into the module and distribution was calculated using the Fraunhofer optical model. Data was collected over 60 s and particle size was expressed as volume percent mean diameter.

3.2. Yield of microparticulates and drug encapsulation efficiency

The yield of microparticulates was obtained by dividing the theoretical weight of polymer and drug used by the weight of the microparticulates obtained. The encapsulation efficiency was determined as follows. Twenty-five milligrams of microparticulates were added to 5 ml of alcohol USP contained in a 20 ml glass vial. After the microparticulates dissolved completely, 5 ml of phosphate buffer pH 6.8 was added to this solution. From this mixture, 0.5 ml was transferred to a HPLC vial, diluted to 1 ml with phosphate buffer, and analyzed by the HPLC method.

3.3. Addition of salts in the precipitating medium

Salts like calcium chloride, sodium sulfate and tween 80 were added at a concentration of 0.25% w/v in cold water during the microparticulate formation process.

3.4. Ratio of polymeric solution to volume of precipitating medium

The ratio of precipitating medium (cold water) was tested at polymeric solution to precipitating medium ratio of 1:2, 1:4 and 1:6.

3.5. Dissolution Studies

In vitro dissolution studies were performed in a dissolution apparatus (Vankel VK 600 Vandekamp Plus, Vankel, Cary, NC) fitted with a 100-ml conversion kit. Phosphate buffer USP pH 6.8 was used as the dissolution medium. The buffer was prepared by mixing 75% of 0.2 M tribasic sodium phosphate and 25% of 0.1 N HCl. The pH was adjusted to 6.8 with 2 M NaOH or 2 M HCl. The temperature of dissolution medium was 37 °C and the rotation speed of the paddles was set to 50 rpm. Microparticulates (125 mg) equivalent to 50 IU of insulin were filled in a size 00 capsule and transferred to the prewarmed dissolution medium. Samples (2 ml) were withdrawn every hour upto 6 h and the volume was replaced immediately by fresh phosphate buffer. Samples were analyzed by the HPLC method.

3.6. In vitro dissolution stability in the presence of enzyme inhibitors

The dissolution set up was the same as above except for the following modifications. The capsule contained microparticulates of insulin with various amounts of chicken and duck ovomucoid. The dissolution medium contained 0.5 μM trypsin for capsules containing CkOVM and 0.1 μM α-chymotrypsin for capsules containing DkOVM. Samples withdrawn (2 ml) were immediately treated with cold 1% v/v TFA/pH 6.8 buffer (2 ml) to stop the enzymatic activity. The samples were maintained at 8 °C in the autosampler through the duration of the analysis.

3.7. Insulin analysis

Insulin analysis was performed by a gradient RP-HPLC method. Compounds were separated on a C_{18} Vydac 218MS54 column (4.6 × 250 mm)

with a pore size of 300 Å and particle size of 5 μm. The mobile phase consisted of 0.05% v/v TFA/water (A) and 0.05% v/v TFA/acetonitrile (B). The gradient conditions were 27% B for 4 min and 27–36 % B in the next 11 min at a flow rate of 1 ml/min. The wavelength of detection was 210 nm. Under these conditions, the retention time of insulin was 10.8 min.

4. Results and discussion

4.1. Microencapsulation by coprecipitation

The coprecipitation technique involves dissolving the polymer and drug in an organic solvent and then adding a non-solvent to precipitate the drug and polymer. During the precipitation process, the polymer particles encapsulate the drug. Coprecipitates have been successfully prepared for drug polymer combinations of ibuprofen/Eudragit S100 (Khan et al., 1995), indomethacin/mixture of Eudragit RS100 and RL100 (Karnachi et al., 1995) and Ketprofen and Eudragit S100 (Khan et al., 1996). In these studies, alcohol was used as a solvent for dissolving polymer and drug. Cold water was used as a non-solvent for precipitation. The drug to polymer ratio used was as high as 10:1 and the particle size obtained was less than 800 μm.

The approximate range of intestinal transit of a dosage form in small intestine is between 3 and 6 h (Davis et al., 1986). The objective of this work was to evaluate coprecipitation technique for preparing microparticulates of insulin at low drug concentration for sustained release over 6 h. Extensive preliminary experiments were done to determine the effect of various formulation and process factors mentioned earlier. These factors were evaluated with respect to percentage yield of microparticulates, drug encapsulation efficiency and dissolution studies.

Our preliminary experiments indicate that insulin remains in solution with the polymer in 32% v/v mixtures of 0.01 N HCl and alcohol. However, below 32% v/v 0.01 N HCl, insulin is in the form of suspension in the polymeric solution. The stirring time of 15 min was fixed after initial

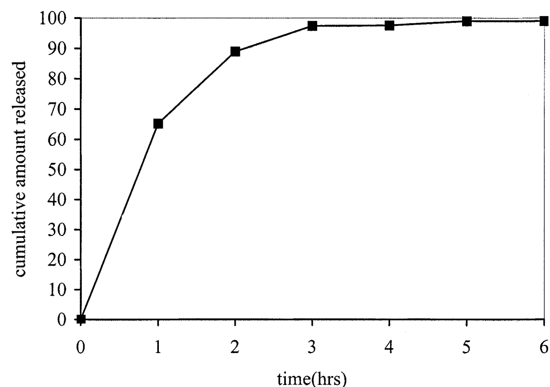

Fig. 2. Representative dissolution profile of insulin microcapsules (insmc) at pH 6.8.

experiments. It is important that the polymer be added slowly over a period of 10 min to avoid the formation of clumps. The additional 5 min of stirring time helps in the preparation of a clear polymeric solution containing the drug. The magnetic stirrer was used during mixing to avoid exposing the protein to high shear rates involved in the homogenizer. The addition of polymeric solution to the cold water was done with the help of a peristaltic pump to control the rate of addition. Precipitation of polymer microparticulates could be achieved by stirring with a homogenizer or a magnetic stirrer. The microparticulates obtained while precipitating with a magnetic stirrer had a tendency to agglomerate and stop the stirrer midway during the precipitation process. This problem could be averted by the use of homogenizer. A representative dissolution profile of a batch is shown in Fig. 2. The encapsulation efficiency for this batch was $95 \pm 2\%$ and volume of precipitating medium was 100 ml (ratio of 1:4 with respect to the polymer solution). This profile has been used for to evaluate the effect of variables.

Insulin was not detectable in the sample analyzed from the supernatant of microparticulates soaked in 0.1 N HCl for 2 h at 37 °C. This reveals that insulin is not present on the surface of the microparticulates and the microparticulates are enteric in nature.

The particle size distribution of the microparticulates was normal as indicated by a typical plot

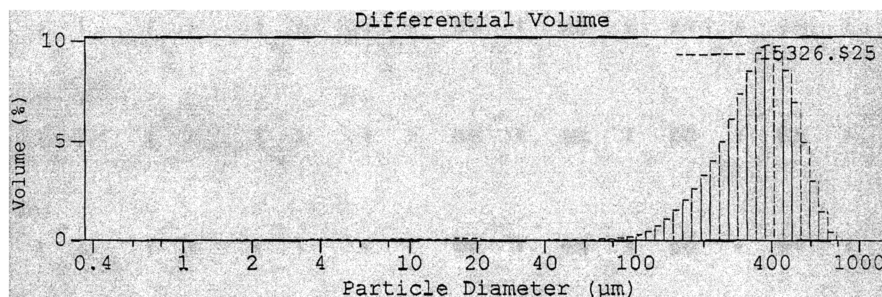

Fig. 3. Particle size distribution of microparticulates.

shown in Fig. 3. The average diameter of the microparticulates was 358.1. The range of particle size was between 184.6–541.6 μM.

4.2. Microparticulate formation efficiency

The yield of microparticulates obtained was only about 80%. The loss may be attributed to the polymeric solution sticking to the glass beaker used in transferring the solution to the precipitating medium. The encapsulation efficiency determined from an average of three runs was $95 \pm 2.0\%$. The chromatogram revealed the formation of desamido insulin that eluted at 11.8 min. The proportion of the peak area with respect to the insulin peak area was close to 5%. The proportion of buffer used in alcohol is critical for accurate determination of encapsulation efficiency.

4.3. Effect of salts in the precipitating medium

The addition of salts and surfactant in the precipitating medium was done with an intention to decrease agglomerate formation. In the microparticulate formation, electrolytes such as calcium chloride, sodium sulfate and surfactant such tween 80 were used at 0.25% w/v concentration in the precipitating medium. Comparison of dissolution profiles is shown in Fig. 4. As shown in the figure, both calcium chloride and sodium sulfate are slowing the release of insulin to some extent. This may be due to the increased likelihood of partial neutralization of the negative charge of insulin in phosphate buffer which is at a higher pH (pH 6.8) than the pH for the isoelectric point of insulin (pH 5.5). Similar to floculation of charged particles in the presence of oppositely charged electrolytes, the fraction of insulin microparticulates with increased diameter and less overall surface area may be greater. The decreased dissolution in the presence of surfactant, tween 80, may be due to increased encapsulation efficiency.

4.4. Effect of ratio of precipitating medium with respect to polymeric solution

The ratios of polymeric drug solution versus volume of precipitating medium investigated in our laboratory were 1:2, 1:4 and 1:6. The drug encapsulation efficiency was effected by the ratio. The range of encapsulation efficiency varied from 91.83 to 103.6%. The ratio of water volume to polymeric drug solution is critical for the forma-

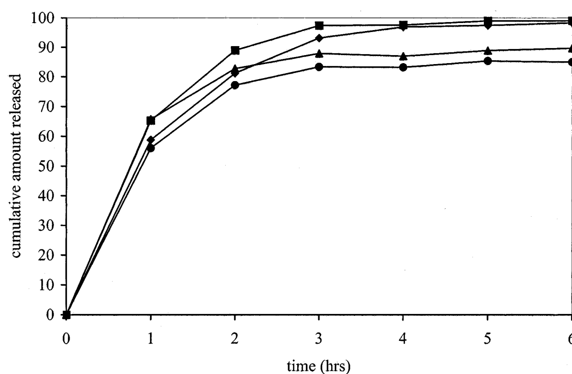

Fig. 4. Effect of salts in the precipitating medium on the dissolution of insulin microcapsules: control (■), sodium sulfate 0.25% (▲), calcium chloride 0.25% (◆) and tween 0.25% (●).

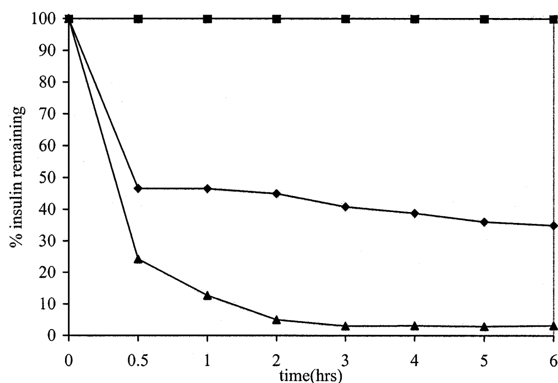

Fig. 5. Degradation of insulin solution (50 IU) in the presence of trypsin and α-chymotrypsin: control (■), trypsin 0.5 μM (◆) and chymotrypsin 0.1 μM (▲).

tion of microparticulates. Microparticulates can start forming only when the volume of water is more than a critical ratio with respect to the volume of polymeric solution. The ratio established for 8% ethanolic solution of Eudragit L100 in water for appearance of turbidity was reported as 1:1.38 (Kachrimanis et al., 2000).

4.5. *In vitro dissolution stability in the presence of enzymes*

In vitro dissolution data was generated for a representative batch whose characteristics are discussed in Section 4.1. Stability of insulin solution in the presence of trypsin and α-chymotrypsin is shown in Fig. 5. Dissolution stability of insulin released from microparticulates in the presence of 0.5 μM trypsin and various ratios of CkOVM is shown in Fig. 6. The control dissolution profiles in Figs. 5 and 6 represent the amount of insulin in the absence of trypsin. For both solution and microparticulate formulations, the area under the curve (AUC) for the dissolution profiles were calculated to compare the percentage of insulin available for absorption in the presence of trypsin. Comparison of the ratio of AUC values for insulin microparticulates at the end of 6 h is shown in Table 1. At the end of 6 h the percentage of insulin available for absorption from microparticulates is negligible. This may be explained by the kinetics of insulin degradation in the presence of trypsin. The degradation of insulin solution is maximum in the first hour and slow thereafter (Fig. 5). Insulin (%) remaining at the end of 1 h is close to 45%. Assuming a linear degradation rate of insulin solution in the first hour, this corresponds to a rate of degradation of 0.46 IU/min. The cumulative percentage insulin released from microparticulates in the first hour in the absence of trypsin is close to 60% (Fig. 6). Assuming a linear rate of release in the first hour, this corresponds to a rate of release of 0.5 IU/min. The rates of degradation and release are comparable, suggesting that insulin is undergoing spontaneous degradation following its release. Due to this extensive degradation in the first hour itself, insulin concentration does not accumulate enough to be detected even at the end of 6 h.

In the presence of chicken ovomucoid, insulin (%) remaining for absorption increases in a concentration dependent fashion. From Table 1 it can be seen that insulin (%) available increases from 17.75% at 1:2 ratio of enzyme to inhibitor to 24.70% at 1:4 ratio. This represents a substantial improvement in the percentage of insulin available for absorption.

Stability of insulin in the presence of α-chymotrypsin is extremely poor. During the course of degradation of insulin solution for 6 h, insulin remaining is almost reduced to a negligible value (Fig. 5). Rate of degradation of insulin solution in

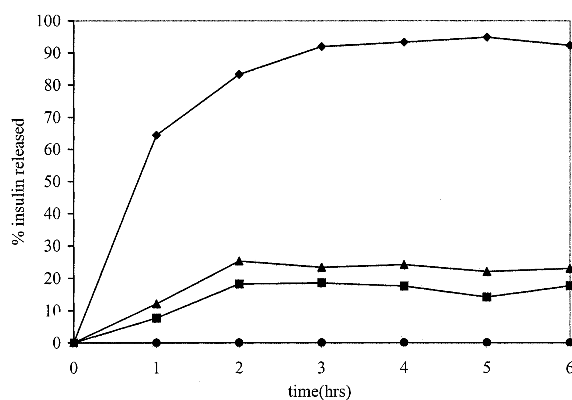

Fig. 6. Dissolution stability of insulin released from microcapsules in the presence of trypsin and CkOVM: insmc (◆), insmc + tryp:ckovm 1: 2 (■), ins + tryp:ckovm 1:4 (▲), insmc + tryp (●).

Table 1
Cumulative amount of insulin available at the end of 6 h in the presence of 0.5 μM trypsin and 0.1 μM chymotrypsin

Formulation	AUC (% h)	Ratio (%)
Solution (sol)		
Sol (no enzymes)	600	
Sol+trypsin (T)	261.33	43.55
Sol+chymotrypsin (CT)	62.24	10.37
Microparticulates		
Control (no enzymes)	479.36	
MC+T	Beyond detection limit	Beyond detection limit
MC+T+CkOVM (1:2)	85.08	17.75
MC+T+CkOVM (1:4)	118.45	24.70
Microparticulates		
MC (no enzymes)	479.36	
MC+CT	Beyond detection limit	Beyond detection limit
MC+CT+ DkOVM (1: 1)	111.71	23.3
MC+CT+ DkOVM (1:2)	190.81	39.40
MC+CT+ DkOVM (1:4)	202.95	42.3

the first hour is 0.75 IU/min while the rate of dissolution remains the same. Insulin solution available for absorption was calculated to be only 10.37% as shown in Table 1. Due to this reason, insulin released from microparticulates is extensively degraded and not detectable in the presence of α-chymotrypsin alone (Fig. 7). The reason may be same as explained above. In the presence of DkOVM, there is a considerable improvement in stability of insulin released from microparticulates at the end of 6 h. Cumulative percentage of insulin remaining in the presence of various ratios of α-chymotrypsin and DkOVM is shown in Fig. 7. As shown in Table 1, the ratio of insulin remaining for absorption steadily increased from 23.3% in the presence of 1:1 concentration of enzyme to inhibitor to 42.3% at 1:4 ratio.

Oral delivery of proteins will be extremely difficult without the use of an absorption modifier. The absorption modifier may be an agent that increases permeability of the protein under study or an enzyme inhibitor that improves stability of the protein in the gastrointestinal tract. An enzyme inhibitor may be required to improve bioavailability of the protein even if permeation enhancement is the goal. There are many reports of bioavailability studies of insulin with enzyme inhibitors in various animal species like rats (McPhillips et al., 1997) and dogs (Ziv et al., 1994). The amounts of enzyme inhibitors used have been selected without performing in vitro stability studies in the presence of pancreatic enzymes. Since variations exist in concentration of pancreatic enzymes with regards to the species under study, it may be helpful to evaluate the stability before hand. In vitro dissolution stability may serve as an effective screening tool to evaluate the effectiveness of various concentrations of inhibitor in the presence of enzymes. This will allow incorporation of practical amounts of enzyme inhibitor targeted towards inhibition of pancreatic enzymes. In vitro dissolution stability of a protein in the presence of enzymes has not been reported. Single time point measurements have been used in some studies to evaluate the in vitro efficacy of inhibitors against specific enzymes (Morishita et al., 1992b; Bernkop-Schnurch et al., 1997). The parameter used in our study, percentage of protein available for absorption, may serve

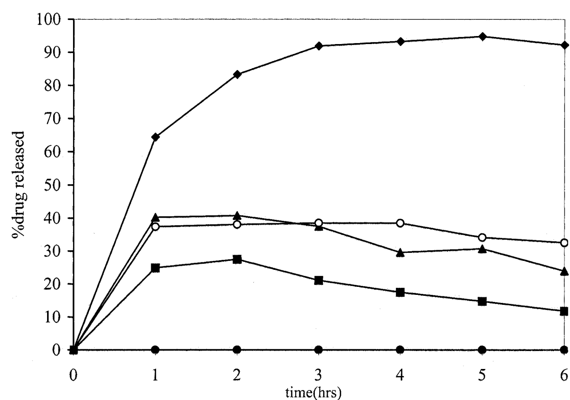

Fig. 7. Dissolution stability of insulin released from microcapsules in the presence of α-chymotrypsin and DkOVM: insmc (◆), insmc + ctryp:dkovm 1:1 (■), insmc + ctryp:dkovm 1:2 (▲), insmc + ctryp:dkovm 1:4 (○), insmc + ctryp (●).

as a better indicator for evaluating the efficacy of inhibitors because it incorporates the complete dissolution profile in the calculation.

5. Conclusions

Microparticulates of insulin have been prepared by the coprecipitation technique with appropriate combination of factors and choice of polymer. Among the factors studied, the addition of salts in the precipitating medium and ratio of polymeric solution to volume of precipitating medium had an effect on the dissolution of insulin and encapsulation efficiency. Dissolution stability studies indicates that the percentage of insulin remaining for absorption increased significantly in the presence of CkOVM and DkOVM. Dissolution stability studies may serve as valuable indicators for judicious choice and concentration of the inhibitor for oral protein dosage forms.

Acknowledgements

Dean Nelson and Dr Smith are acknowledged for their support in the establishment of Center for Drug Delivery and Formulation Development. We acknowledge the timely help of Ed Hoff at Beckman Coulter for particle size analysis.

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