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Polymethacrylate based microparticulates of insulin for oral delivery, part II: solid state characterization

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Received July 7, 2007, accepted August 1, 2007

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Pharmazie 63: 122–128 (2008) *doi: 10.1691/ph.2008.7211*

The objectives of the present study were (A) to characterize insulin microparticles prepared by the coprecipitation process by size exclusion chromatography, differential scanning calorimetry, fourier-transform IR spectroscopy, and powder X-ray diffractometry, and (B) to study the solid state conformation of insulin before and after entrapment in the polymeric carrier. Microparticles were prepared by dissolving insulin in 0.01 N HCl and 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. Insulin powder, microcapsules, and a physical mixture of insulin and Eudragit L100 were then analyzed by SEC-HPLC, DSC, FTIR, and XRD to observe changes in protein conformation as result of the manufacturing process. While DSC, XRD and FTIR results were of limited value due to limits of instrument sensitivity, size exclusion chromatography data indicated that higher order aggregates were not formed during microcapsule formation. It was concluded that formulating insulin into microparticles by the coprecipitation process is an attractive and stable method for protein delivery and might be suitable for oral delivery of insulin.

1. Introduction

There is a growing interest in the oral delivery of therapeutic proteins such as insulin (Agarwal and Khan 2001a). The oral delivery of insulin, however, is limited due to barriers such as enzymatic degradation in the gastrointestinal tract, low epithelial permeability, and instability under formulation conditions (Lee et al. 2000). Extensive research has been reported in the literature that addresses approaches used to overcome these barriers. Among them are polymeric systems such as enteric coated dosage forms and microencapsulation.

Microencapsulation by extrusion/spheronization has the advantage of yielding consistent microparticulates with limited amount of drug. Microcapsules of insulin have been prepared using a variety of polymers, such as microcystalline cellulose (Trenktrog et al. 1996), polyvinyl alcohol (Kimura et al. 1996), and polymethylacrylates (Morishita et al. 1992a). This process, however, suffers from several disadvantages such as the exposure of the protein to intense processing conditions, long processing times, rapid protein release from the microcapsules, and low encapsulation efficiencies.

Alternatively, the coprecipitation technique, which involves the precipitation of freely soluble polymer and drug mixture from a solution by addition of a non-solvent (Kislalioglu et al. 1991) have used to overcome some of the limitations observed with the microencapsulation process. The formation of insulin microparticulates by coprecipitation has been previously reported (Agarwal et al. 2001b). In this preliminary study the coprecipitation process and analysis methods were optimized and the dissolution of insulin from the microparticles was examined. It was found that consistent microparticles could be prepared with well defined and controlled protein release rates.

The processing of insulin into microparticles, however, exposes the protein to harsh processing conditions. There is a possibility that formulation excipients may also interact with the protein, altering its conformation. This may lead to a loss in potency and biological activity. Therefore, it is important to characterize the protein once it is incorporated into a drug delivery system to test for conservation of biological activity. The test method has to be suitable for characterization of the protein in its pure form and also in the presence of excipients. Proteins have to be characterized for change in conformation, size, shape, surface properties and bioactivity upon formulation processing. Changes in conformation, size and shape can be observed by the use of spectrophotometric techniques, X-ray diffraction, differential scanning calorimetry, (DSC) light scattering, electrophoresis, ultracentrifugation and gel filtration. Changes in surface properties can be detected by

the use of electrophoretic and chromatographic techniques. Changes in the bioactivity of the proteins can be observed by bioavailability studies. Selection of a particular technique is based upon sensitivity of the technique, the system under study, and availability of equipment. The interference by formulation excipients may also play a major role in selection of the characterization technique. Theories about selected techniques used for the characterization of proteins have been reviewed by Pearlman and Nguyen (1991) and Hoffmann (2000).

From the above discussion, it can be inferred that a variety of characterization methods are available for characterization of proteins after they were processed into microparticles. For insulin, RP-HPLC and SEC-HPLC would indicate changes in primary structure and DSC, FT-IR and powder X-ray diffraction would indicate changes in secondary and tertiary structure. Changes in primary structure are irreversible whereas denaturation may be reversible or irreversible. The objective of the present investigation was therefore to utilize these techniques to characterize insulin microparticle prepared by the coprecipitation process and study the solid state conformation of insulin before and after entrapment in the polymeric carrier.

2. Investigations, results and discussion

2.1. Differential scanning calorimetry (DSC)

DSC is useful to detect changes in secondary and tertiary protein structure when incorporated in polymer matrices. As a protein is heated, the transition from native to folded state is accompanied by appearance of an endothermic peak on a DSC. The transition temperature, T_m, is analogous to the melting of a crystal and is affected by environmental conditions. A shift in T_m indicates change in the denaturation temperature. This is dependent on the conformation of the protein.

DSC has been used to determine the denaturation endotherms of amorphous and crystalline insulin (Pikal and Rigsbee 1997).

Figures 1–4 represent the DSC thermograms of insulin powder, polymer (Eudragit L100), physical mixture of insulin and polymer, and microparticles of insulin, respectively. The solid lines represent the original thermograms and the dashed lines represent the processed thermograms (first derivative of the original thermogram). From Fig. 1, it can be seen that the insulin thermogram is characterized by broad and weak endotherms within the temperature range of 100–150 °C and a melting endotherm (T_m) in the range of 200–225 °C. Both types of endotherms observed are irreversible. This means that if a sample is scanned through the endotherm and then cooled and re-scanned, the second scan does not show the endotherm. These kinds of broad endotherms have been observed for freeze-dried human growth hormone, bovine somatotropin and several other proteins (Bell et al. 1995). All these thermograms are characterized by the absence of a sharp increase in baseline near the glass transition temperature. Proteins melt in solid state at high temperature due to extensive degradation that occurs due to unfolding.

The advantage of processing of the endotherms by calculating the first-derivative of the original thermogram include improved accuracy, improved peak resolution and quantitative determinations (Ford and Timmins 1989).

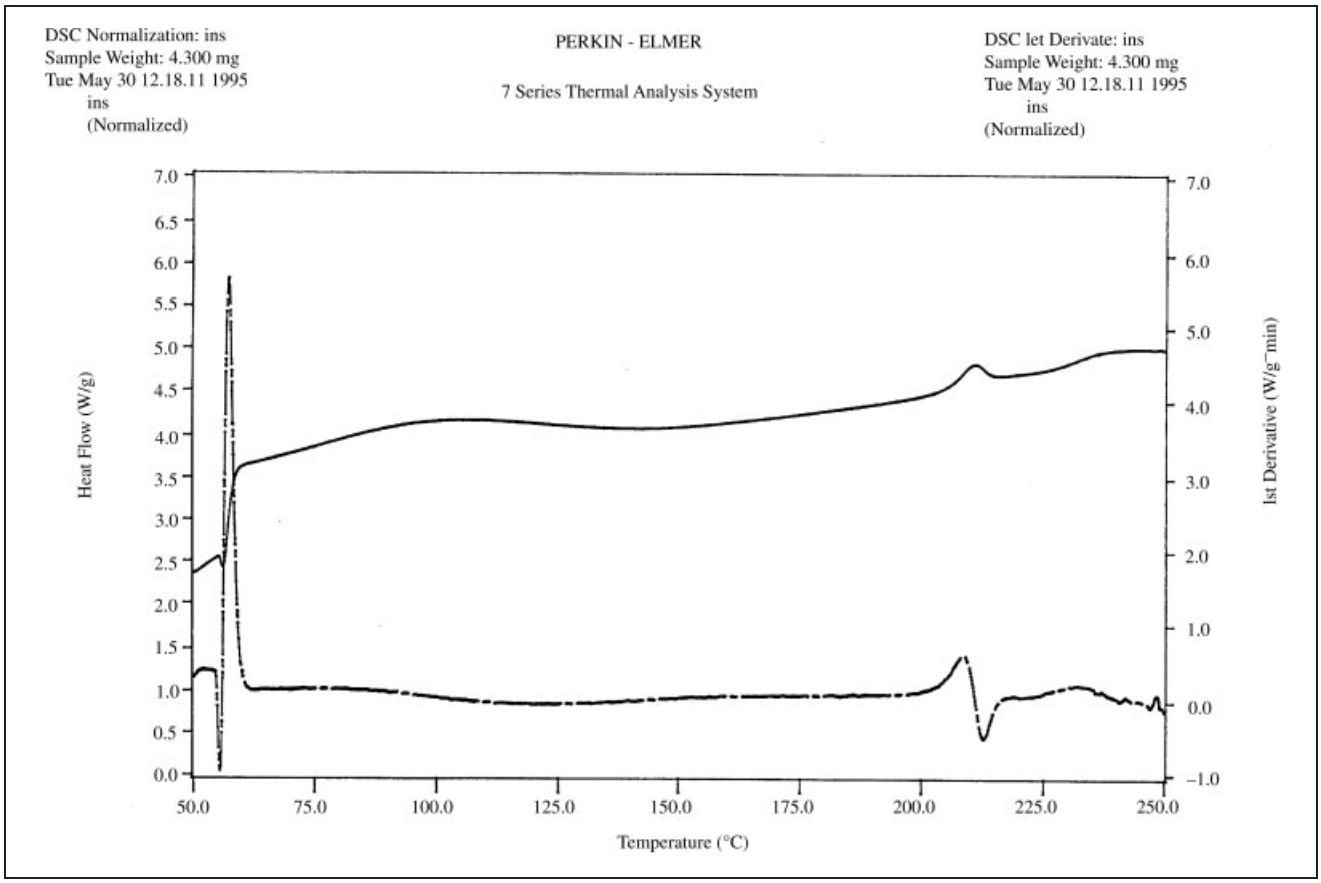

Fig. 1: DSC thermogram of insulin powder. The solid line represents the normal thermogram and the dashed line represents the first derivative processed thermogram

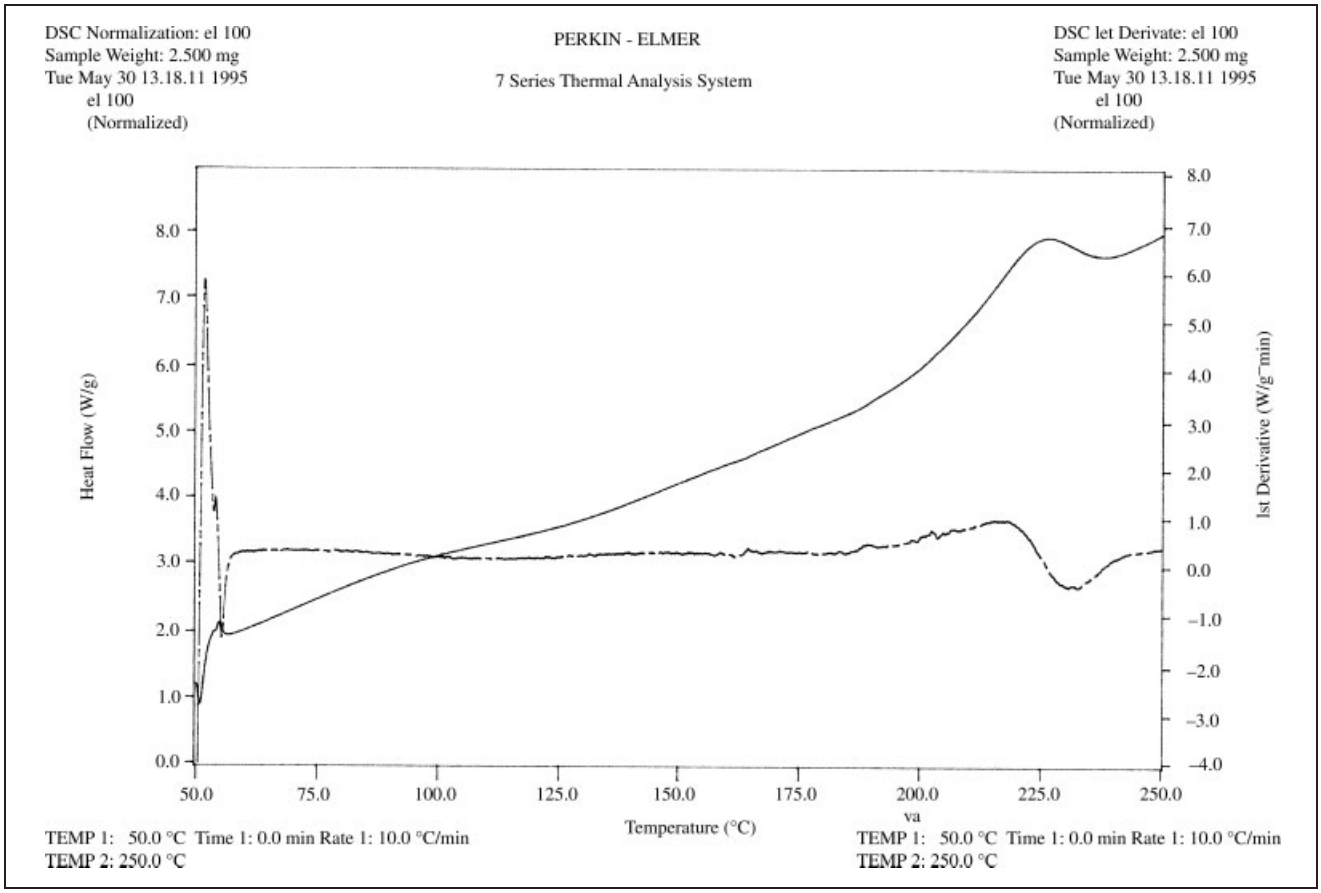

Fig. 2: DSC thermogram of Eudragit L100. The solid line represents the normal thermogram and the dashed line represents the first derivative processed thermogram

Odd derivative curves (1st, 3rd, 5th, etc.) are useful in resolution enhancement of single and overlapping curves. This is especially useful to get the onset of melting temperature in the present case where the thermograms of insulin and polymer overlap in the 200–225 °C region and insulin loading in the physical mixture and microcapsules is extremely low (< 2% of total weight). The calculation of onset of denaturation temperature is difficult from the original thermograms of physical mixtures (Fig. 3) and microcapsules of insulin (Fig. 4). From the processed thermograms, the onset of melting temperature of insulin in pure form was calculated to be 209 °C (Fig. 1). The onset of melting shifted to 201 °C in the case of physical mixture and microcapsules of insulin (Figs. 3 and 4). This indicates that there may be a physical interaction between insulin and polymer in the solid state. However, this interaction should be confirmed by other characterization procedures. Conditions that cause an increase in the T_m for a particular protein promote greater physical stability for the protein by providing greater resistance to thermal denaturation. Excipients that cause an increase in T_m provide for greater physical stability and excipients that cause a decrease in T_m was found to decrease physical stability (Lee and Timasheff 1981; Lee and Lee 1987; Manning et al. 1989). The data from the DSC experiments should be interpreted with caution. It has been reported that some proteins undergo aggregation and precipitation upon thermal denaturation. Also, broadening of peaks leading to a shift in area, onset or peak temperature may be simply due to mixing of components without indicating an interaction (Ford and Timmins 1989).

2.2. Powder X-ray diffraction

The diffraction of X-rays by crystalline substances is of great analytical interest, since two compounds would never be expected to form crystals in which the three-dimensional spacing of planes is totally identical in all directions. A powdered sample will exhibit all possible lattice planes, and the diffraction of the sample will provide information on all possible atomic spacings of the crystal lattice. The pattern consists of a series of peaks at different angles. These angles and their intensities are correlated with the d-spacings (distance between two planes in a crystal) to provide a full crystallographic characterization of the powdered sample. X-rays have wavelengths in the range 10^{-8}–10^{-6} cm which is sufficient to allow determination of interplanar distances between the molecules.

Powder X-ray diffraction chromatograms of proteins are not regularly done due to lack of crystallinity, low intensity peaks, and the requirement of a large sample size. X-ray diffractograms of insulin have been obtained with mixtures of lactose and mannitol to compare the effect of spray drying on the crystalline changes in insulin (Forbes et al. 1998).

Powder X-ray diffractograms of insulin, polymer (Eudragit L100), physical mixture of insulin and polymer and microcapsules of insulin are shown in Fig. 5. The diffractogram of insulin is associated with low intensity or broad peaks indicating lack of crystallinity. This is consistent with the observation that most of the proteins are isolated by lyophilization as amorphous powders. The diffractogram of polymer is devoid of sharp peaks indicating its

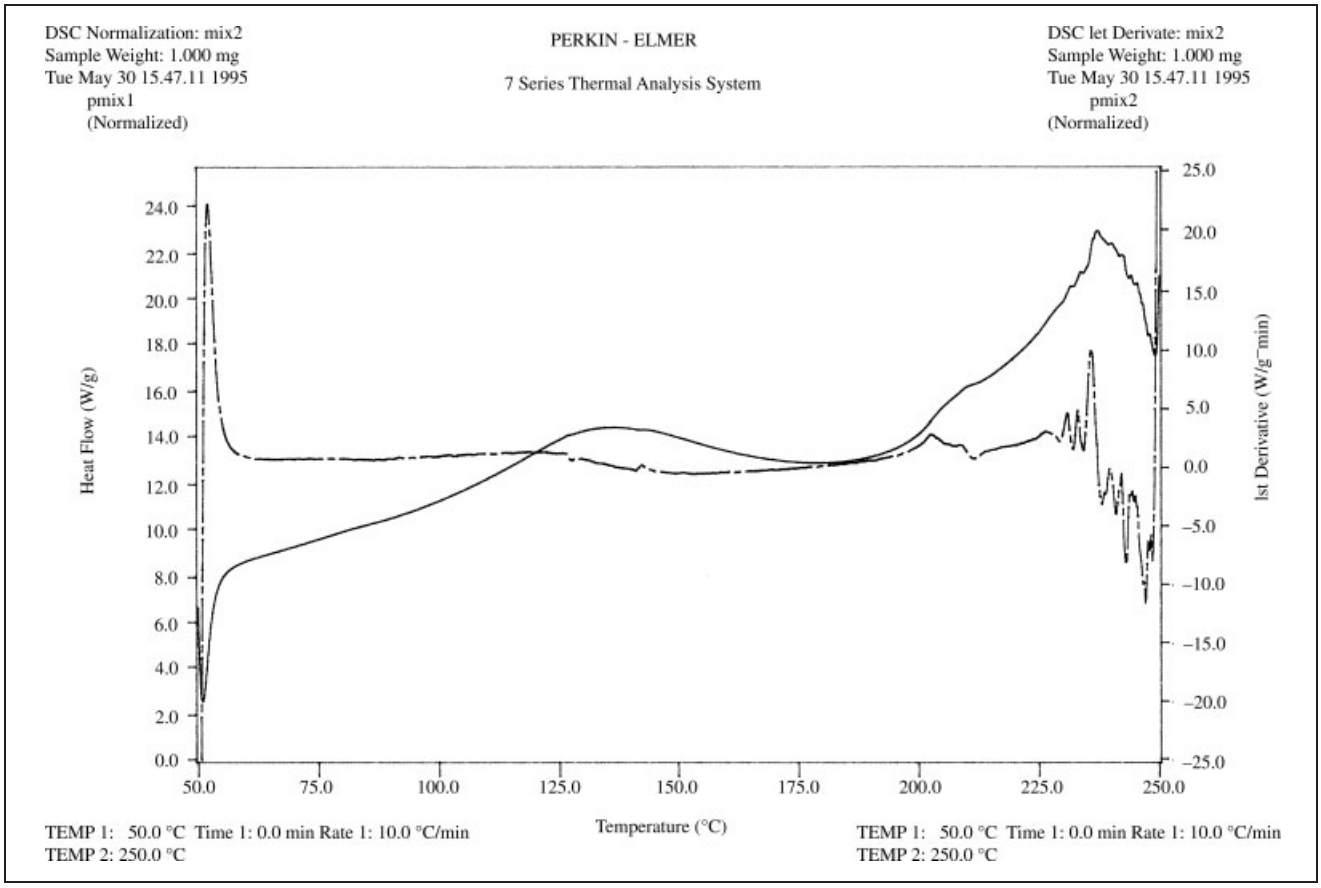

Fig. 3: DSC thermogram of physical mixture of insulin and Eudragit L100. The solid line represents the normal thermogram and the dashed line represents the first derivative processed thermogram

amorphous nature. The diffractograms of physical mixture and microcapsules are almost identical. It is difficult to ascertain from the X-ray data if there is any change in the structure of the insulin in the microcapsules.

2.3. Fourier transform infrared spectroscopy (FT-IR)

FT-IR also provides an estimate of secondary structure composition (Susi and Byler 1986). This method uses special deconvolution methods to separate and integrate overlapping amide I infrared absorption bands associated with α-helix, β-pleated sheet, and random structures. In this method, the spectrum is related to the subtle effects of regular secondary structure on the energetics (vibrational frequency) of amide groups in the peptide linkage. FT-IR also has the advantage of being able to evaluate the structure aspects of the protein in solid state.

FTIR scans of insulin, polymer, 2% insulin in polymer 50 : 50 mixture of insulin and polymer and microcapsules are shown in Fig. 6. The band at 1659 cm^{-1} in the case of insulin corresponds to the α-helix region of the secondary structure. The spectra of polymer shows a characteristic peak of the carboxyl group at 1705 cm^{-1} and of the esterified carboxyl group at 1730 cm^{-1}. From the figure, it can be seen that the spectra of 2% mixture of insulin and polymer and microcapsules of insulin are identical. The band of α-helix is missing from both spectra. This result should be interpreted with caution. Attenuation of alpha-helix band is associated with a change in secondary structure of protein (Pikal and Rigsbee 1997). In the present case, it is possible that insulin loading was below the detection limits of the instrument. To strengthen this argument spectra of a 50 : 50 mixture of insulin and polymer was generated. The appearance of the α-helix band exactly at 1659 cm^{-1} is clearly seen in the figure. This indicates that the presence of polymer does not alter the secondary structure of insulin. The spectra of microcapsules is difficult to interpret due to the low loading of insulin. It does not clearly indicate the presence of insulin α-helix band.

2.4. Size exclusion chromatography

Size exclusion chromatography detects changes in size of the protein under formulation conditions. Based on principles of gel filtration, separation of macromolecules occurs because different sized molecules diffuse into the column matrix pores to a different extent during their passage along the column. Since smaller molecules diffuse into the pores more readily, they elute more slowly than do larger species. It is common to refer to separation of proteins to be based on the "size" of analytes, when in fact the separation also depends on shape of the proteins. This is because the shape also determines entry of the protein into the gel matrix.

Size exclusion chromatography with RP-HPLC has been used to determine the formation of covalent insulin dimers with trace amounts of high molecular weight transformation products after microencapsulating insulin in mixture of poly (DL-lactide-co-glycolide) and poly (L-lactide) (Shao and Bailey 2000). The applications of RP-HPLC arise due to the nature of the interaction between the stationary phase and the surface of the protein. Separation by RP-HPLC involves interaction of the surface hydrophobic

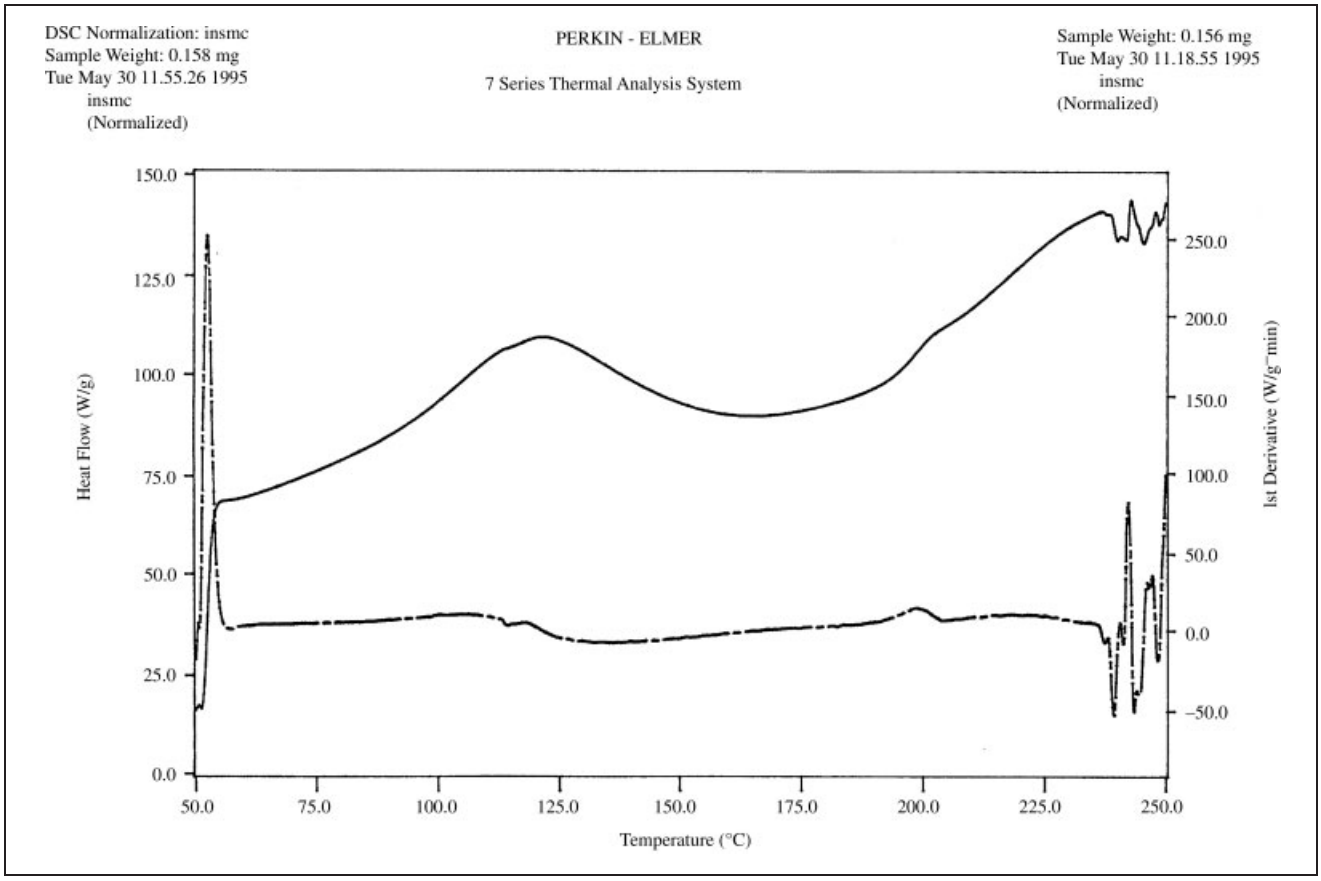

Fig. 4: DSC thermogram of insulin microparticles. The solid line represents the normal thermogram and the dashed line represents the first derivative processed thermogram

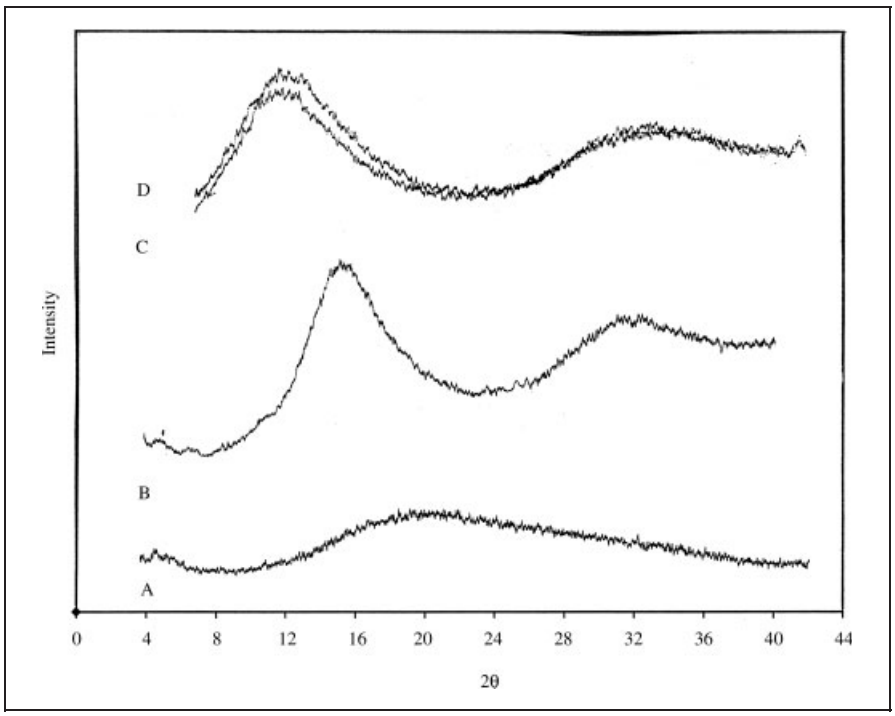

Fig. 5:
Powder X-ray diffractograms of insulin (A), Eudragit L100 (B), physical mixture of Eudragit L100 and insulin (C) and microcapsules of insulin (D)

areas of proteins with alkyl-bonded stationery phase. Elution of adsorbed proteins is produced by an increasing gradient of organic modifier such as isopropanol or acetonitrile. An ion-pairing agent such as triflouroacetic acid (TFA) is added to minimize interactions between the protein and unreacted silanol groups on the stationery phase. Reverse Phase HPLC is routinely employed for analysis of insulin. It has also been used to separate insulin from desamido insulin, higher order aggregates and other derivatives. The USP RP-HPLC method is an acceptable alternative to the rabbit bioassay for insulin, except in the case of highly purified insulins.

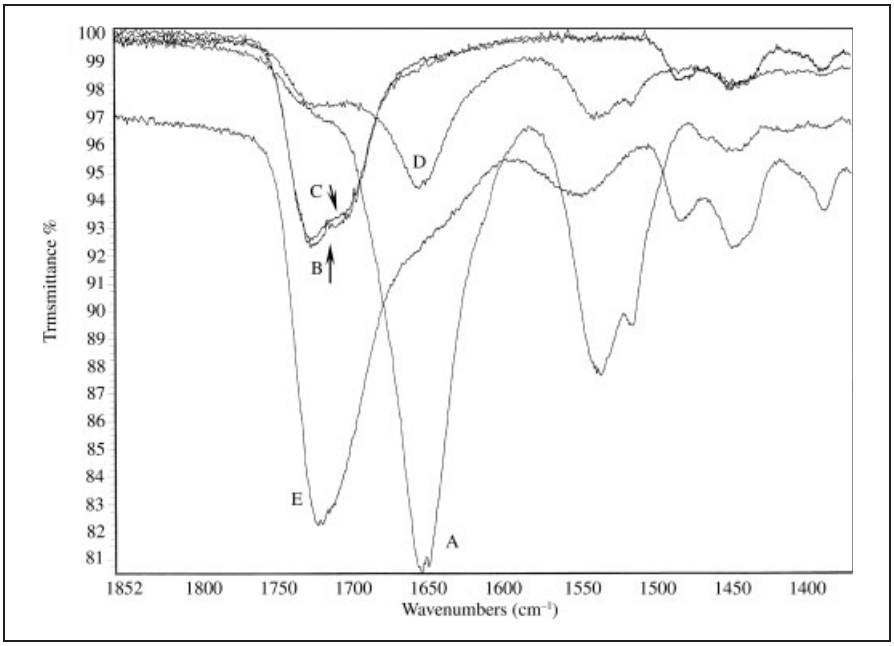

Fig. 6:
FT-IR spectra of insulin (A), polymer (B), physical mixture of 2% insulin and polymer (C), physical mixture of 50 : 50 insulin and polymer (D), and insulin microcapsules (E)

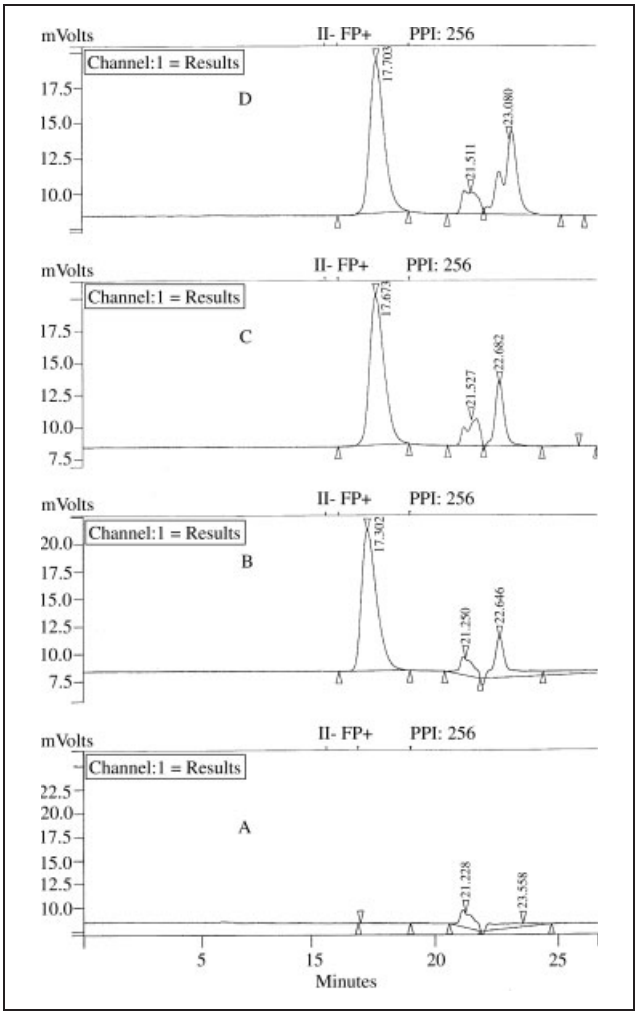

Fig. 7: SEC chromatogram of blank (A), insulin (B), physical mixture of insulin and Eudragit L100 (C) and insulin extracted from microcapsules in pH 6.8 buffer (D)

SEC chromatograms of insulin, 2% insulin and polymer mixture and insulin extracted from microcapsules in phosphate buffer is shown in Fig. 7. The insulin peak at 17.3 min corresponds to the monomeric form. Dimers and higher order aggregates have not been observed at the concentration studied. The presence of polymer or processing conditions could have led to formation of covalent aggregates in the mixture or microcapsules. If they were formed, the dimers would have eluted before the peak of insulin. Formation of dimers and higher order aggregates have been observed during the formation of polyester microspheres of insulin (Shao and Bailey 2000) under identical chromatography conditions. From the chromatograms of insulin in the physical mixtures and that extracted from the microcapsules, it can be seen that there are no additional peaks. This indicates that aggregate formation did not occur due to the presence of polymer or processing conditions.

3. Experimental

3.1. Materials

Insulin was purchased from Intergen Company (Purchase, NY). Eudragit L100 was provided by Röhm Pharma (Parsippany, NJ). Alcohol (USP grade) was purchased from Aaper Co. (Shelbyville, KY). Solvents used were 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.

3.2. Microencapsulation by coprecipitation

Insulin microparticles were prepared by the coprecipitation technique previously reported by Agarwal et al. (2001b). Briefly, insulin was dissolved in 0.01 N HCl to obtain a concentration of 100 IU/ml. Eight milliliter of this solution were 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 g Eudragit L100 was added over a period of 10 min. The polymeric solution was stirred 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 10,000 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 transferred 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.

3.3. Differential scanning calorimetry

DSC was performed using DSC 7 (Perkin Elmer, Waltham, MA) pre-calibrated with indium standards. Analysis was performed on insulin powder,

physical mixture of insulin and Eudragit L100 and insulin microparticle. Three to 20 mg samples were accurately weighed in aluminum pans. Pans were covered with aluminum lids and then sealed. An empty aluminum pan was used as a reference. Samples were heated from 50 °C to 250 °C at a scan rate of 10 °C per minute under an atmosphere of nitrogen. After completion of the run, the thermograms were normalized to one milligram and the melting endotherms of the peaks were recorded.

3.4. Powder X-ray diffraction

The powder X-ray diffraction (PXRD) patterns of insulin, Eudragit L100, physical mixture, and microparticles obtained using a Philips Norelco diffractometer (Philips Analytical Inc., Natick, MA) fitted with a copper target. Before analysis, insulin (50 mg) was suspended in acetone and deposited on a glass slide until the acetone evaporated. Measurements were carried out using 40 kV voltage and 20 mA current. Samples were scanned from 10° 2θ to 40° 2θ at a rate of 2θ/min.

3.5. Fourier transform infrared spectroscopy

FT-IR spectroscopy was done with an Attenuated Total Reflectance (ATR) accessory. The samples analyzed were insulin powder, physical mixture of insulin and polymer and insulin microparticles. The samples were run on a on-bounce diamond ATR accessory DurasampleIR (SensIR Technologies, Danbury, CT). The insulin sample (4 mg/mL) was prepared by dissolving in a 50 : 50 mixture of 0.1 N HCl and alcohol. The polymer sample (4 mg/mL) was prepared by dissolution in alcohol. A 2% solution and a 50% solution of insulin with polymer was prepared to represent the physical mixture. Microcapsules (5 mg equivalent to 69.86 μg/mL) were dissolved in a 50 : 50 mixture of alcohol and phosphate buffer pH 6.8. A drop of liquid was applied to the center of the crystal and allowed to dry for 2 min. The scanning range was 4000–400 cm^{-1} at a resolution of 1 cm^{-1}. Spectra were represented as % transmittance on a common scale. Samples were run on a FTIR model Impact 410 (Thermo Fisher Scientific, Inc., Waltham, MA)

3.6. Size exclusion chromatography

Insulin stock solution was prepared by dissolving insulin powder in 0.01N HCl to obtain a stock solution of 100 IU/mL. Diluted solutions were made with pH 6.8 buffer in the concentration range 2–10 IU/mL. Physical mixture of insulin and Eudragit L100 was prepared by adding 47.5 mg Eudragit to 10 mL of 2 IU/mL stock solution of insulin. Microcapsule samples were prepared by dissolving 50 mg of microparticles in 10 mL of pH 6.8 buffer. Chromatographic analysis was performed at 275 nm using Waters HMWP C_{18} (7.8 × 300 mm) column by running a mobile phase consisting of a mixture of water 65%, glacial acetic acid 15%v/v and acetonitrile 20% v/v containing 0.1% L-arginine at a flow rate of 0.5 mL/min.

Acknowledgement: We thank Dr. Necip Guven at Texas Tech University and Dr. Darren Williams at West Texas A & M University for their assistance with powder XRD and FTIR instrumentation.

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