In vitro and *in vivo* evaluation of insulin microspheres containing protease inhibitor

Mitra Jelvehgari[1,2], Parvin Zakeri Milani[1,3], Mohammad Reza Siahi-Shadbad[1,2], Farnaz Monajjemzadeh[1], Ali Nokhodchi[4], Zahra Azari[1], Hadi Valizadeh[1,5]

[1] Department of Pharmaceutics, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Liver and gastrointestinal diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[4] Medway School of Pharmacy, Universities of Kent and Greenwich, Chatham Maritime, Kent, UK
[5] Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran

Correspondence to: Hadi Valizadeh, Pharm. D., Ph. D., Assoc. Professor, Department of Pharmaceutics, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran; e-mail: valizadeh@tbzmed.ac.ir

Key words

- Double-emulsion solvent diffusion
- Enzymatic degradation
- Insulin
- Microspheres
- Oral delivery
- Protease inhibitor

Arzneimittelforschung 2011;61(1):14–22

Abstract

The aim of this study was to investigate the applicability of microspheres containing protease inhibitor for oral delivery of insulin (CAS 9004-10-8). Microspheres of insulin were prepared by water-in-oil-in-oil ($w/o_1/o_2$) double emulsion solvent evaporation method. Formulations with different drug/polymer ratios were prepared and characterized by drug loading, loading efficiency, yield, particle size, scanning electron microscopy (SEM), Fourier Transform Infrared spectroscopy (FTIR). The *in vitro* release studies were performed in pH 1.2 and 7.4. *In vivo* studies on rats were conducted in order to investigate the bioavailability and performance of oral microspheres. The best polymer to drug ratio in microspheres was 15.6 : 1 (F_2 formulation). The loading efficiency was 77.36 %, production yield was 54.55 % and mean particle size was 222.4 µm. SEM studies showed that the microspheres were spherical and porous in nature. Data obtained from *in vitro* release were fitted to various kinetic models and high correlation was obtained in the first order model. The results of enzymatic degradation indicated that insulin could be protected from trypsinic degradation in the microspheres. Our results indicate that the microspheres containing aprotinin (CAS 9087–70-1) have the advantage of high loading efficiency, pH responsive and prolonged release carrying insulin to the optimum site of absorption as well as the enhanced insulin absorption and biological response.

1. Introduction

Water-in-oil-in-oil ($W/O_1/O_2$) emulsion has been proposed to enhance the enteral bioavailability of drugs, including peptides [1–3]. An oral insulin (CAS 9004-10-8) administration could have an advantage by achieving portal insulin delivery in a convenient way. The development of an oral dosage form providing adequate bioavailability of insulin would revolutionize the treatment of diabetes. The problems encountered with oral dosage forms of insulin are rapid enzymatic degradation in the gastrointestinal tract and poor membrane permeability. In order to develop an oral insulin dosage form, it is necessary that these enzymes be inhibited. Recent studies have shown that some protease inhibitors amplify the biological effect of insulin injected directly into the lumen of the intestine [4–6]. These studies suggested that it was feasible to absorb insulin from the intestine in the presence of the protease inhibitor. The emulsion has advantages for enteral peptides delivery because it protects peptides against proteolysis [1, 3] and enhances the absorption of normally non-absorbed water soluble substances [1–3]. Further, multiple emulsions are easy to handle and drink due to their low viscosity. Thus, there is a possibility for developing an oral dosage form of insulin by using the emulsion. The most convenient administration route of insulin for patients would be a peroral ingestion of a suitable dosage form. The advantages offered by multiple emulsions as carriers of drugs for oral administration include protection against enzymatic hydrolysis and enhanced absorption

Arzneimittelforschung 2011;61(1):14–22

through the intestinal wall. One of the strategies of oral insulin delivery is usage of enzyme-inhibiting agents. Enzyme inhibiting agents are becoming increasingly popular as a delivery strategy for oral insulin. Insulin, like many other protein and peptide drugs, is degraded in the gastrointestinal tract (GIT) by digestive enzymes such as pepsin, proteases, peptidases, and other proteolytic enzymes. The proper selection of protease inhibitors depends on both the peptide and protein drug to be delivered and on the type of proteases and peptidases used to protect the drug in the GIT. Further attempts to increase the hypoglycemic levels in diabetic animals were made by introducing enzyme inhibitors and protease inhibitors (aprotinin (CAS 9087-70-1) or the Bowman-Birk inhibitor) to prevent the degradation of insulin in the intestine by pancreatic enzymes [7, 8].

In one study, the use of a trypsin inhibitor caused greater hypoglycemic effect in a gelatin microspheres formulation containing insulin [9]. It has been suggested that aprotinin or bacitracin (protease inhibitors) may cause a significant and prolonged plasma glucose reduction in diabetic rats [10]. Morishita *et al.* used microspheres for insulin delivery in rats [11]. Their study showed that microspheres carrying insulin and aprotinin enhanced insulin absorption. The results from *in situ* closed small and large intestinal loops in rats suggest that protease inhibitors could increase insulin absorption more effectively in the large intestine than in the small intestine [7]. Side effects such as systemic intoxication and disturbed digestion of food proteins are regularly associated with the use of insulin with enzyme inhibitors [12]. Aprotinin is mostly used in extensive cardiac surgery and liver by intravenous injection. Aprotinin has few side effects but may occasionally cause allergic/anaphylactic reactions [13]. These side effects have prompted caution in the use of enzyme inhibitors in oral insulin formulations. The above mentioned side effects can be reversed, however, if oral insulin formulations are designed to contain an inhibitor that releases insulin and an inhibiting agent at the same time and limit its action to a certain area within the intestine.

In situ intestinal experiments on rats indicate that a water-in-oil-in-water emulsion containing docosahexaenoic acid may improve insulin absorption without causing any serious damage to the intestinal epithelial cells [12]. A group of researchers developed a strategy to use both an absorption promoter (sodium lauryl sulfate) and an enzyme inhibitor (aprotinin) separately in their formulation and reported a reduction in blood glucose levels in beagle dogs [14]. The process produced insulin loaded polymeric microspheres with desirable release characteristics such as low initial burst, narrow particle size distribution, high loading, and preserved integrity of the encapsulated protein.

In this work, microspheres containing protease inhibitors as enteral carriers of insulin were prepared with the intention of enhanced intestinal absorption of insulin. The physicochemical properties of microspheres, hypoglycemic effect of microspheres as well as bioavailability of insulin from the microspheres in rabbits were investigated.

2. Material and methods

2.1 Materials

Recombinant insulin was obtained from *E. coli* (28.3 U/mg, Eli Lilly, Indianapolis, IN, USA). Aprotinin (Sigma-Aldrich, St. Louis, MO, USA), Eudragit S100 (Röhm Pharma, Weiterstadt, Germany), ethyl alcohol, hydrochloric acid, liquid paraffin, gelatin, sodium chloride, potassium chloride, phosphate buffer saline (pH 7.4). Trypsin (Gibco, Ontario, Canada), pepsin and streptozocin (Sigma-Aldrich), methyl cellulose (Shin-Etsu, Tokyo, Japan), Insulin Isophane (NPH, Exir Company, Boroujerd, Iran), glucose and insulin EIA kit (Diaplus Co., San Francisco, CA, USA).

2.2 Experimental methods

Microspheres were prepared by using water-in oil-in oil ($W/O_1/O_2$ double emulsion solvent diffusion method) using different ratios of insulin (with or without protease inhibitor) to Eudragit S100 ratios (1 : 7.8, 1 : 15.6, 1 : 31.2, 1 : 62.4 and 1 : 124.8 as shown in Table 1). Eudragit S100 (500 mg) was dissolved in 5 ml of ethanol. The initial w/o emulsion was prepared by adding 300 µl of 0.1 N HCl containing insulin (32 mg) with or without protease inhibitor (aprotinin) to this ethanolic polymer solution while stirring at 500 rpm. This w/o primary emulsion was slowly added to 50 ml of light liquid paraffin, while stirring by a paddle propeller at 1200 rpm, immersed in an ice water bath. After 2 h, 50 ml of gelatin solution (0.5 % w/w) was added to harden the microspheres and stirring was continued for a further 1 h and the hardened microspheres were collected by filtration and washed with three portions of 50 ml of n-hexane and then freeze-dried [8, 15–17].

Table 1: Insulin microsphere formulations prepared by double-emulsion solvent diffusion method ($W/O_1/O_2$).

Formulations	Drug : polymer ratio	Initial emulsion (W/O_1)				Secondary phase oily (O_2)	
		Aqueous phase (w)		Initial organic phase (O_1)		Liquid paraffin (ml)	Gelatin 0.5 % (ml)
		Insulin (mg)	Hydrochloric acid (ml)	Eudragit S100 (mg)	Ethanol (ml)		
F_1	1 : 7.8	32	0.3	250	5	50	50
F_2	1 : 15.6	32	0.3	500	5	50	50
F_3	1 : 31.2	32	0.3	750	5	50	50
F_4	1 : 62.4	32	0.3	1000	5	50	50
F_5	1 : 124.8	32	0.3	1500	5	50	50

2.3 Determination of drug content of microspheres

Drug amount in microspheres was determined by dissolving 10 mg of each sample in 10 ml of the mixed solvent system consisting of ethanol and saline phosphate buffer (pH 7.4) in 1 : 1 ratio. The drug concentration was determined spectrophotometrically (UV-160, Shimadzu, Kyoto, Japan) at 276 nm. The production yield of the microspheres was determined by calculating accurately the initial weight of the raw materials and the last weight of the microspheres obtained [16–18]. All of the experiments were performed in triplicate and the mean of the values was reported.

2.4 Scanning electron microscopy (SEM)

The morphology of microspheres was examined with a scanning electron microscope (LEO 440i, Cambridge, England) operating at 15 kV. The samples were mounted on a metal stub with double adhesive tape and coated with platinum/palladium alloy under vacuum.

2.5 Fourier-Transform Infrared spectroscopy (FTIR)

The infrared spectrum of the drug, microspheres containing the drug were obtained in potassium bromide discs (0.5 % w/w) using a FTIR (Bomen, Quebec, Canada) spectrophotometer [19–23].

2.6 Fourier self deconvolution (FSD)

Fourier self deconvolution is a method of computationally resolving overlapping lines that cannot be instrumentally resolved due to their intrinsic linewidth. This can be useful for more accurate determination of the number of peaks in a trace region, the band positions, and areas. This technique can be used to accurately determine starting parameters for applications such as curve fit. This application is based on the method described by Griffiths and Pariente [24, 25]. Two filters are employed in this method. An exponential filter is used to sharpen spectral features; the constant γ is varied to change the filter shape. The γ parameter equals the full width at half height (FWHH) of the widest resolved peak. Realize that the filters are boxcare and Bessel, and are mathematically described below. The deconvolution filter is a simple exponential filter of the form $e^{2\pi\gamma X}$ where γ is deconvolution filter constant and X is the array (i. e. data file) whose X range is normalized between 0 and 1. This function is multiplied by the Fourier transformed trace, and the data is then reverse Fourier transformed to give the result [24–25].

2.7 Circular dichroism spectrophotometry

Circular dichroism (CD) spectroscopy is remarkably sensitive to the secondary structure of biological polymers. The effect of encapsulation on the conformation of insulin was evaluated with CD spectrophotometery. The samples for CD analysis were prepared as follows:

(1) Insulin alone was dissolved in HCL solution (pH 2.5), as a standard control.

(2) Lyophilized insulin microspheres were dissolved in 0.1 mol/L NaOH solution. The resultant solution then centrifuged at 12 000 revolutions per minute for 30 min. The supernatant was collected and the concentration of insulin adjusted using deionized water as the sample.

In the above solutions, the final concentration of insulin was 0.2 mg/ml for each and confirmed using a UV spectrometer at 276 nm. The control solutions or sample solutions were measured using a CD spectrophotometer (Jasco J-810, Tokyo, Japan). The measurement conditions were as follows: temperature, 20 °C; a 0.2-cm cuvette; wavelength range, 200–250 nm; resolution, 2 nm; and scanning speed, 200 nm/min with a 1 s response time. Noise reduction, blank buffer subtraction, and data analysis were performed using a standard analysis and temperature/wavelength analysis program [26].

2.8 Particle size analysis

A laser light scattering particle size analyzer (SALD-2101, Shimadzu, Kyoto, Japan) was used to determine the particle size of the drug and microparticulate formulations. Samples of freeze-dried microspheres were dispersed in 2-methyl propane-1-ol (iso-butyl-alcohol) after treatment in an ultrasonic disperser for 5 min to bring about disaggregation of the microspheres [27].

2.9 *In vitro* release study

Dissolution studies were carried out using a USP basket method at 37 °C and 150 rpm. 200 ml of 0.1 N HCl (with 1 mg methyl cellulose to prevent adsorption to the vessel glass) were equilibrated at 37 ± 0.5 °C. Microspheres (100 mg drug) were placed in the apparatus and 4 ml aliquots of medium were withdrawn at pre-set times over 2 h and replaced by 4 ml of fresh medium. The samples were filtered through 0.45 μm filters and used for the spectroscopic determination of the drug. Dilution with the same buffer solution was carried out if necessary. After 2 h, the basket was washed with purified water and replaced in the same beaker containing 200 ml of saline phosphate buffer (pH 7.4) at 37 °C with constant stirring at 100 rpm [8]. At appropriate interval, 4 ml samples were taken from the medium and filtered. Drug concentration in the samples was measured by UV spectrophotometric analysis at 276 nm for acidic and enteric buffers. Each experiment was repeated three times [8].

2.10 Enzymatic degradation study

Pepsin degradation was done for all microsphere formulations. A 100 mg sample of the microspheres was incubated for 1 h at 37 °C with reciprocal shaking (50 strokes/min) in 10 ml of pepsin solution (weight ratio of insulin to enzyme, 200 : 1; glycine buffer, pH 1.3). The microspheres were then collected by filtration and washed with purified water. The collected microspheres were then placed in 10 ml of phosphate buffer (pH 7.8) and dissolved completely. Similarly, incubation with non-incorporated insulin was conducted under the same experimental conditions [8].

For the trypsinic degradation study, the respective enzymatic solutions (weight ratio of insulin to enzyme, 1 : 0.5, 1 : 1, 1 : 2; phosphate buffer, pH 7.8) were used. A 20 mg sample of microspheres containing aprotinin was incubated for 3 h at 37 °C with reciprocal shaking (90 strokes/min) in 5 ml of trypsin solution [8].

For control experiments, all preparations were incubated in enzyme-free solution under the same conditions. The protective efficiency of the preparation towards enzymatic degradation was expressed as the relative percentage of the residual insulin amount to the control. Determination of insulin was performed by an enzyme immunoassay (EIA) using the insulin EIA kit [8].

2.11 *In vivo* studies

Adult male Wistar rats were obtained from the Animal Centre of the local University, Tabriz, Iran. They were housed in air-

Arzneimittelforschung 2011;61(1):14–22

conditioned quarters under a photoperiod schedule of 12 h light/12 h dark. They received standard laboratory diet and tap water, available *at libitum*. Wistar rats of male sex, weighing 250 ± 10 g, were selected. Diabetes was induced in rats by an injection of streptozocin (70 mg/kg). Rats were considered diabetic when glycemia was higher than 300 mg/dl one week after streptozocin administration. Rats were fasted over 12 h before starting the test and were divided into 8 groups of 10 animals. In all of experiments, a dose of insulin (20 IU/kg body weight) was administered by subcutaneous injection or the oral route. Insulin microspheres with and without aprotinin was administered via gavages with a feeder needle into the stomach of normal and diabetic rats in phosphate buffer. Blood samples were collected from the tail vein; blood glucose level was measured before injection and 0.5, 1, 2, 4, 6 and 8 h post injection using a glucometer (Roche diagnostics, Germany). Serum was separated by centrifugation at 3000 rpm for 3 min and kept frozen until analysis [28]. The absorption of intact biologically active insulin was evaluated by measuring the hypoglycemic effect. The areas under the curves (AUC) of the glucose concentration-time profiles were calculated with the linear trapezoidal method. Serum glucose levels after insulin administration were expressed as a percentage of the initial level. The cumulative percentage of change in serum glucose level was calculated by summing the areas below baseline levels (negative values) using the trapezoidal method. The relative bioavailability was calculated by the ratio of the respective AUC of oral to subcutaneous administrations [27, 29]. Approval for the study was obtained from the local research ethics committee (Tabriz University of Medical Sciences, Tabriz, Iran) in advance. In all animal studies "Guide to the care and use of experimental animals" by the Canadian Council on Animal Care, was followed [30].

3. Results and discussion

3.1 The effect of drug/polymer ratio on the physical properties of microspheres

Microspheres were prepared by the emulsion-solvent evaporation (ESE) method using different drug-polymer ratios (1 : 7.8, 1 : 15.6, 1 : 31.2, 1 : 62.4 and 1 : 124.8). The drug loading was at the range of 53.36 % and 108.68 % (Table 2).

An SEM photograph of microspheres (F_2) is shown in Fig. 1. The surface of microspheres appeared spherical. The loading of the drug depended on the solubility of the drug in the solvent and continuous phase. Insulin loading percentage using Eudragit S100 as matrix materials were 77.36 % for F_2 microspheres with 1: 15.6 ratio significantly ($p < 0.05$).

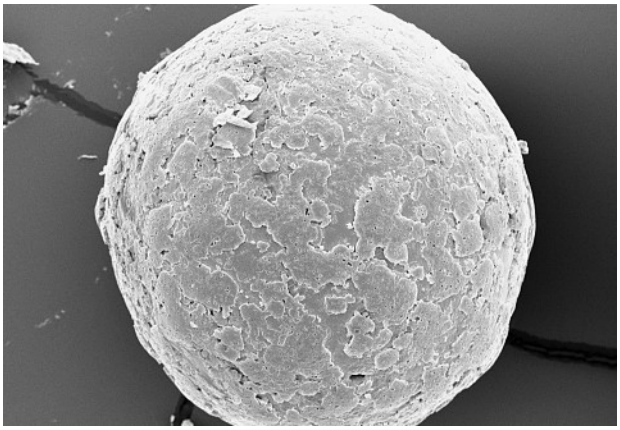

Fig. 1: SEM of insulin microspheres containing drug/polymer ratio 1 : 15.6 (F_2) at 500 × magnification.

Microspheres in the size range 209 – 377.93 µm were obtained (Table 2). It is clear from Table 2, generally, as the polymer amount increased, particle size was increased. The drug/polymer ratio affected the particle size for insulin microspheres.

FTIR patterns of insulin powders, polymer S100, physical mixture of F_2, lyophilized insulin microspheres are illustrated in Fig. 2 A. FTIR spectra were assigned as follows: (1) Insulin: amide band II at 1534 cm^{-1} and C=O stretching 1654.63 cm^{-1}; (2) Eudragit S100: C=O stretching band at 1731.7 cm^{-1} (–COOH); (3) Physical mixture: C=O stretching band at 1731.02 cm^{-1}; (4) F_2 microspheres: C=O stretching band at 1732.82 cm^{-1}. After insulin had been encapsulated into the microspheres, the characteristic peaks for insulin were covered by the stronger intensity peaks of matrix materials [26]. On the other hand, the C=O stretching bands of Eudragit S100 in microspheres (at 1731 cm^{-1}) were merged into one band at 1732.82 cm^{-1}, thus leading to a peak shifting from 1731 cm^{-1} to 1732.82 cm^{-1}.

FSD was applied to obtain the position of the overlapping components of the amide band and to assign them to different secondary structures. The area under the peaks of second derivative spectra can be used to determine the percentage composition of the various structures. The results of FSD peaks (Fig. 2 B) revealed that in the microspheres C=O stretching band of insulin at 1654.63 cm^{-1} was shifted to 1662.77 cm^{-1} and 1737.16 cm^{-1} indicating that some interaction between

Table 2: Effect of different drug/polymer ratio on the drug content, production yield, and particle size of insulin microspheres.

Formulation	Drug : polymer ratio	Production yield (%)	Theoretical drug content (%)	Mean amount of drug entrapped (% ± SD)	Loading efficiency (% ± SD)	Mean particle size (µm ± SD)
F_1	1 : 7.8	60.28	11.35	6.06 ± 0.11	53.36 ± 2.73	209 ± 1.712
F_2	1 : 15.6	54.55	6.01	4.65 ± 0.089	77.36 ± 2.73	222.388 ± 1.715
F_3	1 : 31.2	49.87	4.09	3.67 ± 0.074	89.73 ± 5.80	355.764 ± 1.723
F_4	1 : 62.4	45.91	3.10	3.19 ± 0.032	102.96 ± 8.73	377.929 ± 1.724
F_5	1 : 124.8	41.83	2.5	2.72 ± 0.019	108.68 ± 3.88	359.714 ± 1.718

insulin and Eudragit S100 takes place during encapsulation.

Conformation of insulin inside microspheres was evaluated using circular dichroism spectrophotometery (Fig. 2C). The circular dichroism spectrum of standard insulin showed ellipticity troughs at 208 and 222 nm, respectively. The circular dichroism spectrum of insulin microspheres demonstrated that the matrix materials and/or formulation procedure influenced insulin conformation [3, 7–8].

The parameters of the secondary structure of insulin conformation (i.e. α-helix, β-sheet, β-turn, and random coil) are shown in Table 3. When compared with the conformation parameters of intact insulin, encapsulation with Eudragit S100 microspheres (F_2) obviously altered the secondary structure of insulin as follows: α-helix decreased by 11.7 %, β-sheet decreased by 1.1 %, β-turn increased by 4.6 % and random coil increased by 8.2 %, respectively (Table 3).

There was a remarkable decrease in α-helix conformation, accompanied by equivalent increase in unordered structures in the microspheres. New peaks at 1662.77 and 1737.16 cm^{-1} emerged in microspheres, which could be assigned to random conformation. This contributed to the increase in unordered structures. Random peak was indicated by FTIR, FSD and CD analysis in all the microspheres, though the peak was too small to appear in the second derivative spectra. A major increase in unordered structure came from increase in turn conformation. Therefore the observations indicate that the formulation process affects the α-helix conformations of the insulin resulting in a conformational transition to random and turn structures, β-sheet structures mostly remaining unchanged. The helix to random coil transition is a thoroughly studied phenomenon. This transition is very abrupt and can occur upon change of temperature, solvent etc. [31–33].

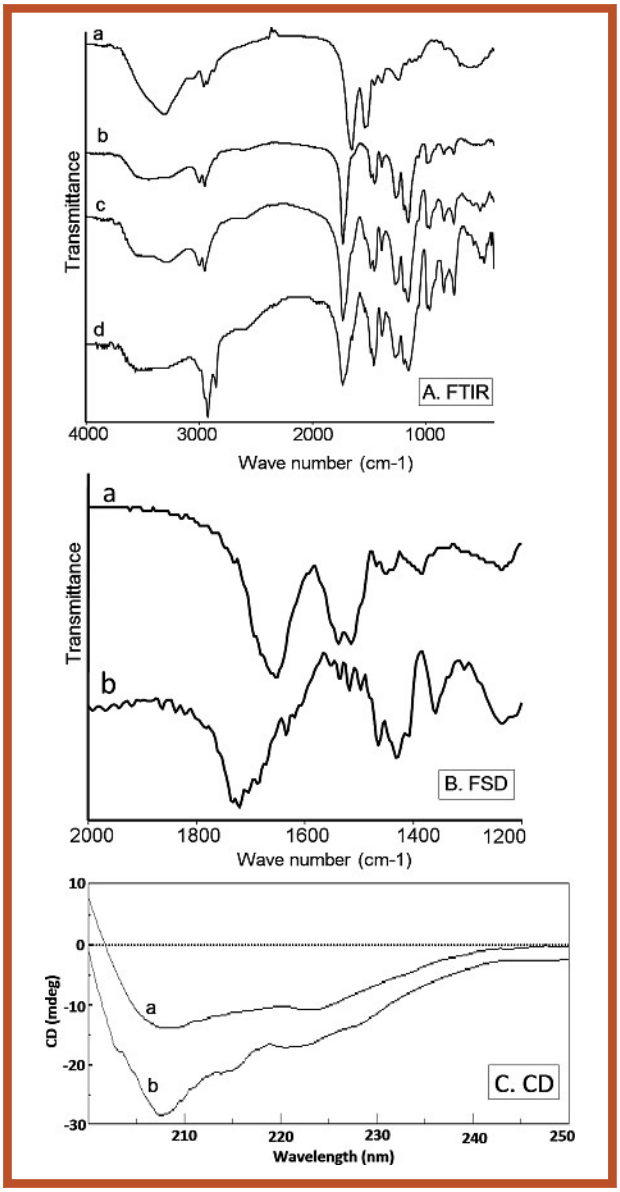

Fig. 2: A) FTIR spectra of insulin (a); Eudragit S100 (b); physical mixture (c); F_2 microspheres (d). B) FSD spectrum performed on FTIR spectrum of insulin microsphere (F_2) with γ = 1 and 30 % smoothing: insulin (a); F_2 microspheres (b). C) Circular dichroism spectra of standard insulin (a) and insulin-Eudragit S100 microspheres (b). The insulin concentration was 0.2 mg/ml.

3.2 *In vitro* release studies

The release profiles of insulin from microspheres of different drug/polymer ratio are shown in Fig. 3. Burst release of insulin from microsphere formulations was low. When microspheres are prepared by double-emulsion ($W/O_1/O_2$) method, water-soluble drugs do not have a tendency to migrate to the organic medium, thereby concentrating at the surface of the particles and the resulting burst effect will be low [34]. Moreover, the burst release could also be explained by the imperfect encapsulation of the drug inside microspheres whereas decreased burst release may be attributed to the increased thickness of the polymer layer surrounding the drug.

Table 3: Observed parameters of secondary structure of standard insulin, and insulin encapsulated in Eudragit S100 microspheres.

Samples	α-Helix (%)	β-Sheet	β-Turn (%)	Random coil (%)
Insulin in pH 1.2 HCl, as a standard control	28.5	24.5	20.5	26.5
Insulin in Eudragit S100 microspheres (F_2)	16.8 (– 11.7)*	23.4 (– 1.1)*	25.1 (+ 4.6)*	34.7 (+ 8.2)*

* Numbers in parentheses are the changed percentage values, compared with the value of the standard control; positive and negative values represent the increased and decreased percentage value, respectively. The concentration of insulin was 0.2 mg/ml.

Arzneimittelforschung 2011;61(1):14–22

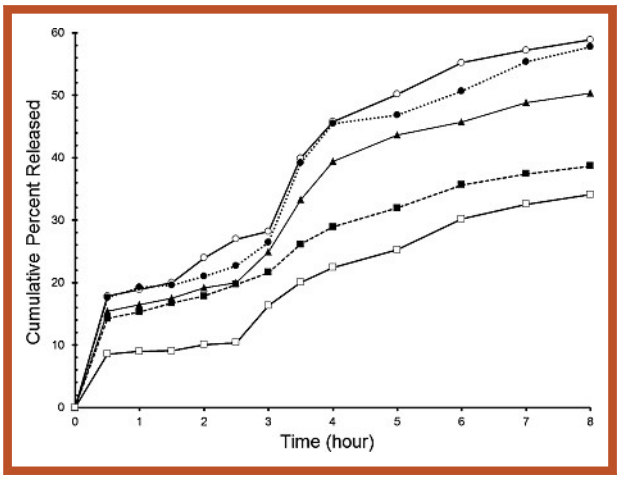

Fig. 3: Cumulative percent release of insulin microspheres prepared with different drug/polymer ratios. F_1, 0.25 : 1 ○, F_2, 0.5 : 1 (●), F3, 0.75 : 1 (▲), F4, 1 : 1 (■), F5 (□). Each data point represents the mean ± SD (n = 3).

Similar behavior was observed in verapamil prepared with Eudragit RS100 [14]. In most cases, a biphasic dissolution profile was observed at pH 1.2: the initial rapid drug leakage generally ended very early (within first 120 min after the change of dissolution medium pH to 7.4); for the remaining time, nearly linear behavior was observed. It is assumed that the first portion of the curves is due to insulin dissolution, which starts immediately after the beginning of the test for the portion of drug on the surface of microspheres. After such a phase, two phenomena can combine in enhancing in the diffusion of the remaining dispersed drug into the bulk phase as well as the formation of pores within the matrix due to the initial drug dissolution; particle wetting and swelling which enhances the permeability of polymer to the drug [26] (Fig. 3). The release rate at pH 7.4 was faster than at pH 1.2. Comparing the drug release from microspheres shows that the drug release during both gastric and enteric stages for formulations F_1 and F_2 was higher than for other formulations ($p < 0.05$).

Model independent analysis of data was performed by comparison of DE (dissolution efficiency), $t_{50\,\%}$ (dissolution time for 50 % release) and similarity factor (Table 4) [35]. The DE was calculated from the area under the dissolution curve at time ti (measured using the trapezoidal rule) and expressed as percentage of the area of the rectangle described by 100 % dissolution in the same time [16, 23, 28, 36–39]. Among the formulations insulin microspheres with 1 : 7.8 drug/polymer ratios showed the highest dissolution efficiency, 39.29. Release profile of formulation F_2 was similar to those of F_1 and F_3 and there was no significant difference between their dissolution profiles (similarity factor of 80.55 and 67.97 % respectively). Mathematical models have been used ex-

Table 4: Comparison of various release characteristics of insulin from different microsphere formulations.

Formulation	Q_2[a] (%)	Q_8[b] (%)	DE[c]	$t_{50\,\%}$[d] (h)	f_2[e]
F1	23.96 ± 0.64	58.89 ± 2.29	39.29	5	80.55
F2*	21.02 ± 0.340	57.74 ± 1.51	37.38	6	100
F3	19.20 ± 0.540	50.80 ± 4.23	33.30	8	67.97
F4	17.85 ± 1.07	38.79 ± 1.57	26.5	–	47.29
F5	10.09 ± 0.13	34.09 ± 1.78	20.45	–	39.07

[a] Q_2 = amount of drug release after 2 h; [b] Q_8 = amount of drug release after 8 h; [c] DE = dissolution efficiency; [d] $t_{50\,\%}$ = dissolution time for 50 % fractions; [e] f_2 = similarity factor. * Formulation F_2 was selected as reference.

Table 5: Fitting parameters of the *in vitro* release data to various release kinetic models.

Order	Formulation	F_1	F_2	F_3	F_4	F_5
Zero f = kt	K RSQ D(SS)%	0.0644 0.9386 504.9048	0.0621 0.9258 490.6668	0.0551 0.9301 492.6239	0.0368 0.9710 626.1257	0.0399 0.9582 339.7816
First Ln(1 – f) = kt	k RSQ D(SS) %	0.1083 0.9539 256.5814	0.1017 0.9428 250.1645	0.0837 0.9429 292.7039	0.0506 0.9763 492.7832	0.0507 0.9640 239.0918
Weibull ln[– ln(1 – f)] = blnk + blnt	b k RSQ D(SS) %	0.5233 0.0958 0.8831 159.6803	0.6358 0.0830 0.8387 195.4734	0.6237 0.0635 0.8581 175.3277	0.4818 0.0245 0.9041 109.1997	0.6691 0.0284 0.8444 205.6553
Peppas Lnf = lnk + blnt	b k RSQ D(SS) %	0.5233 0.1952 0.8935 149.1783	0.5123 0.1876 0.8464 184.7657	0.5195 0.1653 0.8653 166.4475	0.4173 0.1549 0.9114 103.4488	0.6052 0.0887 0.8473 200.1464
Higuchi f = kt 0.5	k RSQ D(SS) %	0.2339 0.9334 233.2359	0.2239 0.9078 259.5510	0.1996 0.9181 255.8154	0.1332 0.9560 104.8783	0.1426 0.9221 572.8286

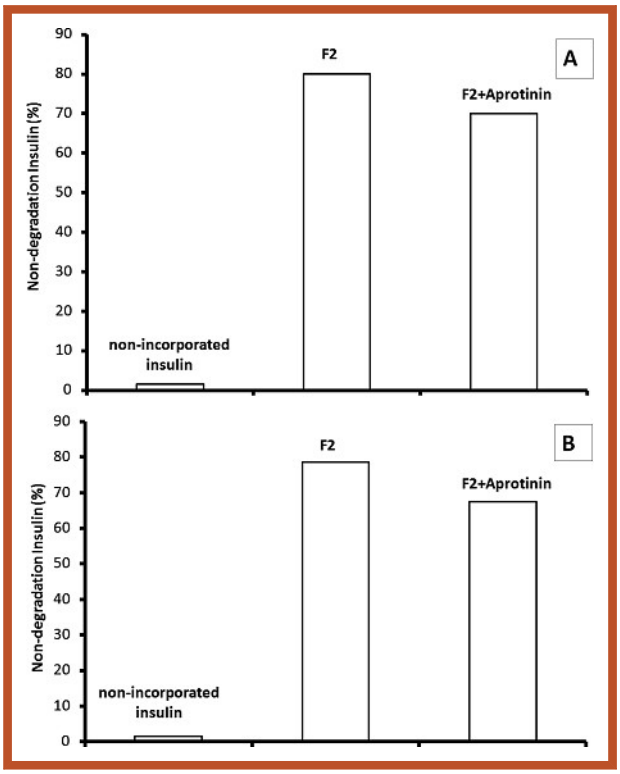

Fig. 4: Protection of insulin towards pepsinic (A) and trypsenic (B) degradation.

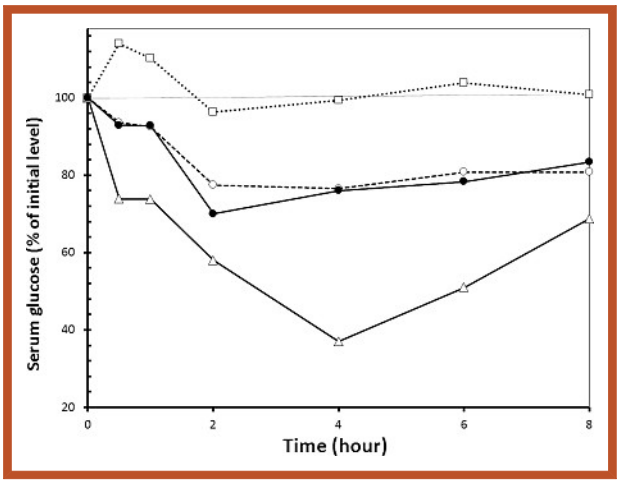

Fig. 5: Hypoglycemic effect of insulin microspheres administered orally to diabetic rats. Control diabetic without treatment (□), diabetic with oral insulin microspheres without protease inhibitor (○), diabetic with oral insulin microspheres with protease inhibitor (●), control diabetic with subcutaneous insulin (△). Each point represents the mean of six experiments.

tensively for the parametric representation of dissolution data [14, 31, 38]. The fit parameters to Higuchi, first-order, Peppas and zero-order equations are presented in Table 5. Applying first order kinetics, a high correlation coefficient was observed for the release profile of all formulations. The data obtained were also put in the Korsemeyer-Peppas model in order to find out the n value, which describes the drug release mechanism. The n value of microspheres of different drug/polymer ratio was between 0.4173–6052, indicating that the mechanism of the drug release was diffusion and erosion controlled (Table 5) [38].

3.3 Enzymatic degradation studies

The results of pepsinic and trypsinic degradation studies are shown in Fig. 4. Insulin solution was degraded during incubation in pepsin solution. However, a considerable amount of insulin remained unchanged in microspheres after the acidic incubation of F_2 with and without aprotinin (71.91 % and 70.87 %, respectively) (Fig. 4 A). After trypsinic incubation, complete insulin degradation was observed for insulin solution in the absence of protease inhibitor. The amount of insulin remaining after the trypsinic incubation of formulation F_2 with and without aprotinin was 76.95 % and 70.87 %, respectively (Fig. 4 B).

3.4 Relative bioavailability and hypoglycemic effect of oral insulin microspheres in diabetic rats

Average serum glucose level vs time profiles after administration of subcutaneous (s. c.) insulin solution and oral microspheres (F_2) to diabetic rats are shown in Fig. 5. Following s. c. administration, the serum glucose levels were lower than the initial level and that of orally administered microspheres. The profiles of serum glucose levels after the administration of microspheres with and without aprotinin were nearly similar ($p > 0.05$). In comparison to control group oral administration of microspheres decreased serum glucose level (Fig. 5).

However, the fall in blood glucose may induce the release of counter regulatory hormones such as glucagon, growth hormone and cortisol [7, 40]. In particular, glucagon plays a major role in the recovery from hypoglycemia in the normal state. However, this phenomenon might not occur in diabetic rats. On the other hand, the marked change in serum glucose levels observed in diabetic rats may be attributed to the absence of these phenomena [7].

The relative efficacy of F_2 microspheres with and without aprotinin in comparison to s. c. administration to diabetic rats, the percentages of change in serum glucose levels are demonstrated in Fig. 5. The results indicated that the hypoglycemic effect of insulin microspheres containing aprotinin was slightly more than that of microspheres without aprotinin (oral to s. c. administration efficacy ratio of % 2.98 and % 2.72, respectively) although the difference was not statistically significant ($p > 0.05$). This could be partly ascribed to the fact that aprotinin may be destroyed by oral intake.

Arzneimittelforschung 2011;61(1):14–22

4. Conclusion

Insulin-Eudragit S100 microspheres were prepared using double emulsion ($w/o_1/o_2$) solvent diffusion method. The drug : polymer ratio influenced the sphericity of the microspheres. The entrapment efficiency was high for all formulations. Insulin release from the microspheres followed the first order kinetic model controlled by diffusion and erosion mechanism. The results indicate that the process of microsphere formation is affecting the a-helix conformations of insulin resulting in a conformational transition to random and turn structures, β-sheet structures mostly remaining unchanged. The results of enzymatic degradation showed that regardless of the existence of aprotinin inside microspheres the encapsulation of insulin inside Eudragit S100 microspheres protects insulin from pepsinic and trypsinic degradation. The findings of *in vivo* experiments demonstrated an enhanced hypoglycemic effect of insulin following oral administration of insulin microspheres; however, this hypoglycemic effect is not significantly dependent on the existence of protease inhibitor inside the microsphere.

Acknowledgement

The financial support from the drug applied research center of Tabriz University of Medical Sciences is greatly acknowledged.

Conflict of Interest

All the authors report no conflicts of interest.

References

[1] Cunha AS, Grossiord JL, Puisieux F, Seiller M. Insulin in w/o/w multiple emulsions: preparation characterization and determination of stability towards proteases in vitro. *J Microencapsul.* 1997;14(3):311–9.

[2] Engel RH, Riggi SJ, Fahrenbach MJ. Insulin: intestinal absorption as water-in-oil-in-water emulsions. *Nature.* 1968; 219(5156):856–7.

[3] Schilling RJ, Mitra AK. Intestinal mucosal transport of insulin. *Pharm Res.* 1990;62(1):53–64.

[4] Fujii S, Yokoyama T, Ikegaya K, Sato F, Yokoo N. Promoting effect of the new chymotrypsin inhibitor FK-448 on the intestinal absorption of insulin in rats and dogs. *J Pharm Pharmacol.* 1985;37(8):545–9.

[5] Kidron M, Bar-On H, Berry EM, Ziv E. The absorption of insulin from various regions of the rat intestine. *Life Sci.* 1982;31(25):2837–41.

[6] Ziv E, Lior O, Kidron M. Absorption of protein via the intestinal wall. A quantitative model. *Biochem Pharmacol.* 1987;36(7):1035–9.

[7] Morishita I, Morishita M, Takayama K, Machida Y, Nagai T. Hypoglycemic effect of novel oral microspheres of insulin with protease inhibitor in normal and diabetic rats. *Int J Pharm.* 1992;78(1):9–16.

[8] Morishita M, Morishita I, Takayama K, Machida Y, Nagai T. Novel oral microspheres of insulin with protease inhibitor protecting from enzymatic degradation.novel oral microspheres of insulin with protease inhibitor protecting from enzymatic degradation. *Int J Pharm.* 1992;78(1–3):1–7.

[9] Narayani R. Oral delivery of insulin making needles needless. *Trends Biomater Artif Organs.* 2001;15:12–6.

[10] Kimura T, Sato K, Sugimoto K, Tao R, Murakami T, Kurosaki Y, Nakayama T. Oral administration of insulin as poly(vinyl alcohol)-gel spheres in diabetic rats. *Biol Pharm Bull.* 1996;19(6):897–900.

[11] Morishita I, Morishita M, Takayama K, Machida Y, Nagai T. Enteral insulin delivery by microspheres in 3 different formulations using Eudragit-L100 and Eudragit-S100. *Int J Pharm.* 1993;91(1):29–37.

[12] Suzuki A, Morishita M, Kajita M, Takayama K, Isowa K, Chiba Y, *et al.* Enhanced colonic and rectal absorption of insulin using a multiple emulsion containing eicosapentaenoic acid and docosahexaenoic acid. *J Pharm Sci.* 1998;87(10):1196–202.

[13] Verstraete M. Clinical application of inhibitors of fibrinolysis. *Drugs.* 1985;29(3):236–61.

[14] Kraeling ME, Ritschel WA. Development of a colonic release capsule dosage form and the absorption of insulin. *Methods Find Exp Clin Pharmacol.* 1992;14(3):199–209.

[15] Morishita I, Morishita M, Machida Y, Nagai T. Controlled release microspheres based on Eudragit L100 for the oral administration of erythromycin. *Drug Des Deliv.* 1991;7(4): 309–19.

[16] Jelvehgari M, Valizadeh H, Rezapour M, Nokhodchi A. Control of encapsulation efficiency in polymeric microparticle system of tolmetin. *Pharm Dev Technol.* 2010;15(1): 71–9.

[17] Jelvehgari M, Rezapour M, Nokhodchi A, Valizadeh H. Effect of formulation and processing variables on the characteristics of tolmetin microspheres prepared by double emulsion solvent diffusion method. *Ind J Pharm Sci.* 2010; 72(1):72–8.

[18] Kilicarslan M, Baykara T. The effect of the drug/polymer ratio on the properties of the verapamil HCl loaded microspheres. *Int J Pharm.* 2003;252(1–2):99–109.

[19] Valizadeh H, Zakeri-Milani P, Barzegar-Jalali M, Mohammadi G, Danesh-Bahreini MA, Adibkia K, *et al.* Preparation and characterization of solid dispersions of piroxicam with hydrophilic carriers. *Drug Dev Ind Pharm.* 2007;33(1):45–56.

[20] Valizadeh H, Nokhodchi A, Qarakhani N, Zakeri-Milani P, Azarmi S, Hassanzadeh D, *et al.* Physicochemical characterization of solid dispersions of indomethacin with PEG 6000, Myrj 52, lactose, sorbitol, dextrin, and Eudragit E100. *Drug Dev Ind Pharm.* 2004;30(3):303–17.

[21] Monajjemzadeh F, Hassanzadeh D, Valizadeh H, Siahi-Shadbad MR, Mojarrad JS, Robertson TA, *et al.* Compatibility studies of acyclovir and lactose in physical mixtures and commercial tablets. *Eur J Pharm Biopharm.* 2009;73(3): 404–13.

[22] Monajjemzadeh F, Hassanzadeh D, Valizadeh H, Siahi-Shadbad MR, Mojarrad JS, Robertson T, *et al.* Assessment of feasibility of maillard reaction between baclofen and lactose by liquid chromatography and tandem mass spectrometry, application to pre formulation studies. *AAPS PharmSciTech.* 2009;10(2):649–59.

[23] Barzegar-Jalali M, Nayebi AM, Valizadeh H, Hanaee J, Barzegar-Jalali A, Adibkia K, *et al.* Evaluation of in vitro-in vivo correlation and anticonvulsive effect of carbamazepine after cogrinding with microcrystalline cellulose. *J Pharm Pharm Sci.* 2006;9(3):307–16.

[24] *http://www.thermo.com/com/cda/resources/resources-detail/1,2166,13411,00.html;* 2009.

[25] Shingel KI. Determination of structural peculiarities of dexran, pullulan and gamma-irradiated pullulan by Fourier-transform IR spectroscopy. *Carbohydr Res*. 2002;337(16): 1445–51.
[26] Li MG, Lu WL, Wang JC, Zhang X, Zhang H, Wang XQ, *et al.* Preparation and characterization of insulin nanoparticles employing chitosan and poly(methylmethacrylate/methylmethacrylic acid) copolymer. *J Nanosci Nanotechnol*. 2006; 6(9-10):2874–86.
[27] Jain D, Panda AK, Majumdar DK. Eudragit S100 entrapped insulin microspheres for oral delivery. *AAPS PharmSciTech*. 2005;6(1):E100–7.
[28] Dastmalchi S, Garjani A, Maleki N, Sheikhee G, Baghchevan V, Jafari-Azad P, *et al.* Enhancing dissolution, serum concentrations and hypoglycemic effect of glibenclamide using solvent deposition technique. *J Pharm Pharm Sci*. 2005;8(2):175–81.
[29] Damge C, Maincent P, Ubrich N. Oral delivery of insulin associated to polymeric nanoparticles in diabetic rats. *J Control Release*. 2007;117(2):163–70.
[30] Ernest D, Alfert E, Brenda M, Cross BM, McWilliam AAE. CCAC, Guide to the care and use of experimental animals. Canadian Council on Animal Care; 1993.
[31] Dong A, Huang P, Caughey WS. Protein secondary structures in water from second-derivative amide I infrared spectra. *Biochemistry*. 1990;29(13):3303–8.
[32] Susi H, Byler DM. Protein structure by Fourier transform infrared spectroscopy: second derivative spectra. *Biochem Biophys Res Commun*. 1983;115(1):391–7.
[33] Rastogi SK, Singh J. Passive and iontophoretic transport enhancement of insulin through porcine epidermis by depilatories: permeability and fourier transform infrared spectroscopy studies. *AAPS Pharm Sci Tech*. 2003;4(3):E29.
[34] Jameela SR, Suma N, Jayakrishnan A. Protein release from poly(epsilon-caprolactone) microspheres prepared by melt encapsulation and solvent evaporation techniques: a comparative study. *J Biomater Sci Polym Ed*. 1997;8(6):457–66.
[35] Moore JW, Flanner HH. Mathematical comparison of dissolution profiles. *Pharm Technol*. 1996;20:64–74.
[36] Nokhodchi A, Okwudarue ON, Valizadeh H, Momin MN. Cogrinding as a tool to produce sustained release behavior for theophylline particles containing magnesium stearate. *AAPS PharmSciTech*. 2009;10(4):1243–51.
[37] Nokhodchi A, Nazemiyeh H, Khodaparast A, Sorkh-Shahan T, Valizadeh H, Ford JL. An in vitro evaluation of fenugreek mucilage as a potential excipient for oral controlled-release matrix tablet. *Drug Dev Ind Pharm*. 2008;34(3):323–9.
[38] Barzegar-Jalali M, Adibkia K, Valizadeh H, Shadbad MR, Nokhodchi A, Omidi Y, *et al.* Kinetic analysis of drug release from nanoparticles. *J Pharm Pharm Sci*. 2008;11(1):167–77.
[39] Azarmi S, Farid J, Nokhodchi A, Bahari-Saravi SM, Valizadeh H. Thermal treating as a tool for sustained release of indomethacin from Eudragit RS and RL matrices. *Int J Pharm*. 2002;246(1–2):171–7.
[40] Paquot N, Scheen AJ, Franchimont P, Lefebvre PJ. The intra-nasal administration of insulin induces significant hypoglycemia and classical counterregulatory hormonal responses in normal man. *Diabetes Metab*. 1988;14(1):31–6.

Arzneimittelforschung 2011;61(1):14–22