Drug Development and Industrial Pharmacy, 27(9), 981–989 (2001)

RESEARCH PAPER

Enhancement of Absorption of Insulin-Loaded Polyisobutylcyanoacrylate Nanospheres by Sodium Cholate After Oral and Subcutaneous Administration in Diabetic Rats

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ABSTRACT

Polyisobutylcyanoacrylate (PIBCA) nanospheres were employed as biodegradable polymeric carriers for oral (p.o.) and subcutaneous (s.c.) delivery of insulin. The polymerization technique used was able to hold 65%–95% of insulin added 30 min after initiation of polymerization. The percentage drug loading was monomer concentration dependent. Insulin adsorption to the nanospheres was measured by radioimmumoassay. Although Pluronic F68 (0.5%) did not significantly alter the in vitro insulin degradation half-life $T_{50\%}$*, sodium cholate (0.5%) increased the degradation* $T_{50\%}$ *of insulin by 56% (from* 13.6 ± 1.6 *to* 22.1 ± 2 *min). This study also investigated the in vivo performance of insulin-loaded PIBCA in aqueous suspension with or without sodium cholate (0.5%) and Pluronic F68 (0.5%) surfactants after oral and subcutaneous administration to alloxan-induced diabetic rats. Insulin absorption was evaluated by its hypoglycemic effect. Insulin associated with PIBCA nanospheres retains its biological activity up to 15 h and 24 h after oral and subcutaneous administrations, respectively. Administered orally, insulin-loaded (75 U/kg) nanospheres, in the presence of surfactants, significantly reduced the mean blood glucose level from* 392 ± 32 *to* 80 ± 13 *mg/dl within 2 h and maintained it at 100 mg/dl or less for more than 8 h. On the other hand, the subcutaneous*

administration of insulin-loaded (25 U/kg) nanospheres significantly decreased the blood glucose level from 406 ± 33 to 88.5 ± 12.8 mg/dl within 1 h, and the lowered glucose level was maintained at 100 mg/dl or less for more than 12 h; it returned to its initial value 24 h after administration. Insulin-loaded nanospheres with surfactants showed significant ($P < .05$) pharmacological availability (PA%) of 37.6% ± 3.7% and 65.2% ± 2.7% after oral and subcutaneous dosages, respectively. The existence of surfactants with PIBCA nanospheres improved the oral PA% by 49.2%. These findings suggest that the developed PIBCA, in the presence of surfactants, would be useful not only in improving insulin gastrointestinal absorption, but also in sustaining its systemic action by lowering the blood glucose to an acceptable level.

Key Words: *Absorption enhancer; Insulin; Nanospheres; Polyisobutylcyanoacrylate; Sodium cholate*

INTRODUCTION

Bioactive proteins and peptides are a rapidly growing class of therapeutic agents, and even though there are only a few of them currently marketed, there are hundreds in clinical testing. Most of these, however, can be given parenterally because, with oral administration, they are degraded by proteolytic enzymes in the gastrointestinal (GI) tract or are impermeable to the intestinal mucosa due to their hydrophilic characteristics and large molecular size (1). Moreover, injections are given frequently because the in vivo half-lives are generally no more than several hours (2).

Various approaches have been proposed and demonstrated, such as incorporation of protease inhibitors (3–5), absorption enhancers (3,4,6–9), chemical modification (10,11), and dosage forms (12,13) to overcome the problems encountered with the delivery of peptides and proteins via the GI tract.

Encapsulating insulin in liposomes has been used as a means to target insulin selectively to the liver, enhance its oral absorption, and prolong its action. The high doses of liposome-entrapped insulin required perorally, coupled with extreme variability in the glycemic response to peroral liposomes, limit the value of peroral liposomal insulin as a viable diabetic therapy (14).

Recent studies have demonstrated the potential of polyalkylcyanoacrylate (PACA) as a colloidal carrier of drugs (15,16). Nanoparticles of PACA were first developed by Couvreur et al. (17). Drugs with diverse physicochemical properties can be adsorbed or entrapped by these ultrafine particles, less than 1 μm in diameter (18–21). PACA has also been investigated as a promising carrier for peptides and proteins (22–28). When insulin was associated to these nanocapsules and administered orally to streptozocin-induced diabetic rats, it reduced fasting glycemia for 1–3 weeks as a function of the dose of insulin administered (22). In addition, nanospheres protect insulin against proteolytic degradation and induce delayed urinary excretion of the hormone (23). It has been proposed that the prolonged effect of encapsulated insulin could be explained in part by a progressive arrival of nanocapsules from the stomach to the gut, leading to delayed absorption, and by progressive release of insulin after slow degradation of the polymer (22).

Miglyol 812 (a triglyceride containing C8 and C10 fatty acids) was incorporated in insulin-loaded nanocapsules either during the preparation (22,23) in the presence of Poloxamer 188 (Pluronic F68) or as a final dispersion medium (26) containing Poloxamer 188 and deoxycholic acid. However, the exact role of each compound remains unclear (26).

Insulin was chosen as a model polypeptide in this study for two reasons. First, this peptide is among the most widely studied for possible absorption through alternative routes. Second, improved insulin delivery could significantly influence diabetes treatment. The lifelong existence of diabetes has made the parenteral route extremely inconvenient, particularly for elderly and juvenile patients who are unable to self-administer the drug routinely. There was no report about the absorption enhancement effect of sodium cholate on insulin-loaded PIBCA in alloxan-induced diabetic rats. Therefore, after physical characterization of the prepared nanospheres, this study was carried out to examine the feasibility of PIBCA

nanospheres to protect and control the in vivo performance of peptides after oral and subcutaneous administration in the presence of sodium cholate in alloxan-induced diabetic rats.

EXPERIMENTAL

Materials

Isobutylcyanoacrylate, crystalline bovine insulin (24.4 U/mg), and α-chymotrypsin were purchased from Sigma Chemical Company (St. Louis, MO). Cholic acid sodium salt was obtained from Serva Feinbiochemica and Company (Heidelberg, Germany). Alloxan monohydrate was purchased from Winlab, Wilfrid Smith Limited (Middlesex, UK). Pluronic F68 Prilled was obtained from Ruger Chemical Company, Incorporated (Irvington NJ). All other reagents and chemicals were analytical grade and were used as received.

Nanosphere Preparation

Nanospheres were prepared by polymerization technique in continuous aqueous phase according to a previously described method (20) with a slight modification. In brief, the monomer solution (200 µl) was slowly added, using mechanical stirring, to 19 ml of 2×10^{-3} M HCl and 0.5% dextran 70 (pH 2.5) solution. Polymerization was carried out in a cold room, at 5°C. After 30 min of the addition of the monomer, 400 U of insulin solution (1 ml) was added dropwise. After 1 h, the resulting milky suspension was neutralized with 0.1 M NaOH. Each batch was prepared in triplicate.

Drug Loading

The sorption of insulin on PIBCA nanospheres was assessed immediately after preparation of the spheres. The suspension was centrifuged at 18,000 rpm for 30 min and filtered through a 0.22-µm filter. An aliquot of the filtrate was added to 4% bovine serum and analyzed by radioimmunoassay using a Coat-A-Count insulin kit provided by Diagnostic Products Corporation (Los Angeles, CA). The resulting free concentration was subtracted from the theoretical concentration (free + encapsulated) to estimate the drug loading. Nanospheres in the sediment were freeze-dried at −70°C. The lyophilized powder was weighed before storage at −20°C.

To determine the amount of insulin in the nanospheres, 2 mg of nanospheres in 1 ml of 0.5 M NaOH and 4 ml of 4% bovine serum were analyzed by radioimmunoassay as stated above. The results were compared with the analysis of drug in the filtered supernatant as described above.

Nanosphere Size

Scanning electron microscopy (Joel Scanning Microscopy, JSM840, Tokyo, Japan) was used to study the size and surface characteristics of the nanospheres.

In Vitro Stability of Insulin in the Presence of Sodium Cholate

α-Chymotrypsin was dissolved in 100 mM Tris buffer containing 1 mM $CaCl_2$ (pH 8). Insulin (10 mg) was prepared in Tris buffer containing 0.2% trifluoroacetic acid (TFA) (pH 2.45). Aliquots of insulin solution alone or containing sodium cholate (0.5% or 1%) were diluted to 2 ml with the Tris buffer. The solution was preequilibrated at 37°C for 15 min. Enzyme solution, 40 µl, was then added to insulin solution and maintained at 37°C. Before the addition of α-chymotrypsin solution, insulin solution was vortexed at high speed, and a 40-µl sample was withdrawn and diluted with 60 µl of cold Tris buffer solution containing 0.2% TFA (pH 2.45) as the zero time sample.

Samples (40 µl) were withdrawn, after mixing the solution, at 0, 1, 2, 5, 10, and 15 min after the addition of the enzyme solution. The collected samples were immediately diluted as described above to arrest the reaction. The samples were vortexed at high speed for 10 s and stored at −30°C until assayed by high-performance liquid chromatography (HPLC). Each experiment was carried out in triplicate.

High-Performance Liquid Chromatography System

The Waters HPLC system was equipped with a Waters Lambda Max 461 variable UV absorbance detector (set at 215 nm) and a Waters 710 B autosampler. A Waters 510 solvent delivery system was used to adjust the flow through a Zorbax C8 column (3.9×300 mm) packed with 5-µm spherical particles. The mobile phase was acetonitrile (31.5%)

containing triethylamine (0.15%); TFA was used to adjust the pH to 2.2. The flow rate was 1.3 ml/min, and the sample run time was 18 min. The injection volume was 50 µl. The assay was fully validated, and the mean relative standard deviations (RSD%) of the results of within-day precision and accuracy of insulin were less than 12%.

Induction of Diabetes

Diabetes was induced in male Sprague-Dawley rats (175–250 g) by an intravenous injection, in the tail vein, of alloxan monohydrate (80 mg/kg dissolved in normal saline). Rats were considered diabetic when fasting glycemia was 300 mg/dl or higher after 3 days of alloxan administration.

Insulin Preparation for Oral and Subcutaneous Administration

Immediately before administration, the specified weight of lyophilized insulin-loaded nanospheres (200 or 400 U) was dispersed in a normal saline solution containing 0.5% Pluronic F68 and 0.5% sodium cholate (surfactants) in the final solution. Insulin solution was also prepared in normal saline without any surfactant for use as a reference.

Animal Dosing

The 36 diabetic rats were randomly divided into six groups, and each group ($n = 6$) was housed in one cage. Animals were fasted for about 12 h prior to experiments, but water was available ad libitum at all times. The first two groups of diabetic rats were given 75 U/kg as an aqueous suspension of insulin-loaded nanospheres, in the presence or absence of surfactants, by oral tubing. The third group received the same suspension, but as 25 U/kg subcutaneously. Insulin solution in the absence or presence of surfactants was given orally to the fourth and the fifth groups, respectively. The last group received a subcutaneous injection (5 U/kg) of insulin solution containing the surfactants.

Blood Sampling

Blood samples (about 20 µl) were collected from the orbital venous plexus under light ether anesthesia. After overnight fasting, glycemia was measured in all animals at 0, 1, 2, 4, 8, 12, 15, and 24 h after insulin administration as described above.

Glucose Concentration

Insulin absorption was estimated by its hypoglycemic effect. Therefore, the blood glucose level was immediately measured using Haemo-Glukotest® reagent test strips (Boehringer Mannheim GmbH, Mannheim, Germany) with a Reflolux® S blood glucose monitor (Boehringer Mannheim Diagnostics). The sample size was approximately 20 µl whole blood. The precision of the assay was found to be within ±5%, and the measurable glucose levels ranged within 10–500 mg/dl.

Data Analysis

All data are presented as mean plus or minus the standard error (SE) or standard deviation (SD). The area under the percentage blood glucose remaining–time profile from time zero to the last time, in hours, (AUC_{0-24}) was calculated by the linear trapezoidal rule following oral and subcutaneous administration of insulin solution or insulin-loaded nanosphere aqueous suspension to rats. The effect of formulation or route of administration on the hypoglycemic action of insulin was assessed by the pharmacological availability (PA%), which indicates the extent of insulin action as follows:

$$PA\% = \frac{(AUC_{0-24,test} - AUC_{0-24,po})}{AUC_{0-24,po}} * 100$$

where $AUC_{0-24,test}$ is the AUC from time zero to 24 h, the last measured time, after the specified formulation or route of administration. Since the AUC after oral administration of insulin solution in the absence of surfactant ($AUC_{0-24,po}$) showed the highest AUC, it was selected as the reference in the above equation.

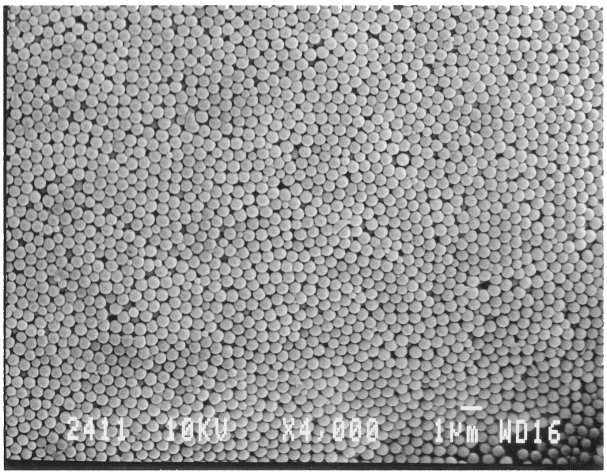
Linear regression was used to calculate the insulin degradation rate (according to zero-order kinetics) for concentration-time profiles, excluding the zero time. The degradation rate constant k, as percentage degraded per unit time, was calculated from the slope of the linear regression analysis of the percentage insulin remaining versus time curve. The degradation $T_{50\%}$ was determined from $(0.5\ A_0)/k$.

Statistical Analysis

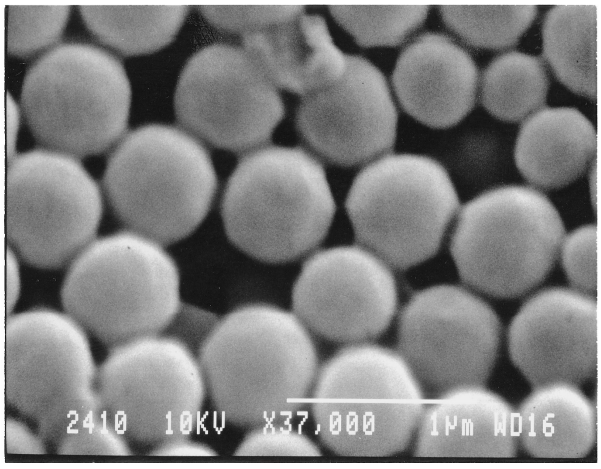
A one-way analysis of variance test and a Student t test were used for statistical evaluation. Differences were assumed to be statistically significant when $P < .05$.

RESULTS AND DISCUSSION

Insulin-loaded nanospheres were prepared by emulsifier-free polymerization in aqueous media.



(a)



(b)

Figure 1. (a) Electron scanning micrograph of insulin-loaded PIBCA nanospheres showing the mean diameter of the nanospheres, < 500 nm. (b) Electron scanning micrograph of insulin-loaded PIBCA nanospheres showing their spherical and smooth surface at higher magnification.

Figures 1a and 1b represent the electron scanning micrographs of insulin-loaded PIBCA nanospheres and show their homogeneous size distribution, with a mean diameter of less than 500 nm, which is in agreement with values in the literature (17). Higher magnification reveals the uniform spherical shape of the nanospheres and their smooth surface.

It was found that the addition of insulin 30 min after initiation of polymerization resulted in a substantial increase (from 2% to 95%) in drug loading. This polymerization technique was able to hold 65%–95% of insulin added 30 min after initiation of polymerization. Therefore, insulin throughout this study was added 30 min after initiation of polymerization. The percentage drug loading was monomer concentration dependent.

It was noticed that $27.1\% \pm 3\%$ of insulin degraded, in the presence of α-chymotrypsin, within 1 min, and 30% or more of the insulin remained at 15 min under the test conditions. The correlation coefficient between the percentage insulin remaining and time from 1 to 15 min was more than 0.97, with rapid degradation within the first minute. Therefore, insulin data were fitted to zero-order degradation kinetics from 1 to 15 min for insulin alone or in combination with sodium cholate (0.5% or 1%) or Pluronic F68 (0.5% or 1%). Table 1 represents the degradation $T_{50\%}$ of insulin in the absence or presence of surfactants. Accordingly, insulin degradation $T_{50\%}$ was 13.6 ± 1.6 min. Although Pluronic F68 (0.5%) did not significantly alter the in vitro insulin degradation $T_{50\%}$, sodium cholate (0.5%) increased the degradation $T_{50\%}$ of insulin by 56% (from 13.6 ± 1.6 to 22.1 ± 2 min). Increasing sodium cholate from 0.5% to 1% did not significantly change the degradation $T_{50\%}$ of insulin. Therefore, in this study,

Table 1

Effect of Sodium Cholate and Pluronic F68 on the Kinetics of Insulin Incubated with α-Chymotrypsin at 37°C (Mean ± SD)

Compound	Degradation $T_{50\%}$, min (SD)
Insulin	13.6 (1.6)
Sodium cholate	
0.5%	22.1 (2.0)
1%	22.2 (1.8)
Pluronic F68	
0.5%	12 (1.2)
1%	9 (0.7)

sodium cholate and Pluronic F68 surfactants were used at 0.5% as an absorption enhancer for insulin.

There is increasing interest for utilizing non-invasive routes for the developed bioactive proteins and peptides. It was suggested that the ileum seemed to be the most useful region in the small intestine for insulin absorption (29). However, insulin must be protected from proteolysis to enhance its absorption. In addition, absorption promoters could increase insulin efficacy more effectively in the colon than in the small intestine. The bile salt sodium cholate was selected as an absorbance promoter not only due to its being a normal endogenous bile salt in the body, but also because the recovery rate of the mucosal membrane from its damaging effect is much faster than that from deoxycholate (12,30,31). When sodium cholate was used alone, however, it was less effective than protease inhibitors in promoting oral insulin absorption (31). Therefore, a surface-active agent, Pluronic F68 (0.5%), was also used.

Figure 2 shows the mean blood glucose concentration-time profiles after oral (75 U/kg; Fig. 2A) and subcutaneous (5 and 25 U/kg; Fig. 2B) administration of insulin solution or insulin-loaded nanosphere suspensions in the presence or absence of surfactants. Table 2 represents the pharmacological availability (PA%) of insulin in alloxan-induced diabetic rats as a function of either the route of administration or the formulation used. There was no reduction in blood glucose levels in the absence of surfactants in insulin solution. However, a significant ($P < .05$) blood glucose reduction, from 373 ± 66 to 97 ± 79, was observed after 1 h of insulin solution oral administration in the presence of surfactants. The addition of surfactants to insulin solution showed a $9.6\% \pm 1.9\%$ increase in PA%, but the glucose level increased to more than 200 mg/dl after 2 h. However, it was reported (26) that insulin dispersed in Poloxamer 188 and deoxycholic acid in water or in Miglyol 812 had no effect on glycemia in rats.

A slow reduction in blood glucose levels was observed after oral administration of insulin-loaded nanospheres in the absence of surfactants. The highest reduction was obtained after 15 h, with a return to the original level within 24 h. This formulation increased the PA% by $25.2\% \pm 1.2\%$. It was suggested that the association of insulin to the polymer preserved its biological activity, and the absorption

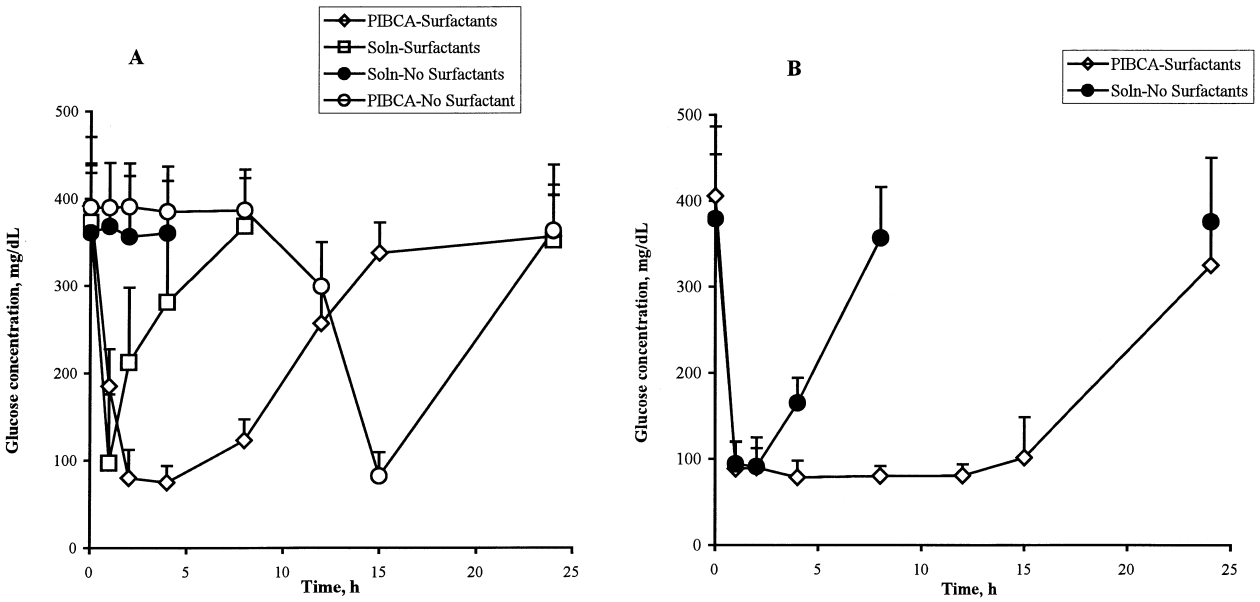


Figure 2. (A) Mean (+SE) blood glucose concentration-time profiles after oral (75 U/kg) administration of insulin-loaded nanosphere suspension (PIBCA-surfactants) and insulin solution in the presence (Soln-Surfactants) or absence (Soln–No Surfactants) of 0.5% Pluronic F68 and 0.5% sodium cholate. (B) Mean (+SE) blood glucose concentration-time profiles after 5 and 25 U/kg subcutaneous administration of insulin solution (Soln–No Surfactants) and insulin-loaded nanospheres suspension (PIBCA-Surfactants).

Table 2

Pharmacological Availability (Mean ± SE) of Insulin in Alloxan-Induced Diabetic Rats (n = 6) as a Function of Either the Route of Administration or the Formulation Used

Formulation	Dose (U/kg)	AUC (SE)	PA% (SE)
Oral			
PIBCA, no surfactants	75	1799 (30)	25.2 (1.2)
PIBCA, surfactants	75	1500 (90)	37.6 (3.7)
Solution, surfactants	75	2175 (46)	9.6 (1.9)
Solution, no surfactants	75	2405 (45)	—
Subcutaneous			
PIBCA, surfactants	25	838 (65)	65.2 (2.7)
Solution, no surfactants	5	1983 (36)	17.6 (1.5)

of intact insulin-loaded nanospheres (≤200 nm in diameter) in the presence of the surfactants cannot be excluded (26). On the other hand, the absorption of the released insulin from the nanospheres at the site of absorption also cannot be excluded. Without encapsulation, due to the presence of absorption promoters sodium cholate and Pluronic F68, insulin solution did reduce the blood glucose level, as stated above.

A fast decline (77%–91%) in blood glucose level was observed after 1 to 2 h of insulin-loaded nanosphere administration in the presence of surfactants. This may be attributed to the fact that, in aqueous medium, there is an initial surge (burst release) of insulin, enhanced by the presence of surfactants, from the surface of the nanospheres. These results are consistent with a previous report by Damge et al. (26). They showed that the fasting glycemia level decreased from the second hour by 50%–60% in streptococin-induced diabetic rats when insulin nanosphere dispersion medium was composed of Poloxamer 188 and deoxycholic acid in water or in Miglyol 812. However, there are reports (22,23,29) that show that oral administration of insulin-loaded nanocapsules to streptococin-induced diabetic rats markedly reduced fasting glycemia from the second day. The difference in the onset of action between the above reports could be attributed to the difference between nanocapsules and nanospheres in the methods of manufacture or characterization.

Insulin associated with PIBCA nanospheres in the presence of surfactants retains biological activity after oral and subcutaneous administration up to 12 and 24 h, respectively. The blood glucose levels were significantly reduced from 392 ± 32 to 80 ± 13 mg/dl within 2 h, maintaining an acceptable blood glucose level for more than 8 h, and it returned to its initial value after 15 h of oral administration. On the other hand, the subcutaneous administration of insulin-loaded nanospheres decreased the blood glucose level from 406 ± 33 to 88.5 ± 12.8 mg/dl within 1 h, and the glucose level was maintained at the lower level up to 24 h. After oral dosing, in the presence of surfactants, PIBCA improved insulin PA% by about 2.9 times compared to results without encapsulation.

It should be mentioned that Damge et al. (26) reported that fasting glycemia was about 150 mg/d from the second hour up to the second day after insulin administration, as mentioned above. However, the present study showed that the glucose concentration was ≤100 mg/dl for 8 and 12 h after oral and subcutaneous administration, respectively. This indicates that the developed nanosphere system produced an extended acceptable blood glucose level in alloxan-induced diabetic rats.

The difference in experimental design and the chemicals used in induction of diabetes in different animal species could account for the controversial observations between results.

In conclusion, polyisocyanoacrylate nanospheres were successfully employed in our laboratory for oral and subcutaneous administration in alloxan-induced diabetic rats. PIBCA not only enhanced the oral and subcutaneous absorption of insulin, but also prolonged its action up to 15 and 24 h, respectively. Sodium cholate seems to enhance the absorption of insulin solution more than deoxycholate. The factors affecting this fascinating system are being thoroughly investigated, in vitro and in vivo, using different markers.

ACKNOWLEDGMENT

I wish to express gratitude to Dr. Ahmed M. Ibrahim for his endless support during this study. I am also grateful to Dr. Robert Bayton for insulin analysis, Mr. Azad Moorj for lyophilization of the nanospheres, and Mr. Jeff McClintock for providing the electron scanning micrographs at King Faisel Specialist Hospital and Research Centre.

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