JPP Journal of Pharmacy and Pharmacology

JPP 2002, 54: 473–480

Received August 7, 2001
Accepted November 26, 2001
ISSN 0022-3573

Research Paper

In-vitro release and oral bioactivity of insulin in diabetic rats using nanocapsules dispersed in biocompatible microemulsion

Suchat Watnasirichaikul, Thomas Rades, Ian G. Tucker and Nigel M. Davies

Abstract

This study evaluated the potential of poly(*iso*-butyl cyanoacrylate) (PBCA) nanocapsules dispersed in a biocompatible microemulsion to facilitate the absorption of insulin following intragastric administration to diabetic rats. Insulin-loaded PBCA nanocapsules were prepared in-situ in a biocompatible water-in-oil microemulsion by interfacial polymerisation. The microemulsion consisted of a mixture of medium-chain mono-, di- and tri-glycerides as the oil component, polysorbate 80 and sorbitan mono-oleate as surfactants and an aqueous solution of insulin. Resulting nanocapsules were approximately 200 nm in diameter and demonstrated a high efficiency of insulin entrapment (> 80 %). In-vitro release studies showed that PBCA nanocapsules could suppress insulin release in acidic media and that release at near neutral conditions could be manipulated by varying the amount of monomer used for polymerisation. Subcutaneous administration of insulin-loaded nanocapsules to diabetic rats demonstrated that the bioactivity of insulin was largely retained following this method of preparing peptide-loaded nanocapsules and that the pharmacodynamic response was dependent on the amount of monomer used for polymerisation. The intragastric administration of insulin-loaded nanocapsules dispersed in the biocompatible microemulsion resulted in a significantly greater reduction in blood glucose levels of diabetic rats than an aqueous insulin solution or insulin formulated in the same microemulsion. This study demonstrates that the formulation of peptides within PBCA nanocapsules that are administered dispersed in a microemulsion can facilitate the oral absorption of encapsulated peptide. Such a system can be prepared in-situ by the interfacial polymerisation of a water-in-oil biocompatible microemulsion.

R&D Institute, Government Pharmaceutical Organization, 75/1 Rama VI, Rajtevee, Bangkok 10400, Thailand

Suchat Watnasirichaikul

Formulation and Drug Delivery Group, School of Pharmacy, University of Otago, P.O. Box 913, Dunedin, New Zealand

Nigel M. Davies, Thomas Rades, Ian G. Tucker

Correspondence: N. M. Davies, School of Pharmacy, University of Otago, P.O. Box 913, Dunedin, New Zealand. E-mail: nigel.davies@stonebow.otago.ac.nz

Acknowledgement and Funding: This study was financially supported by the New Zealand Pharmacy Education and Research Foundation. The authors wish to thank Mark Gould of the South Campus Electron Microscope Unit, University of Otago for the sample preparation for TEM. We acknowledge Abitec corporation, Columbus, Ohio for donation of Capmul MCM, Croda Surfactant NZ for providing Crodamol GTCC and Loctite, Dublin, Ireland for donation of *iso*-butyl cyanoacrylate. Acknowledgement is also given to the Thai Government Pharmaceutical Organization (GPO) and School of Pharmacy, University of Otago, for a PhD scholarship to S.W.

Introduction

The oral route has received much attention for the delivery of macromolecules, such as proteins and peptides, owing to its convenience and acceptability. However, major barriers exist for the oral delivery of these bioactives. Proteolytic enzymes present in the stomach and small intestine result in their digestion and the absorptive epithelium effectively excludes absorption of such hydrophilic macromolecules (Zhou & Li Wan Po 1991). Various approaches have been used in an attempt to overcome these barriers and increase the oral bioavailability of proteins and peptides including the use of polymeric nanoparticles and microemulsions (Damgé et al 1988, 1997; Constantinides et al 1994; Trenktrog et al 1995; Vranckx et al 1996). Encapsulation of the protein or peptide within nanoparticles can physically protect the bioactive from proteolytic enzymes (Lowe & Temple 1994;

Damgé et al 1997). Further, nanoparticles have been shown to translocate across the intestinal mucosa and hence can facilitate the absorption of peptides from the gut lumen (Aprahamian et al 1987; Jani et al 1990; Florence et al 1995). Under such circumstances, the polymer used for the preparation of the particulate carrier must be biodegradable. Lipid-based microemulsions have also proven beneficial for improving the oral delivery of proteins and peptides (Ritschel 1991; Constantinides et al 1994; Trenktrog et al 1995). The mechanism by which microemulsions enhance the oral absorption of peptides may not only be a result of their effect on membrane structure and fluidity (Ritschel 1991; Constantinides et al 1994; Trenktrog et al 1995) but may also result from their ability to enhance lymph uptake thereby overcoming hepatic first-pass metabolism (Ritschel 1991; Porter et al 1996).

We recently reported a method by which biodegradable, aqueous cored nanocapsules suitable for the entrapment of proteins and peptides can be prepared by interfacial polymerisation of water-in-oil microemulsions using alkylcyanoacrylates (Watnasirichaikul et al 2000). The use of microemulsions as a template for polymerisation has advantages over the use of size-reduced kinetically stabilised water-in-oil dispersions which have also been used for the preparation of aqueous cored nanocapsules (Vranckx et al 1996). As microemulsions are thermodynamically stable, they form spontaneously upon combining the oil, surfactant and aqueous mixture. As such, they require only a minimal input of energy for their formation in contrast to size-reduced kinetically stabilised water-in-oil dispersions. Further, microemulsions have a small and uniform droplet size and form reproducibly yielding nanocapsules having similar characteristics (Watnasirichaikul et al 2000). If biocompatible oils and surfactants are used for the formation of the microemulsion, the necessity of isolating the nanocapsules from the reaction matrix following polymerisation is removed. Poly(alkyl cyanoacrylate) (PACA) nanocapsules can therefore be prepared in-situ in an oily microemulsion matrix which may prove beneficial for the delivery of encapsulated peptide exploiting the benefits of both nanocapsules (translocation and protection) and microemulsions (permeation enhancing effects and lymph delivery).

The aims of this study were to investigate whether the biological activity of a polypeptide (insulin) is retained following the interfacial polymerization of microemulsions with alkyl cyanoacrylates and to what extent the release of peptide could be controlled by varying the monomer mass used for polymerization. Further, the study investigated whether PACA nanocapsules dispersed in a biocompatible microemulsion could facilitate the oral delivery of insulin in rats.

Materials and Methods

Materials

Caprylic and capric triglycerides (Crodamol GTCC), polysorbate 80 (Crillet 4) and sorbitan mono-oleate (Crill 4) were supplied by Croda Surfactants NZ (Auckland, NZ). Caprylic and capric mono- and diglycerides (Capmul MCM) were a gift from Abitec Corp. (Columbus, OH). *Iso*-butyl cyanoacrylate was a gift from Loctite (Dublin, Ireland). Human insulin (Humulin R) was obtained from Eli Lilly (Auckland, NZ). Streptozotocin was purchased from Sigma (St Louis, MO). Acetonitrile (HPLC grade) and methanol (AR grade) were purchased from BDH (Poole, Dorset, UK). Distilled water was used throughout. A proprietary glucose test kit (Advantage) was purchased from Roche Diagonostics (Auckland, NZ). Male Wistar rats (180–220 g) were used for the pharmacodynamic study which was approved by the University of Otago Animal Ethics Committee in compliance with the Institutional Code of Ethical Conduct, as required by The Animals Protection Regulations 1987 which regulate the use of animals in research, testing and teaching in New Zealand.

Preparation of nanocapsules

Microemulsions were prepared at 4°C by mixing 1.4 g of the surfactant blend (polysorbate 80 and sorbitan mono-oleate, 3:2 weight ratio), 7.6 g of the oil mixture (medium-chain triglycerides and medium-chain mono- and diglycerides, 3:1 weight ratio) and 1.0 g of a proprietary human insulin aqueous solution having a concentration of 100 units mL^{-1} (Humulin R). Liquid *iso*-butyl cyanoacrylate monomer (100, 150 or 200 mg) was slowly added to 10 g of the microemulsion under mechanical stirring. The system was stirred for at least 12 h at 4°C for polymerisation.

Characterisation of nanocapsules

The size of the nanocapsules was determined by laser diffraction (Mastersizer-X, fitted with a small-volume dispersion unit and a 45-mm lens; Malvern Instruments Ltd, UK). For particle size analysis, the nanocapsule dispersion was diluted with the microemulsion to obtain the desired obscuration. Measurements were carried out at room temperature.

The internal structure of the nanocapsules was visualized by freeze-fracture transmission electron micro-

scopy (Philips 410LS) as previously described (Watnasirichaikul et al 2000). Briefly, nanocapsules were collected by centrifugation at 51 500 *g* for 60 min at 25°C (Beckman J2/MC Centrifuge, JA20.1 rotor) and sandwiched between two copper grids. The grids were snap-frozen by immersion in liquid propane (−180°C) and freeze-fractured using a Balzers BAF 300. Fractured samples were shadowed with platinum (45°) and carbon (90°). The replicas were then washed in 50% chloroform in methanol followed by distilled water. Dried replicas were viewed at an accelerating voltage of 80 kV.

Determination of encapsulated insulin

The polymerized insulin microemulsion (1.6 g) was diluted to 10 mL with water adjusted to pH 2.5 by addition of hydrochloric acid. A sample of the dispersion (300 μL) was thoroughly mixed with 300 μL of 80% v/v methanol in water (pH 2.5). Nanocapsules and oil were separated from the methanolic aqueous phase by centrifugation (12 000 *g* for 12 min at room temperature). The concentration of insulin in the aqueous supernatant, representing the insulin not associated with the nanocapsules, was determined by HPLC as previously described (Watnasirichaikul et al 2000). The amount of insulin encapsulated was estimated from the difference in concentration of insulin detected in the supernatant of a polymerized microemulsion following processing and the concentration of insulin in the supernatant of an unpolymerized microemulsion following the same processing to which an equivalent concentration of insulin had been added.

Release of insulin from nanocapsules

The release of insulin from nanocapsules was carried out by diluting 3.2 g of the polymerized microemulsion containing insulin to 20 mL with phosphate buffer pH 6.8. The diluted systems were rotated longitudinally at 50 rev min^{-1} in a water-bath (37°C). Samples of 200 μL were removed at various times and diluted with 200 μL of 80% v/v methanol in water and centrifuged to separate the aqueous fraction from the poorly water-soluble components. The aqueous supernatant was analysed for insulin using HPLC as described for determination of encapsulated insulin. Zero-order release rates for insulin were estimated from the linear portion of the release rate versus time profile.

Release was also studied as described above under conditions simulating those of the gastrointestinal tract. The polymerized microemulsions containing insulin (3.2 g) were diluted to 20 mL with water adjusted to pH 1.2 with hydrochloric acid. Release was monitored for 2 h at this pH. The pH was then adjusted to 6.8 using a solution of Na_3PO_4 (0.5 M) and the release monitored for a further 8 h. The pH of the dissolution medium was monitored throughout the study. Samples of 200 μL were removed at various times and released insulin was again analysed by HPLC as described.

Pharmacodynamic study in diabetic rats

Induction of diabetes

Diabetes was induced in male Wistar rats, 180–220 g, by intravenous injection of streptozotocin (70 mg kg^{-1}) in citrate buffer pH 4.5 via the tail vein. The solution of streptozotocin was aseptically prepared and filtered through a 0.22-μm membrane filter (Millex-GV, Millipore). Rats were considered diabetic when fasted plasma glucose levels were greater than 300 mg dL^{-1} (typically 7 days after streptozotocin injection).

Subcutaneous administration

Diabetic rats, fasted overnight for 10 h, were injected subcutaneously with a single dose of insulin (10 units kg^{-1}) administered in one of five different formulations: an aqueous solution of insulin (IN), insulin-free nanocapsules (prepared using 150 mg monomer) dispersed in the microemulsion (NC), the microemulsions containing insulin (INME), nanocapsules (prepared using 150 mg monomer) containing insulin dispersed in the microemulsions (INNC 150 mg) and nanocapsules (prepared using 200 mg monomer) containing insulin dispersed in the microemulsions (INNC 200 mg). Five rats were used for each treatment group.

Intragastric administration

Four different formulations as described above (IN, NC, INME and INNC 150 mg) were administered intragastrically in a single dose to diabetic rats that had been fasted overnight for 10 h. Five rats were used for each treatment group. The dose of insulin used for intragastric administration was 50 units kg^{-1}.

Blood glucose determination

Baseline glucose levels were obtained following a 12-h fasting period, 24 h before administration of insulin in either study. Blood samples were taken from the tip of the tail vein using an automatic lancing device (Softclix II finger pricker, Roche). For both subcutaneous and intragastric administration, blood samples were taken 2, 4, 6, 8, 10, 12, 16, 36 and 60 h following administration. Blood samples were analysed for blood glucose using a proprietary glucose test kit (Advantage, Roche). All rats had free access to water throughout the study

and access to food during the periods 16–24 h and 36–48 h post administration of insulin. As such, all blood samples were taken after at least 12 h of fasting.

Statistical analysis

Data were analysed by a one-way analysis of variance followed by Tukey's pairwise comparisons at a 95% confidence interval to test for significant differences between formulations at each time point with regards to blood glucose levels.

Results and Discussion

Characterisation of nanocapsules

The mean of the number size distribution of three different poly(*iso*-butyl cyanoacrylate) (PBCA) nanocapsules formulations prepared using different masses of monomer ranged from 200 to 210 nm with the average size slightly increasing with increasing mass of monomer used for polymerisation (Table 1). Transmission electron micrographs of freeze-fractured samples showed the particles to have a central cavity surrounded by a polymer wall (Figure 1). The average size of nanocapsules determined from the micrographs was approximately 200 nm ($n > 100$) in agreement with the results from dynamic laser light scattering.

Table 1 Effect of monomer mass on size of PBCA nanocapsules, insulin entrapment and zero-order rate constants of insulin release.

Monomer mass (mg)	Size (nm)	Entrapment (%)	Rate constant (% min^{-1})
100	200	84.3	0.089 (60–360)
150	210	94.7	0.055 (60–360)
200	210	97.2	0.048 (120–360)

Values represent means, n = 2. Values in parentheses represent the time (min) over which zero-order release was observed.

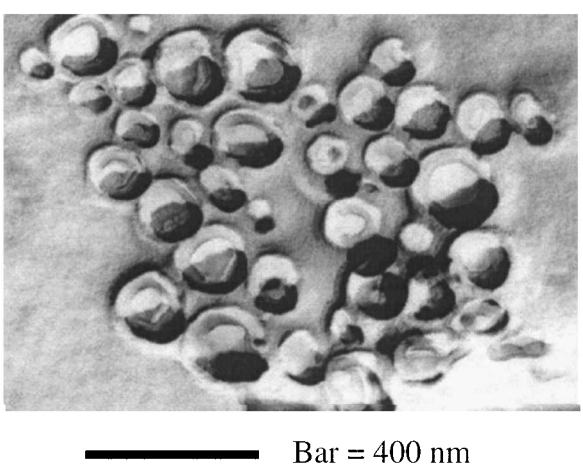

Figure 1 Freeze-fracture transmission electron micrograph of PBCA nanocapsules.

Nanoencapsulation of insulin

The entrapment of insulin within nanocapsules was in all cases greater than 80% and increased with increasing the mass of monomer used for polymerisation. Entrapment efficiencies reached values of greater than 90% for nanocapsules prepared using 150 and 200 mg of *iso*-butyl cyanoacrylate when added to 10 g of microemulsions containing 10% aqueous solution of insulin (Table 1). A similar relationship between entrapment efficiency and mass of monomer used for polymerisation was reported by El-Samaligy et al (1986) for the entrapment of fluorescein within nanocapsules prepared by interfacial polymerization of kinetically stabilized water-in-oil emulsions. The higher entrapment of this hydrophilic marker noted with increasing monomer mass was believed to be a result of the increased resistance of thicker coats to the washing process.

Release of insulin from nanocapsules

Repeated measures analysis of variance of the release data demonstrated that the percentage of insulin released was significantly influenced by the mass of mono-

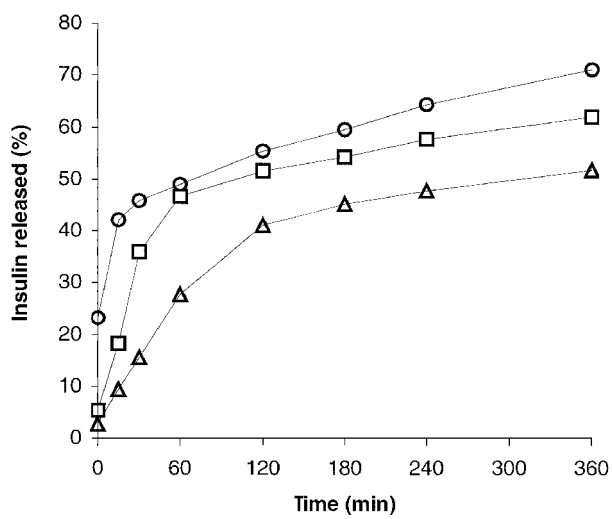

Figure 2 Release of insulin in pH 6.8 medium from PBCA nanocapsules prepared using 100 mg (○), 150 mg (□) or 200 mg (△) of *iso*-butyl cyanoacrylate added to 10 g of microemulsion. Values represent means, n = 2.

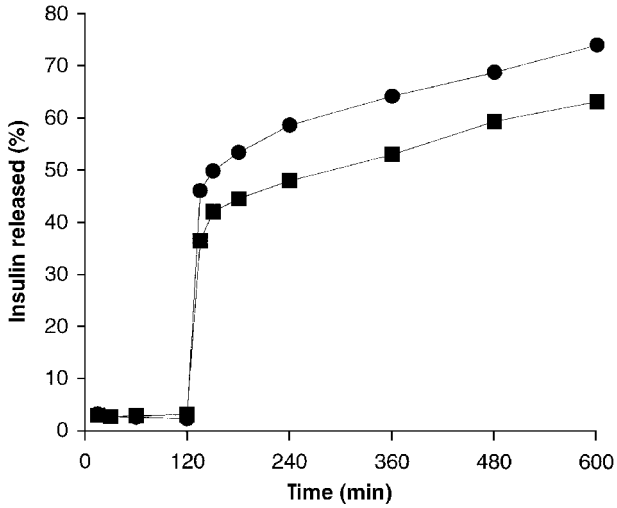

Figure 3 Release of insulin from PBCA nanocapsules prepared using 100 mg (●) and 150 mg (■) of *iso*-butyl cyanoacrylate added to 10 g of microemulsion under conditions simulating the pH in the gastrointestinal tract (pH was changed from 1.2 to 6.8 at 120 min). Values represent means, n = 2.

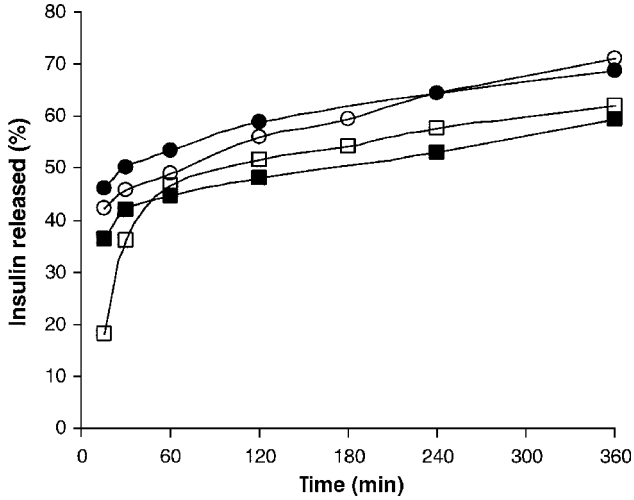

Figure 4 Release of insulin from PBCA nanocapsules prepared using 100 mg (circles) and 150 mg (squares) of *iso*-butyl cyanoacrylate in pH 6.8 medium with (filled symbols) or without (open symbols) a 2-h exposure to pH 1.2 release medium.

mer used for polymerisation ($P < 0.01$; Figure 2). In all cases, an initial rapid rate of insulin release was observed followed by a period of slower release which appeared zero order when release rate was plotted against time. The zero-order release rates noted (Table 1) support the postulated capsule nature of the nanocapsules produced by this technique as previously evidenced by freeze-fracture transmission electron microscopy. The decrease in the release rate of insulin with increasing monomer mass may be due to formation of nanocapsules having a thicker polymer wall.

The pH of the release media was also shown to influence the rate of insulin release from PBCA nanocapsules. Release of insulin from PBCA nanocapsules was suppressed in an acidic release medium (pH 1.2) simulating the pH of the stomach over a 2-h period (Figure 3). Changing the pH of the release medium to 6.8 after this time resulted in an immediate biphasic release profile as observed when release was monitored in only a pH 6.8 release medium. Only slight differences in the release profiles at pH 6.8 were observed between nanocapsules exposed for 2 h to an acidic release medium and those which had not been exposed to acid (Figure 4). These slight differences were only observed during the initial burst release (first 30 min) where release from PBCA nanocapsules which had been exposed to the acidic medium was slightly higher. The lack of release under acidic conditions and hence potential protection of insulin from the hostile environment of the stomach is in agreement with the results of Scherer et al (1994) and could be a result of either the stability of the PBCA under acidic conditions or due to the formation of poly(cyano acrylic acid) following hydrolysis of the butyl side-chain which could behave as an enteric-coating polymer. The similarities between release profiles for nanocapsules exposed for 2 h to an acidic release medium and those which had not been exposed to acid would suggest that suppression of release is a result of the stability of the PBCA. However, the possibility of some hydrolysis occurring over the 2-h period cannot be discounted and may explain the slight differences noted in the burst release profiles (Figure 4).

Subcutaneous administration

The process used for the preparation of nanocapsules could potentially denature the protein or peptide being entrapped or alternatively the protein or peptide could become involved in the polymerisation. Either of these processes could render the protein or peptide inactive. The activity of insulin nanocapsules was thus first evaluated following subcutaneous administration. Further, two batches of nanocapsules were prepared using different masses of monomer (150 and 200 mg) to investigate whether the difference in release profiles observed in-vitro from nanocapsules prepared using different masses of monomer translated to different release profiles in-vivo.

All formulations containing insulin (IN, INME, INNC 150 mg or INNC 200 mg) effectively reduced

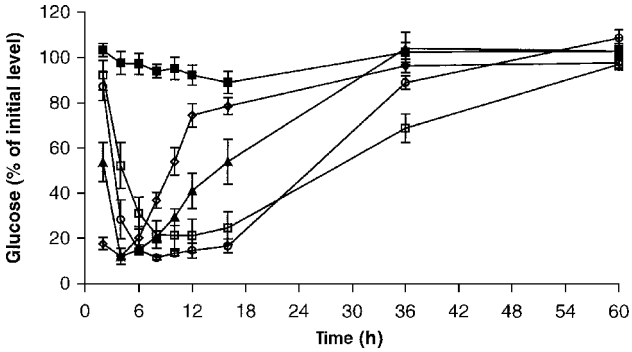

Figure 5 Blood glucose response in diabetic rats following the subcutaneous administration of insulin-free nanocapsules (prepared using 150 mg monomer) dispersed in the microemulsion (NC; ■), an aqueous solution of insulin (IN; ◇), microemulsion containing insulin (INME; ▲), nanocapsules (prepared using 150 mg monomer) containing insulin dispersed in the microemulsions (INNC 150 mg; ○) or nanocapsules (prepared using 200 mg monomer) containing insulin dispersed in the microemulsions (INNC 200 mg; □). Values represent means±s.e.m., n = 5.

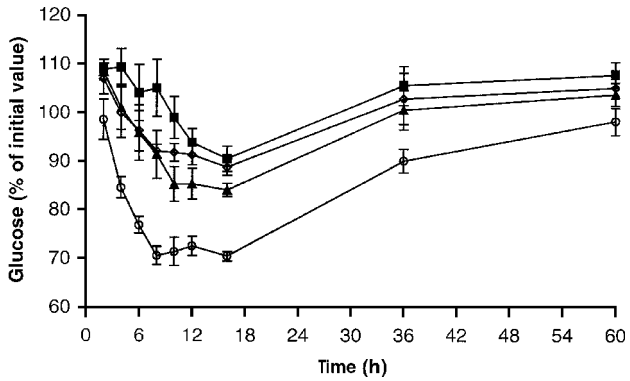

Figure 6 Blood glucose response in diabetic rats following the intragastric administration of insulin-free nanocapsules (prepared using 150 mg monomer) dispersed in the microemulsion (NC; ■), an aqueous solution of insulin (IN; ◇), microemulsion containing insulin (INME; ▲) or nanocapsules (prepared using 150 mg monomer) containing insulin dispersed in the microemulsions (INNC 150 mg; ○). Values represent means±s.e.m., n = 5.

blood glucose in diabetic rats following subcutaneous administration (Figure 5). The pharmacodynamic effect was delayed when insulin was administered in the microemulsion compared with insulin administration as an aqueous solution ($P < 0.05$ at 2 h). The effect was further delayed when insulin was encapsulated within nanocapsules and administered dispersed in the microemulsion. The extent of this delay was dependent on the mass of monomer used for polymerisation. Nanocapsules prepared using 200 mg of monomer added to 10 mL of a microemulsion containing 10% insulin solution not only reduced the extent of the initial drop in blood sugar but significantly prolonged the pharmacodynamic effect compared with nanocapsules prepared using 150 mg monomer ($P < 0.05$ at 36 h). The subcutaneous study therefore showed that the bioactivity of insulin is largely retained following the interfacial polymerisation of a microemulsion using alkyl cyanoacrylates and that the release of insulin can indeed be controlled by varying the amount of monomer used for polymerisation, hence varying the polymer wall thickness of the nanocapsules.

Intragastric administration

The resulting blood glucose (as percentage of baseline value) following the intragastric administration of empty nanocapsules dispersed in microemulsion (NC), an aqueous insulin solution (IN), insulin formulated in a microemulsion (INME) and insulin encapsulated within nanocapsules prepared using 150 mg monomer (INNC 150 mg) and administered dispersed in the microemulsion are shown in Figure 6. For empty nanocapsules, blood glucose was noted to decrease with time, as was noted for the same formulation in the subcutaneous study, and was attributed to the continued fasting status of the diabetic rats. No significant difference in blood glucose reduction (% of initial value) was observed between administration of an aqueous insulin solution and empty nanocapsules at any of the measured time points ($P > 0.05$). A significant difference in blood glucose reduction between empty nanocapsules and the insulin microemulsion was observed only at 10 h post-administration ($P < 0.05$). A significant difference in blood glucose levels was observed between 4 and 16 h for INNC 150 mg and empty nanocapsules and between 6 and 16 h for INNC 150 mg and either the aqueous insulin solution or the insulin microemulsion. Thus, a formulation in which insulin is encapsulated within nanoparticles and administered dispersed in a microemulsion is more effective in improving the oral bioavailability of insulin than a microemulsion alone. Whether this is a result of enhanced protection against proteolytic enzymes or the ability of nanocapsules to translocate across the intestinal mucosa and hence facilitate intestinal absorption remains to be determined, as is the importance of administering the nanocapsules dispersed in a microemulsion.

The release studies carried out in-vitro demonstrated that PBCA nanocapsules could suppress the release of insulin under acidic conditions (pH 1.2) and hence may

protect the entrapped protein during its passage through the stomach where it has been reported that up to 30% of protein digestion can occur (Verhoef et al 1990). When exposed to media having a pH similar to that expected in the intestine (pH 6.8), a significant proportion of the insulin is immediately released (approx 40% burst release). As mentioned, microemulsions incorporating medium-chain glycerides have been reported to enhance peptide absorption (Ritschel 1991; Constantinides et al 1994). The release of insulin in the intestine in the presence of the microemulsion excipients could therefore facilitate the intestinal absorption of some of this released insulin before enzymatic digestion and lead to the early absorption of insulin as noted in this investigation, with effects being noted as early as 2 h post intragastric administration.

The prolonged duration of the effect on blood glucose noted in this study lasting up to 60 h post intragastric administration, however, would suggest that the sustained release of insulin entrapped in nanocapsules plays a role in the overall response and that the gastrointestinal transit of the nanoparticles is either delayed or the particles must passage across the intestinal mucosa. A prolonged reduction in blood glucose in diabetic rats of 10–13 days was reported by Damgé et al (1997) following the intragastric administration of insulin-loaded nanospheres dispersed in an oily medium (Miglyol 812) containing surface active agents. Using radiolabelled polymer and insulin, these workers investigated the tissue distribution of nanospheres and insulin following intragastric administration and concluded that the prolonged effect was due, in part, to the progressive arrival of nanospheres from the stomach to the intestine and a prolonged mucoadhesion of nanospheres, leading to a delayed absorption and also to the absorption of intact nanospheres or at least small oligomers linking insulin. The difference in persistence of the pharmacodynamic effect of greater than a week observed between this study and that of Damgé et al (1997) is difficult to interpret but could be a result of the difference in release characteristics between the nanocapsules used in this investigation and the nanospheres used by Damgé et al, difference in the vehicle used to disperse and administer the nanoparticles, difference in the amount of insulin oligomers formed in the two methods of nanoparticle preparation or difference in dose of insulin administered (50 units kg^{-1} cf 100 units kg^{-1}).

The findings of this study further lend support to the importance of the vehicle used to disperse and administer the nanoparticles. Both Lowe & Temple (1994) and Damgé et al (1997) have reported that nanocapsules dispersed in water are ineffective in enhancing the oral absorption of entrapped peptide. Damgé et al (1997) proposed that the dispersion of the nanocapsules in an oily vehicle containing surfactants was a prerequisite for oral-absorption-enhancing effects for encapsulated peptides. However, using the more commonly used methods for the preparation of PACA nanocapsules, the peptide-loaded nanoparticles must first be isolated from the polymerisation vehicle before they can be dispersed in such a vehicle. By using a biocompatible water-in-oil microemulsion as a template for polymerisation as described in this study, peptide-loaded nanocapsules are formed in-situ in a vehicle reportedly capable of promoting intestinal absorption. This overcomes the need for isolation and renders the process very amenable to scale-up.

Conclusion

Varying the mass of monomer used for polymerisation can control the rate of release of insulin from PBCA nanocapsules prepared by the interfacial polymerisation of a water-in-oil microemulsion as demonstrated both in-vitro and in-vivo following subcutaneous administration. The release of insulin from PBCA nanocapsules is suppressed in acidic conditions and release in near neutral conditions is not dramatically altered by pre-exposure to an acidic environment. This would suggest that PBCA would remain largely intact during transit through the stomach. The formulation of insulin within nanocapsules dispersed in the water-in-oil microemulsion has been shown in this study to significantly increase the oral bioavailability of insulin in diabetic rats following intragastric administration compared with an aqueous insulin solution or a water-in-oil insulin microemulsion. Whether this is a result of enhanced protection against proteolytic enzymes afforded by encapsulation, the ability of nanocapsules to translocate across the intestinal mucosa and hence facilitate intestinal absorption or some synergistic effect of the microemulsion vehicle remains to be determined and is the subject of continuing investigations.

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