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Quantitative estimation of the effects of bile salt surfactant systems on insulin stability and permeability in the rat intestine using a mass balance model

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Abstract

The oral delivery of peptides and proteins is compromised by chemical and proteolytic instability as well as by permeability limitations. The aim of this study was to delineate the relative contributions of simple bile salt and bile salt:fatty acid mixed micellar systems to protein stability vs permeability enhancement in the rat intestine. Insulin disappearance from the rat intestine was evaluated when administered in simple micellar systems of sodium cholate (NaC), sodium taurocholate (NaTC) and sodium glycocholate (NaGC), and in mixed micellar systems of these bile salts and linoleic acid (LA). In-vitro stability studies were used to evaluate the extent of insulin degradation in the different micellar systems. After correction for insulin degradation in all systems a mass balance model was used to estimate the fractions of insulin absorbed for all systems. Mass balance estimates for the extent of insulin absorption in control perfusion systems were consistent with previously reported predictions of the model for ileal insulin absorption. Mass balance estimates for NaGC suggested no significant effects on the fraction of insulin absorbed relative to control. However, insulin absorption was estimated to occur to a significantly greater extent for NaTC simple micellar systems and was coincident with increased permeability of the hydrophilic marker molecule PEG 4000. The mass balance model estimated higher fractions of insulin absorbed for all mixed micellar systems in line with enhanced plasma insulin levels and higher PEG 4000 permeabilities for these systems.

Introduction

The relative or absolute deficiency of insulin manifests itself as a disease known as diabetes mellitus which can generally be divided into two categories: type I (juvenile onset or insulin-dependent diabetes mellitus) or type II (adult onset or non-insulin-dependent diabetes mellitus). Despite the recent advances in the development of non-invasive insulin delivery systems (Owens et al 2003; Foss et al 2004), long-term treatment of type I diabetes relies predominantly on subcutaneous injection of the hormone. Subcutaneous administration of insulin differs from physiological secretion of insulin in at least two major ways; the kinetics do not mimic the normal rapid rise and decline of insulin in response to secretion of nutrients and the insulin diffuses into the peripheral circulation instead of being released into the portal circulation; the preferential effect of secreted insulin on hepatic metabolic processes is then eliminated (Brange & Vølund 1999). Insulin absorbed from the intestinal tract, like insulin secreted by the pancreas, drains into the hepatic portal vein to exert its initial effect on the liver. Therefore, several methods have been investigated with a view to enhancing the passage of insulin across the intestinal membrane.

Bile salts and bile salt:lipid mixed micelles containing fatty acids or mono- and diglycerides with bile salts have been extensively studied to promote drug absorption (Sugiyama et al 1997; Sakai et al 1999). These formulations have been administered nasally, intratracheally, orally and to specific regions of the gastrointestinal tract (Muranishi 1985; Kararli et al 1992; Morimoto et al 1998). Although enhanced oral absorption of insulin in bile salts and bile salt:fatty acid systems has been reported (Mesiha et al 1994; Hosny et al 1998), the extent to which such improvements in bioavailability reflect enhanced permeability as opposed to insulin stability has not been clearly elucidated.

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There are limited reports of attempts to theoretically model the intestinal absorption of peptides and proteins following intestinal administration. Using mass balance approaches, Sinko et al (1993) simulated insulin absorption in the rat small intestine. This model described the total extent of peptide or protein disappearance from the small intestine as F_L. F_L was composed of F_A, the fraction of protein absorbed and F_R, the fraction of protein degraded. The primary objective of this study was to delineate the relative contributions of simple and mixed bile salt micellar systems to peptide stability vs absorption towards an understanding of the mechanism by which they modulate intestinal insulin permeability when administered to the rat small intestine. Allowing for enzymatic degradation and insulin metabolism, the maximal extent of insulin absorption in the duodenum was calculated to be 1.6% and in the ileum 13% (Sinko et al 1993). Evidence for the duodenal value was provided from the value of 2% for polyethylene glycol (PEG) absorption data reported by Donovan et al (1990), but to date, literature data to verify the extent of the ileal absorption of insulin is lacking. A further aim of this study, therefore, was to compare our estimated values with previously predicted values for ileal insulin intestinal absorption from the mass balance model.

In-vitro stability studies were used to assess the stability of insulin when incubated with the simple and mixed micellar systems in effluent collected from the rat gut. The degradation rate constants for insulin in the presence of micellar systems were subsequently applied to calculate the fraction degraded (F_R) for each system and to correct the observed extent of insulin disappearance (F_L) when perfused with these systems in the rat small intestine for estimation of the fraction of insulin absorbed from the intestine (F_A).

A perfused rat model was used as it offered the advantage of maintaining an intact blood supply, accessing a specific intestinal region, and controlling input into the segment. The bile salts studied and their respective critical micellar concentration (CMC) were the trihydroxy unconjugated bile salt, sodium cholate (NaC, CMC 11 mM), and the conjugated bile salts, sodium taurocholate (NaTC, CMC 4 mM) and sodium glycocholate (NaGC, CMC 11 mM). The effects of simple bile salt micellar systems (40 mM) and mixed micelles of 40 mM bile salt:40 mM linoleic acid (LA) on the disappearance of insulin from the rat intestine and appearance in plasma were determined. Polyethylene glycol (PEG) 4000 was included in the perfusion systems as a marker of paracellular absorption.

Materials and Methods

Materials

Human insulin (28.1 IU mg^{-1}), linoleic acid and the sodium salts of cholic acid, glycocholic acid and taurocholic acid were obtained from Sigma Chemical Company. [C^{14}]PEG 4000 was obtained from Amersham UK. All other reagents were of analytical grade.

Preparation of simple and mixed micellar systems

Bile salt micellar systems were prepared by weighing accurately the appropriate quantity of the salt and dissolving in phosphate buffer or rat gut perfusate. Dissolution of the salt was achieved by constant stirring at 37°C. After dissolution the system was allowed to stand for approximately 30 min. The preparation of mixed micellar systems incorporating fatty acid involved preparing the simple micellar system in the same manner. The fatty acid was added incrementally to the highly concentrated simple micelle system with stirring at 37°C.

In-situ absorption studies

All animal experiments were performed at Trinity College, Dublin, in association with the BioResources Unit, which is registered with the Department of Health, the competent authority designated under EU directive 86/609. The facility is under the full-time direction of a veterinary surgeon who maintains the health and welfare programme. Ethical approval was obtained through the veterinary surgeon.

In-situ absorption studies were conducted according to the rat gut perfusion method described by Komiya et al (1980). Male Wistar albino rats (250–300 g) were used and were fasted overnight, but allowed free access to water. An intraperitoneal injection (50 mg kg^{-1}) of pentobarbital sodium (60 mg mL^{-1}) was used to anaesthetize the animals. The proximal jejunum was exposed via a midline abdominal incision, and was cannulated with a glass cannula (i.d. 2 mm, o.d. 4 mm). A 33.3-cm length (l) of intestine was used for all experiments. After gentle washing the distal end of the intestine was cannulated. [C^{14}]PEG 4000 was included in all systems under investigation as a marker of paracellular permeability as reported by Lane et al (1996). A flow rate (Q) of 0.2 mL min^{-1} was used. Perfusion experiments were carried out in Sørenson's phosphate buffer pH 7.4 adjusted to isotonicity using an osmometer (Model 110, Fiske Associates) where necessary. The concentration of insulin in perfusion solutions was 0.1 mg mL^{-1}. Experiments were carried out for 3 h with perfusate collection at 10-min intervals. Collection sample vials were weighed before use and after sample collection to check the flow rate and to determine any variation in volumes of liquid collected. Corrections for water flux were carried out according to the method of Peréz et al (2002). Blood samples were collected from the jugular vein at 30-min intervals and centrifuged at 3000 *g* for 20 min in a refrigerated centrifuge. Plasma was collected and stored at −20°C before analysis for insulin.

In-vitro stability studies

For in-vitro stability studies, isotonic phosphate buffer was collected from animals after perfusion through the intestine as for the in-situ studies. The protein concentration of perfusate was diluted to 1 mg mL^{-1} by measurement of total protein content of the perfusate (Lowry et al 1951) as described by Asada et al (1994). Insulin was added to rat gut perfusate alone and to perfusate containing simple and

mixed micellar systems at the same concentration as for the in-situ absorption studies. Stability experiments were carried out for insulin incubated in perfusate over 30 min. Degradation rate constants were calculated for insulin from replicate experiments ($n = 3$) when incubated in perfusate in the presence and absence of all micellar systems.

Analytical methods

Insulin was assayed using a commercial radioimmunoassay kit (Phadaseph RIA, Pharmacia). The detection range of the assay was 1.5–240 μU insulin mL^{-1}. Samples were diluted where appropriate, to fall within the linear range of the assay. The inter- and intra-assay coefficients of variation were both $< 5\%$. [C^{14}]PEG 4000 was determined by liquid scintillation counting (Packard Tri-Carb 2500 TR liquid scintillation analyser). Quench correction was carried out using the method of external standardization.

Calculation of apparent permeability coefficient of PEG 4000

Permeability coefficients for PEG 4000 were calculated from the fraction of solute remaining in the intestinal lumen of length l, and effective lumenal radius r, using equation 1:

$$P_{app} = -Q/2\pi rl \times \ln(C_l/C_o) \quad (1)$$

where C_o was the input perfusate drug concentration, C_l was the outlet perfusate and Q was the flow rate ($mL\,s^{-1}$).

Mass balance estimations of insulin absorption and degradation

In-vitro stability studies were used to calculate the corresponding degradation rate constant k_r for insulin metabolism at the steady-state phase of the absorption studies for each system studied. To estimate the fraction of insulin absorbed (F_A) relative to the fraction degraded (F_R), the total disappearance of insulin from the lumen at steady state (F_L) and the relative degradation constant for insulin (k_r) in each system were applied to the mass balance mathematical model described by Sinko et al (1993). In this model the fraction of drug lost in a tube (F_L) was defined as follows:

$$F_L = 1 - e^{-(2An + Da)} \quad (2)$$

An had been defined previously as the absorption number and was defined as:

$$An = (l/r)(Pe/v_z) \quad (3)$$

where l was tube length, r was radius, Pe was drug permeability coefficient and v_z was fluid velocity.

Da had been defined previously as the Damkohler number and was defined as:

$$Da = k_r(V_L/Q) \quad (4)$$

where k_r was the degradation rate constant calculated from in-vitro stability studies, V_L was tube volume, and Q was flow rate. The fraction of drug absorbed was further defined as:

$$F_A = 2\ An[1 - e^{-(2An + Da)}]/(2An + Da) \quad (5)$$

and the fraction of drug degraded as:

$$F_R = Da[1 - e^{-(2An + Da)}]/(2An + Da) \quad (6)$$

F_L was the total fraction of drug, which was lost from the gut via both absorption and degradation and was directly measured from in-situ perfusion data. The model experimental parameters and in-vitro degradation rate constants were input for each system to calculate Da. This allowed calculation of An for each simple and mixed micellar system in the perfusion experiments from equation 2. An for each system was used to estimate a permeability coefficient for insulin in the different experimental conditions in equation 3.

Statistics

Each value was expressed as the mean or the mean $\pm$ s.d. of the value of the mean. Student's *t*-test was used to test the significance of the difference between two means. Analysis of variance was used when more than two means were compared. *P* values less than 0.05 were considered to be statistically significant. Stability data and mass balance data were fitted to the relevant models using Minsq 4.0 (Micromath Inc.).

Results and Discussion

In-vitro stability studies

The disappearance of insulin when incubated in rat gut perfusate followed first-order kinetics with a degradation rate constant of $0.85 \pm 0.09\,s^{-1}$ (Table 1). This was in line with previous reports of insulin biodegradation as a first-order process (Schilling & Mitra 1991). The degradation of insulin in rat gut effluent in the presence of NaGC and NaTC also followed first-order kinetics with respective degradation rate constants of 0.24 ± 0.01 and $0.48 \pm 0.05\,s^{-1}$, suggesting that NaGC and NaTC promoted insulin stability relative to control systems (Table 1). The ability of NaTC and NaGC to enhance insulin stability had been reported by Bai (1994), the mechanism being a direct inhibitory effect of those compounds on peptidase activity.

When incubated with mixed micelles of NaC:LA in rat gut effluent, insulin was observed to exhibit pronounced instability with a corresponding first-order degradation rate constant of $2.03 \pm 1.02\,s^{-1}$ in rat gut effluent (Table 1). NaGC:LA micelles did not significantly affect insulin stability in rat gut effluent ($k_r = 1.02 \pm 0.12\,s^{-1}$) but NaTC:LA systems significantly enhanced insulin degradation ($P < 0.05$). The proteolytic degradation of porcine zinc insulin by α-chymotrypsin had been reported to follow

Table 1 Degradation rate constant (k_r) for insulin in 40 mM bile salt and 40:40 mM bile salt:fatty acid systems in rat perfusate

Formulation	k_r (s^{-1})
Insulin	0.85 ± 0.09
Insulin/NaGC	0.24 ± 0.01*
Insulin/NaTC	0.48 ± 0.05*
Insulin/NaC:LA	2.03 ± 1.02*
Insulin/NaGC:LA	1.02 ± 0.12
Insulin/NaTC:LA	1.14 ± 0.21*

*$P < 0.05$ compared with control.

Table 2 Apparent permeability coefficients (P_{app}) for PEG 4000 in the presence and absence of 40 mM bile salt systems and 40:40 mM bile salt:fatty acid systems

Perfusion system	P_{app} ($cm\,s^{-1}$) $\times 10^6$
Phosphate buffer pH 7.4	0.58 ± 0.09
Insulin/phosphate buffer pH 7.4	0.71 ± 0.15
Insulin/NaC	$2.02 \pm 0.14^{\dagger}$
Insulin/NaGC	1.10 ± 0.16
Insulin/NaTC	0.86 ± 0.22
Insulin/NaC/LA	$3.42 \pm 0.45^{\dagger}$
Insulin/NaTC/LA	$3.24 \pm 0.83^{\dagger}$
Insulin/NaGC/LA	$3.35 \pm 1.20^{\dagger}$

$^{\dagger}P < 0.05$ compared with from P_{app} in (i) phosphate buffer pH 7.4, and (ii) insulin/phosphate buffer pH 7.4.

first-order kinetics with degradation being accelerated by mixed micelles of NaGC:LA when compared with simple micelles of NaGC (Li et al 1992). Degradation of insulin in rat gut effluent in this study was enhanced by mixed micelles of NaGC:LA and NaTC:LA in comparison with the corresponding simple micellar systems.

In-situ absorption studies

Simple micellar systems

Perfusion experiments were carried out to determine F_L, the extent of insulin disapparance from the lumen at steady state. When perfused over 180 min the extent of insulin disappearance in the NaC and NaTC systems was similar, with approximately 50% of insulin remaining in the perfusate at steady state (Figure 1). For the NaGC system, approximately 80% of insulin remained in the perfusate after 3 h. The in-vitro stability studies suggested that NaGC had a protective effect on insulin in rat gut perfusate and this appears to be supported by the higher insulin levels in NaGC micellar systems relative to perfusion of insulin in phosphate buffer pH 7.4.

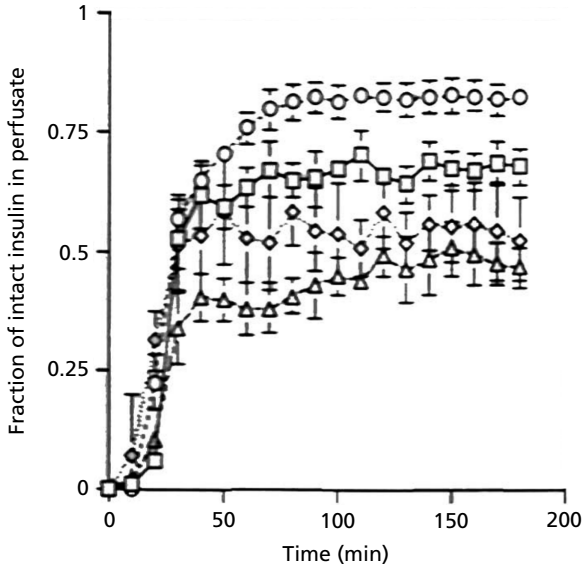

Figure 1 Fraction of intact insulin remaining in perfusate during perfusion with insulin in phosphate buffer (□), 40 mM NaC (◇), 40 mM NaGC (○), or 40 mM NaTC (△).

Neither NaGC nor NaTC significantly altered the permeability of the hydrophilic molecule PEG 4000 (Table 2). Poelma (1989) reported that at a concentration of 10 mM, NaTC exhibited a minor if not negligible effect on the barrier function of the intestinal wall. The P_{app} of PEG 4000 increased threefold for the NaC systems, which was significantly different from the control P_{app} and from the P_{app} in the presence of NaGC or NaTC ($P < 0.05$). Using erythrocyte haemolysis as a model parameter, Martin et al (1992) observed that NaC was more toxic than the conjugated NaTC to the epithelial cell membrane. Conjugation reduces the overall hydrophobicity of the bile salt and Murakami et al (1984) observed that the absorption-promoting efficacy of bile salts in rats increased with increasing hydrophobicity. The greater permeability of PEG 4000 in NaC systems may reflect the difference in polarity between the unconjugated NaC and the conjugated NaGC and NaTC.

This trend was seen in the plasma data as insulin concentrations increased steadily over the perfusion time and at 180 min were highest for NaC, followed by NaGC, and were lowest for NaTC, which was the most hydrophilic of the three bile salts (Figure 2). Plasma levels after perfusion with insulin in NaC and NaGC systems were significantly greater than corresponding levels after perfusion with control at 180 min ($P < 0.05$). The mechanism by which the enhanced insulin plasma concentrations were achieved was consistent with an effect on the paracellular transport route for NaC. NaGC enhanced insulin stability in the in-vitro studies in line with previous reports of its use as an enzyme inhibitor. These enzyme inhibition properties were also likely to contribute to the enhanced plasma insulin concentrations obtained with this system.

The calculated extent of insulin absorption for the control conditions in this study (Table 3) concurred with the maximum predicted value of 13% ileal absorption of insulin reported by Sinko et al (1993). The model appeared to overestimate the contribution of NaTC to insulin absorption

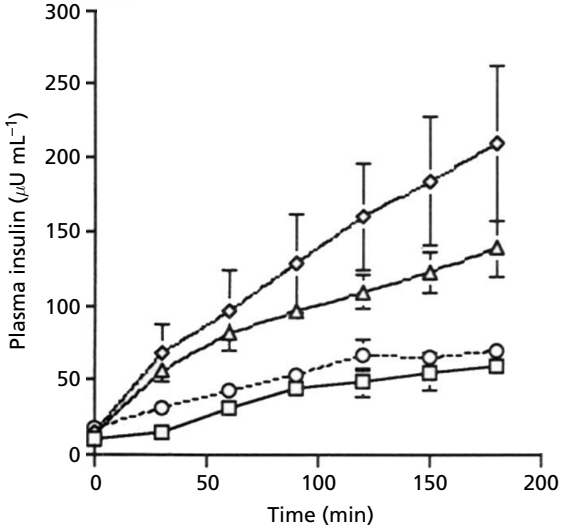

Figure 2 Plasma levels of insulin during perfusion with insulin in phosphate buffer pH 7.4 (□), 40 mM NaC (◇), 40 mM NaTC (○), or 40 mM NaGC (△).

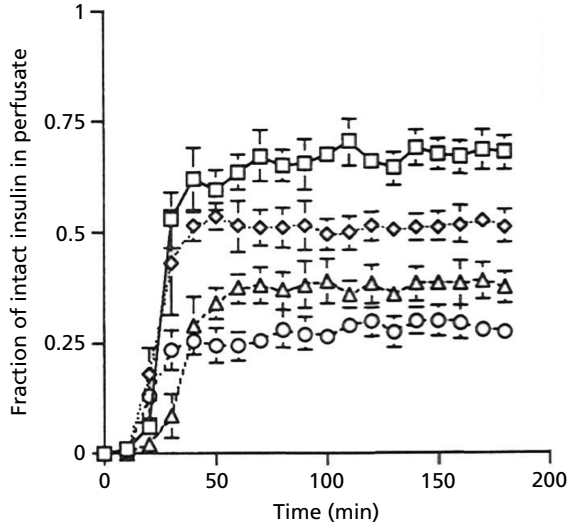

Figure 3 Fraction of intact insulin remaining in perfusate during perfusion with insulin in phosphate buffer pH 7.4 (□), 40 mM NaC:LA (△), 40 mM NaGC:LA (◇), or 40 mM NaTC:LA (○).

Table 3 Experimentally determined fractions of insulin lost (F_L), calculated fractions of insulin degraded (F_R) and absorbed (F_A) when perfused in 40 mM bile salt systems and 40:40 mM bile salt:fatty acid systems

System	F_L	F_R	F_A
Insulin	0.33 ± 0.07	0.21 ± 0.04	0.12 ± 0.05
Insulin/NaGC	0.30 ± 0.04	0.19 ± 0.03	0.11 ± 0.02
Insulin/NaTC	0.55 ± 0.08*	0.12 ± 0.04‡	0.44 ± 0.09†
Insulin/NaC/LA	0.64 ± 0.09*	0.39 ± 0.03‡	0.25 ± 0.05†
Insulin/NaGC/LA	0.49 ± 0.06*	0.22 ± 0.04	0.27 ± 0.03†
Insulin/NaTC/LA	0.72 ± 0.04*	0.20 ± 0.03	0.53 ± 0.02†

*$P < 0.05$ compared with F_L for insulin, ‡$P < 0.05$ compared with F_R for insulin control, †$P < 0.05$ compared with F_A for insulin.

(0.44) when compared with NaGC (0.11). A possible explanation for the overestimation for the NaTC system might be related to the findings of in-vivo studies in anaesthetized rats which reported approximately 30% of NaTC absorbed from proximal jejunum and appearing in bile when the bolus dose had traversed only half the intestine (McClintock & Shiau 1983). Kimura et al (1972) had earlier demonstrated 25.4% absorption of 20 mM NaTC systems over a 1-h absorption study in the rat ileum. Therefore variation in the NaTC concentrations over the course of the perfusion experiments might have contributed to higher estimations of fractional insulin absorption as the model assumed constant levels of NaTC for the calculations.

Mixed micellar systems

Enhanced insulin degradation in mixed micellar systems relative to the simple micellar systems was evident from the in-vitro stability studies (Table 1). All of the mixed micellar systems significantly promoted PEG 4000 disappearance when compared with PEG 4000 alone ($P > 0.05$, Table 2). Previously, we reported (Lane et al 1996) the effects of mixed micellar systems on the permeability properties of hydrophilic probe molecules of different sizes where the relative enhancement of hydrophilic probe permeability was observed to be more pronounced for probe molecules of molecular weights in excess of 400 Da. All mixed micellar systems promoted an almost fivefold enhancement of PEG 4000 permeability when administered with insulin.

After 3-h-perfusion (Figure 3), NaTC:LA and NaGC:LA micellar systems produced significantly higher plasma insulin levels than did the NaC:LA mixed micelles (Figure 4). Scott-Moncrieff et al (1994) reported that mixed micelles of 40 mM NaGC:30 mM LA were extremely effective in enhancing plasma insulin levels in the rat, with a maximum bioavailability of insulin of 41% being reported. In a canine model, those workers observed plasma insulin concentrations of the order of 200 $\mu U\,mL^{-1}$ after intrajejunal administration of 10 $U\,kg^{-1}$ insulin with 30 mM NaGC and 40 mM LA. Lower insulin bioavailability in the dog (1.8%) was ascribed to more rapid dilution of absorption enhancers and a greater exposure of insulin to proteolytic enzymes within the intestine.

The mass balance model estimated higher fractions of insulin absorbed for the NaC:LA and NaGC:LA systems relative to administration of insulin in phosphate buffer pH 7.4. This was in line with the higher plasma concentrations observed for these mixed micellar systems and higher PEG 4000 P_{app} values (Table 2). Similarly, the enhanced plasma insulin levels resulting from co-administration with NaTC:LA systems were in line with significantly higher PEG 4000 P_{app} permeability relative to control. However the higher F_A value for TC:LA relative to

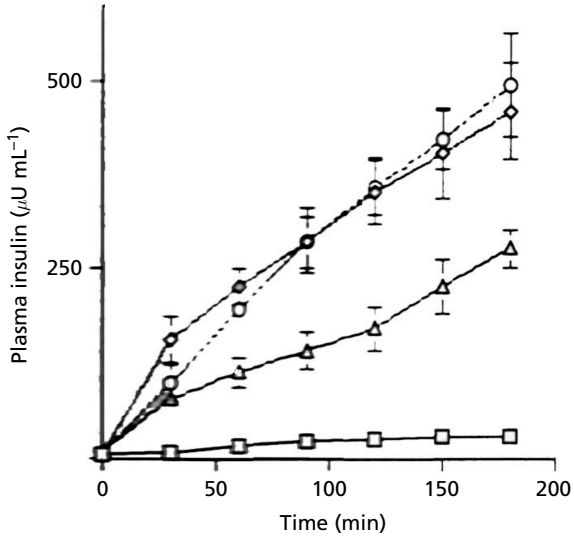

Figure 4 Plasma insulin levels during perfusion with insulin in phosphate buffer pH 7.4 (□), 40:40 mM solutions of NaC:LA (△), 40:40 mM NaGC:LA (◇), or 40:40 mM NaTC:LA (○).

NaC:LA and NaGC:LA suggested an overestimation of F_A for insulin by the model, suggesting a departure from mass-balance conditions in this system. The total amount of insulin lost from the perfusion solutions at steady state was composed of fractions degraded and absorbed (Table 3). Therefore, as for the simple micellar systems, the rate of absorption of TC or TC:LA from perfusion systems might have been a contributing factor to the higher F_A of insulin predicted by the model in this instance.

Biochemical and morpho-cytochemical evidence for insulin absorption via transcellular pathways was reported by Bendayan et al (1994). Insulin was co-administered with sodium cholate and a protease inhibitor, aprotinin, in the duodenum and colon of rats. Maximal plasma insulin values reported by these workers were of the order of 578 pmol L^{-1} in normal rats and 1760 pmol L^{-1} in diabetic rats after duodenal administration of an insulin solution. The integrity of the intestinal wall after insulin administration was retained and application of protein A-gold immunocytochemistry indicated that insulin could be internalized by epithelial cells and transferred through a transcytotic pathway to the interstitial space, and subsequently to the blood circulation. Therefore in this study, the absorption enhancers should have facilitated insulin absorption via paracellular and transcellular pathways.

Conclusions

A theoretical analysis for estimating the extent of intestinal peptide and protein absorption on the basis of a mass balance approach, which incorporated protein permeability and instability, had been reported previously. In this study that model was applied to delineate the effects of simple and mixed micelles of bile salts and linoleic acid on insulin stability and permeability in the rat intestine.

Preliminary stability studies indicated that micellar solutions of NaGC and NaTC promoted insulin stability in contrast to control studies on insulin alone. Mixed micellar systems did not promote insulin stability, and in the case of NaC:LA, appeared to enhance insulin degradation.

Following perfusion through the small intestine, insulin disappearance from the segment was greater with the mixed micelles as compared with the simple micelles. The greater lumenal disappearance was mirrored in enhanced plasma insulin concentrations for the mixed micellar systems. The larger permeation enhancing capacity of mixed micellar systems compared with simple micellar systems was paralleled by an increase in permeability of the paracellular marker molecule PEG 4000. Maximum plasma insulin levels were observed for the NaTC:LA and NaGC:LA mixed micellar systems. The mechanism of enhancement for mixed micellar systems likely involved a possible combination effect on both the paracellular and transcellular routes.

A complex relationship exists between degradation and permeability for peptide and protein drugs. In this study the perfusion model chosen allowed the extent of insulin disappearance in an intestinal segment to be determined. Experimental measurements of insulin disappearance from the rat intestine were corrected for degradation to estimate insulin absorption in the segment studied. Using known and measured anatomical characteristics for the rat intestine and stability data, the fractions of insulin degraded in the perfusion experiments were calculated relative to fractions absorbed. In the perfusion experiments of insulin in buffer, the fraction of insulin absorbed was calculated to be 0.12 and fraction degraded to be 0.21. The fraction absorbed was consistent with the maximal extent of insulin absorption of 13% calculated by Sinko et al (1993). When administered in mixed micellar systems the calculated F_A values were generally higher than for insulin administered in the absence of micellar systems. The plasma insulin data generally reflected the higher F_A values for the micellar systems, with the exception of NaTC and NaTC: LA micellar systems. A possible explanation for this might have been related to the rate of disappearance of NaTC over the time course of the perfusion studies.

The results suggested that the mass balance model provides a quantitative framework within which the fractional masses of protein therapeutics surviving and degraded in the small intestine may be predicted.

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