Development of a Gas Empowered Drug Delivery System for Peptide Delivery in the Small Intestine


Abstract

The aim of this investigation was to design a novel Gas Empowered Drug Delivery (GEDD) system for CO₂ forced transport of peptide drugs together with mucoadhesive polymers to the surface of the small intestine. The GEDD effect of the core tablet was achieved using CO₂ gas to push insulin together with the mucoadhesive excipients poly(ethyleneoxide) (PEO) and the permeation enhancer trimethyl chitosan (TMC) to the surface of the small intestine. The in-vitro insulin release showed that almost 100% of the insulin was released from enterically coated tablets within 30 minutes at pH 6.8. The designed GEDD system was shown to increase the insulin transport by approximately 7 times in comparison with the free insulin across sheep’s intestine ex-vivo. Three different peroral formulations were administered to male rabbits: F1 containing no TMC or PEO, F2 containing PEO but no TMC and F3 containing both PEO and TMC. The administrations of insulin using the formulation F1 resulted in a low FR value of 0.2% ±0.1%, while the formulations F2 and F3 resulted in a much higher FR values of 0.6 ±0.2% and 1.1%±0.4%, respectively. Hence, the insulin permeation achieved by the GEDD system is primarily due to the enhancing effect of TMC and the mucoadhesive properties of PEO both of which synergistically increase the bioavailability of insulin.
1. Introduction

In the past decade, a large number of endogenous peptides and protein drugs are produced in biotechnological companies for the treatment of chronic diseases. The majority of these drugs are administered by parenteral injection which is inconvenient, time and money consuming as well as dangerous. Oral drug delivery is the most convenient way for self administration of drugs, allowing a wide range of dosage adjustments [1]. Hence, a lot of research is focused on design and development of novel oral drug delivery systems for both hydrophilic, large macromolecules such as peptide and protein drugs and also small hydrophilic drug molecules with poor bioavailability.

Effective oral delivery of therapeutic peptides and proteins to the small intestine poses a great challenge in drug delivery system design. The harsh environment of the stomach, the presence of proteolytic enzymes in the small intestine, the hydrophilicity of the peptide molecules and its large molecular size as well as the poor membrane permeability have led to an intense investigation for site-specific drug delivery systems [2-4]. In order to overcome the above obstacles, the ideal oral delivery system must release its contents pH dependently only at the optimal target region, remain in the optimal site long enough for the complete peptide and protein release to be absorbed across the intestinal epithelium, and have a reproducible therapeutic effect. Thus, site specific delivery is required to deploy the peptides and proteins intactly to specifically targeted parts of the body through a platform that can control their release by means of physiological or chemical triggers [5-7].

A number of sophisticated oral novel delivery systems have been developed for the delivery of peptides and proteins [8]. Most of these delivery systems,
however, are only designed to release the hydrophilic macromolecule at the desired site of action but do not take into account appropriate mechanisms to also facilitate absorption of the drug molecule which in most cases is then enzymatically degraded in the small intestine [9].

In order to actively improve drug absorption a lot of focus was given to the mucoadhesive polymers for oral delivery of peptide drugs. Mucoadhesive polymers are hydrophilic macromolecules with numerous hydrogen bond forming groups such as carboxyl, hydroxyl, amide and amine groups that need to be hydrated for swelling and to act as a mucoadhesive polymers on the mucus linings of the gut. Suitable mucoadhesive polymers like chitosans and their quaternary derivatives form intermolecular complexes with the glycoproteins of the mucus linings to form a stable adhesive layer with the mucus. This adhesion can further assist in the absorption of peptide drugs across the mucosal membrane of the gut by reversibly opening of the tight-junctions [10-12].

Chitosan, a biocompatible and biodegradable polymer has been used as an oral drug delivery vehicle. However, chitosan can be used as an enhancer only in the proximal part of the intestine where the pH is close to its pKa value of 5.5. Quaternized derivatives of chitosan were synthesized and shown in contrast to chitosan to be drastically more soluble in neutral and alkaline environments of the intestine and more useful for drug delivery and absorption across the intestinal epithelium of the jejunum and ileum [13, 14]. The permeation enhancing properties of these chitosan derivatives have been attributed to their ion pair interactions with the tight- junctions and cellular membrane components to increase the paracellular permeation of hydrophilic compounds [15]. The polymer charge density, determined by the substitution degree is a key factor in obtaining both the mucoadhesion
and penetration enhancement towards the intestinal epithelium [16, 17]. Among chitosan derivatives, TMC was shown to have the most permeation enhancing effect. Hence, TMC was synthesized from low molecular weight chitosan with a degree of quaternization of approximately 50 ±5% which has been shown to have the highest penetration enhancing effect across the intestinal epithelium in vitro and in vivo [18].

However, the use of mucoadhesive polymers used as delivery platforms in the micro- and nanosize poses the problem that after the release of these mucoadhesive units they firstly have to swell before they can fully exert their mucoadhesive properties. Already during this swelling process the mucoadhesive sites can be de-activated by soluble mucins amply available in the intestinal fluids rendering in systems which are unable both to adhere to the mucous linings of the gut wall and to trigger the opening of the tight junctions. This holds especially for big gut lumina as those of the pig and humans [19, 20]. In order to overcome this drawback, Dorkoosh et al. have developed a time-and site- controlled delivery system that kept the drug mechanically attached to the intestinal site of absorption. They have used superporous hydrogel (SPH) and SPH composite (SPHc) polymers that were able to swell very quickly due to their highly porous structure and incorporated in these conveyor systems suitable delivery systems for the drug octreotide to get a desirable time-controlled release profile, required for absorption of peptides and proteins. They succeeded in getting high absolute bioavailabilities (F= 8 – 16%) depending on the type of delivery system in an in vivo study in pigs [21-24]. However these systems were not suitable for industrial mass production.

In this study, an easier to produce system was developed namely a CO₂ empowered drug delivery (GEDD) system has been designed to quickly
release insulin and the mucoadhesive polymers (polyethylene oxide) (PEO) and trimethyl chitosan (TMC) and to push them by the CO₂ bubbles to the mucous linings without losing their mucoadhesiveness and potency to open the tight junctions. The delivery system is enterically coated to release its contents in the proximal part of the small intestine where the CO₂ gas directs the mucoadhesive PEO polymer to attach to the mucosa of the small intestine. Once attached, the permeation enhancer, TMC, will also bind to the mucosal surface and trigger the opening of the tight junctions of the epithelial cells of the small intestine and open the paracellular pathway to facilitate the transport of the insulin across the mucosal membrane.

Robinson et al. have done studies using CO₂ as absorption enhancing agent in the ex vivo intestine of rabbit and mouse and showed that the direct bubbling of CO₂ can enhance drug permeability by reversibly altering the paracellular pathway [25]. However in the newly designed GEDD systems described here, CO₂ is primarily not used as a penetration enhancer but as pushing agent of the drug containing excipients to the absorbing surface.

Hence, the aim of this study was to investigate 1) the mucoadhesive and permeation enhancing effect of the novel delivery system 2) the in-vitro insulin release from the delivery system in the two different milieus of stomach and intestine 3) to evaluate the effectiveness of the GEDD system as permeation inducer of insulin in the sheep's intestine in ex vivo studies and 4) to determine the bioavailability of peroral insulin using GEDD delivery system in an in vivo study in rabbits.
2. Materials and Methods

2.1. Materials

Chito Clear® chitosan (viscosity 1% w/v solution, 22 mPa.s) was purchased from Primex, Iceland. Human insulin was a generous gift from Exir Pharmaceutical Company (Lorestan, Iran). The Polyethylene oxide (Mw 900,000) was a gift from (Dow Chemicals, U.K.). TMC with the degree of substitution of 50 ± 5% was synthesized in our laboratory as described previously [26]. Cellulose acetate phthalate (CAP) was purchased from Trans Medica (Germany), Ac-Di-Sol was purchased from SMF (Netherlands). All the other materials were of pharmaceutical and analytical grades and used as received.

2.2. Formulation and design of the GEDD system

The delivery system was prepared based on a $2^3$ factorial design. The variables were the percent polyethylene oxide (PEO) as mucoadhesive polymer, percent of citric acid and Na-bicarbonate (for CO$_2$ gas production), and Ac-Di-Sol as super-disintegrant. The response was the CO$_2$ gas production, tablet disintegration time as well as a mucoadhesive mass response. The variables were set as low and high values of 50% and 70%, 2.5% and 5.0% and 5.0% and 10% of the total weight for the amount of acid-base, Ac-Di-Sol and the PEO, respectively. As it was impossible to quantify the response of the CO$_2$ gas produced, designated (+) was used to evaluate the formulations 1-8. Accordingly one plus (+) corresponds to a low response and two (++) corresponds to a high response. The disintegration response (amount of Ac-Di-Sol) was evaluated by the disintegration time of the tablets in PBS of pH 6.8 at 37°C. Finally, the mucoadhesive response was measured by the amount of work (N) required to detach the dosage form from the sheep's intestinal mucosa in vitro as
described in section 2.5. According to the results obtained from the factorial design experiment, the following formulation was used for further studies: (46.7% citric acid, 23.3% Na-bicarbonate, 5% Ac-Di-Sol, 5% Avicel PH102, 10% PEO, 3.0% lactose 80, 1.5% human insulin, 0.5% TMC, and 5.0% Na-benzoate as lubricant).

In a lab-scale formulation, absolute alcohol was used for a wet granulation with citric acid, Na-bicarbonate and lactose. TMC, PEO, Avicel, insulin and the Na-benzoate were then added step-wise to the obtained granules and mixed well using manual mixing. The granules were then pressed to tablets using a single, lab scale press machine with a round, concave 7mm punch at a 150 mg± 7.5% weight and hardness of 4-6 Kpa. Content uniformity of the tablets was determined according to the standard method of USP 30 where 10 tablets were used to assay the amount of insulin. The RSD of the tablets weights was below 6%. The percent of insulin was calculated from the following equation: \( \text{Ru/Rs x 100} = \% \text{ Insulin in each tablet} \).

\( \text{Ru and Rs are the respective areas under the curve for the test and standard samples (data not presented).} \)

**2.3. Preparation of enteric coated tablets**

Once the tablets were pressed, they were enterically coated with a 3.0 mg sub-coat of a 3% solution of polyvinylpyrrolidone (PVP) K30 in absolute isopropanol and subsequently by 11mg of a 6% CAP solution containing diethyl phthalate as plasticizer per single tablet. The PVP layer was a protective layer to prevent the direct binding of the CAP to the tablets to avoid interactions of the acidic CAP with Na-hydrogen carbonate, and to smoothen the tablet surface at the edges to achieve a homogeneous enteric coating. The pan coating method was used to coat the tablets with an
Erweka pan apparatus (Heusenstamm, Germany) with 30 rpm and spraying 1.0mL coating solution per minute for 15-20 minutes. A suitable hot air drier was used to heat and dry the coating. In order to examine the effectiveness of the enteric coat layer, dissolution tests were performed using the standard USP 30 paddle apparatus. The rotation speed was set at 75 rpm and the dissolution medium was 500 mL 0.1N HCl for 2 hours, followed by phosphate buffer saline solution (PBS) of pH 6.8 at 37°C for 60 minutes. Six coated tablets were used and the disintegration behavior was investigated in both the acidic and alkaline medium.

2.4. Insulin release studies from the GEDD system

The insulin release from GEDD system was measured from both the uncoated as well as the enterically coated tablets. The coated tablets were placed in 25 mL tubes that were designed to fit into the Erweka apparatus (DT6, Heusenstamm, Germany). Initially 10 mL of 0.1N HCl was added to the tube and after 2 hrs, a sample was removed and analyzed. Subsequently, the tablets were transferred to tubes containing 10 mL of PBS buffer of pH 6.8. At predetermined time intervals of 5, 15, 30, 45 and 60 min, 0.5 mL of sample was removed and replaced with fresh pre-warmed buffer. The samples were further analyzed for insulin using HPLC as described previously [26]. The temperature and rotation were adjusted to 37°C and 75 rpm, respectively. The release from the uncoated tablets was only measured in the PBS buffer of 6.8. The release studies were repeated at least three times for each sample and the sink condition was considered in all cases.
2.5. Mucoadhesive properties of PEO after its release from the GEDD system

The mucoadhesive properties of the PEO used in the system were evaluated in a piece of sheep’s intestine (jejunum). The experiment was performed according to the method described by Smart et al. [12] with some modifications. Briefly, a small section of fresh sheep's intestine was removed, quickly frozen and was cut into pieces of 3cm lengths, opened longitudinally to expose the mucous surface and gently washed with PBS buffer pH 6.0. A preliminary histological study indicated the absence of any major damage to the tissue caused by the freeze-thawing process. A layer of mucus was shown to be present on the inner surface of the intestine. The sections of intestine were mounted on a platform and secured using a plastic cap, exposing an 11mm diameter of the test surface. The exposed surface was equilibrated in PBS buffer of pH 6.8 for 1 min at 37 °C. The cap was elongated at one side to allow sufficient distance for the tablet to be detached. Using a cyanoacrylate adhesive, the GEDD tablets were individually attached to a 1.5g weight and placed in contact with the adhesive surface for 2 minutes. Subsequently, a brass ring was placed over the 1.5g weight. The ring was connected to the force and position sensor of the rheometer via a pulley system. After 2 min the contact platform was lowered at a rate of 2mm/min and the maximum detachment force was calculated.

2.6. The CO₂ induced insulin penetration ex vivo

The procedure was done according to Eichmann and Robinson [25] with minor modification. Briefly, male albino rabbits weighting between 2-3 Kg were fasted overnight but had access to water ad libitum. The animals were sacrificed by 10 mL i.v. injection of 5% sodium pentobarbital solution in the marginal vein. The abdominal wall was exposed and 8-10 cm of the ileum
was excised and placed on ice cold Krebs buffer solution. The intestine was washed with the Krebs buffer to remove the feces and food debris. The perfusion was set as described by Robinson et al. For the mucosal to serosal transport study, 5.0 mL of the insulin solution (2.0 mg/mL final concentration) was added to the donor chamber. Solutions were kept at 37°C by a circulating water bath. The CO₂ gas was bubbled at 100 mL/min directly onto the epithelial surface. At predetermined intervals of 0, 15, 30, 45 and 60 minutes, 1.0 mL of sample was removed from the receiver chamber and replaced with fresh medium. The insulin transport was measured as described previously [26]. The permeability coefficient (P) was calculated using the following formula:

\[
P = \frac{V}{A x C_0} \left(\frac{dM}{dt}\right)
\]

Where V= volume inside the intestine, A= tissue surface area, C₀= Initial donor concentration, \(dM/dt\) = the amount of transported drug versus time. The experiment was repeated 3-5 times.

2.7. The evaluation of the permeation enhancing effect of chitosan and TMC

The procedure for evaluating the permeation enhancing effect was performed as described by Dorkoosh et al. and applied with some modifications [27]. On the day of the experiment, pieces of intestine were removed from the jejunum of freshly slaughtered sheep and washed with PBS (0.9% NaCl) to remove food residues and feces. The intestine was cut into 15 cm pieces and subsequently transferred to ice cold Krebs-Ringer bicarbonate buffer solution. One side of the intestine was tightly tied with a silk thread and the following solutions were added to different pieces of the
intestine: a) free insulin solution, b) free insulin and chitosan solution, c) free insulin and TMC solution. The insulin and chitosan were dissolved in 0.01% HCl and 0.25% acetic acid, respectively and then mixed together. The pH was slowly raised to 6.0 using 1N NaOH to prevent the precipitation of chitosan or insulin. The TMC was dissolved in water and the pH of the insulin polymer was adjusted to 6.8. Chitosan and TMC were used at a concentration of 0.5% w/v. The amount of insulin and polymer used were the same in the free form and in the GEDD tablet. The filled intestine was hooked onto a stainless steel support to keep it stretched and immersed in a container filled with 50 mL of Krebs buffer medium saturated with 95% O₂ and 5% CO₂. The container was placed in a water bath of 37°C with gentle movements. At predetermined times of 0, 15, 30, 60, 90 and 120 minutes 5mL samples were removed from the serosal part and replaced with fresh medium. The samples were analyzed by HPLC method as described previously [26] and the amount of insulin transported across the intestinal lumen was calculated.

2.8. The efficiency of the GEDD system for insulin permeation using sheep's intestine (ex vivo studies)

The procedure used was as described in section 2.5 and the following formulas were added to the isolated sheep intestine a) free insulin solution (f1), b) free insulin and TMC solution (f2), c) GEDD tablet containing Insulin without TMC (f3), d) GEDD tablet containing both insulin and TMC (f4). Consequently, 8.0 mL of the Krebs buffer pH 6.8 was added to the intestine and the other end of the intestine was also tied carefully. The filled intestine was hooked onto a stainless steel support to keep it stretched and immersed in a container filled with 50 mL of Krebs buffer pH 6.8 medium saturated with 95% O₂ and 5% CO₂. The container was placed in a water bath of 37°C with gentle movements. At predetermined times of 0, 15, 30,
60, 90 and 120 minutes 5mL samples were removed from the serosal part and replaced with fresh medium. The samples were analyzed by HPLC method as described previously [26] and the amount of insulin transported across the intestinal lumen was calculated. The apparent permeability coefficient (Papp) of insulin transported was calculated according to the approximated surface area in the intestinal lumen.

2.9. The in vivo studies using enteric coated GEDD tablets

The investigation was performed in healthy, male rabbits of 2-3 kg body weight. A week prior to the start of the experiment the rabbits were kept in the animal unit for environmental adaptation. The animals were fasted the night before each experiment with access to water ad libitum. The enteric coated GEDD tablets were administered using a custom made applicator via the oral route and placed in the stomach of the animals. Subcutaneous injection of insulin was done in the lateral skin of the hind limb.

The following formulations were administered to the rabbits as a cross-over setup (Table 1). For subcutaneous injection, the desired amount of insulin was initially dissolved in 0.1M HCl and added to 0.9% NaCl. The pH was then adjusted to 7.2 using 0.1M NaOH. The formulations were administered to animals at 48hrs intervals to ensure complete insulin washout. Blood samples were collected at the predetermined intervals of -10, 15, 30, 60, 90, 120, 240 and 300 minutes. The samples were kept on ice and centrifuged at 2000 rpm for 10 min at 4°C. The separated serum was kept at -20°C for further analysis.
Table 1. Administration of different insulin formulations

<table>
<thead>
<tr>
<th>Type of Administration</th>
<th>Insulin Dose</th>
<th>Amount of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td>0.2 IU/Kg</td>
<td>0.5 mL containing 0.016 IU insulin</td>
</tr>
<tr>
<td>F1 Tablet</td>
<td>20 IU/Kg</td>
<td>Dosage form containing 1.6 IU insulin</td>
</tr>
<tr>
<td>F2 Tablet</td>
<td>20 IU/Kg</td>
<td>Dosage form containing 1.6 IU insulin</td>
</tr>
<tr>
<td>F3 Tablet</td>
<td>20 IU/Kg</td>
<td>Dosage form containing 1.6 IU insulin</td>
</tr>
<tr>
<td>F4 Tablet</td>
<td>-</td>
<td>Dosage form without Insulin</td>
</tr>
<tr>
<td>Oral Solution</td>
<td>20 IU/Kg</td>
<td>Solution containing 1.6 IU insulin</td>
</tr>
</tbody>
</table>

(F1 contains: neither TMC nor PEO, F2 contains: PEO but no TMC, F3 contains: both TMC and PEO, F4 contains: all the ingredients of the tablet without PEO, TMC or insulin)

2.10. Measurement of insulin levels in blood plasma

The insulin concentration in serum was measured by a radioimmunoassay kit (Diaplus, USA). The analysis was done according to the manufacturer’s protocol.

2.11. Pharmacokinetic data analysis

Pharmacokinetic parameters, including the area under the curve (AUC) of the plasma concentration curve and mean insulin concentration after each administration ($C_{\text{ins}}$: average absorbed insulin at each time point), were obtained directly from the plasma insulin concentrations. The AUCs for each administration were calculated by the linear trapezoidal rule [28]. The
relative bioavailabilities of insulin after each administration were calculated according to the following formula

\[
F_R = \frac{AUC_{\text{table} t} \times D_{s.c}}{AUC_{s.c} \times D_{\text{tablet}}} \times 100\% 
\]

Where \( F_R \) is the relative bioavailability and \( D \) is the administered dose.

### 2.12. Statistical analysis

The data are expressed as means ±S.D. Non-linear regression was used for ex vivo and in vivo studies. \( P<0.05 \) was considered significant.

### 3. Result and Discussion

#### 3.1. Formulation and design of the GEDD system using factorial design

In Table 2 the results of the factorial design experiments obtained from formulations 1-8 are presented. They were designed as follows: F1 (low acid-base, Ac-Di-Sol, and PEO content), F2 (high acid-base, low Ac-Di-Sol, and low PEO content), F3 (low acid-base, high Ac-Di-Sol, and low PEO content), F4 (low acid-base, low Ac-Di-Sol, and high PEO content), F5 (high acid-base, high Ac-Di-Sol, and low PEO content), F6 (high acid-base, low Ac-Di-Sol, and high PEO content), F7 (low acid-base, high Ac-Di-Sol, and high PEO content), F8 (high acid-base, high Ac-Di-Sol, and high PEO content). The \( \text{CO}_2 \) production could only be described as slow (+) and satisfactory (++) and the amount of gas produced is also dependent on the amount of gas forming agents in the tablet. As the gas is primarily intended to push the additional excipients of the GEDD system to the absorbing
surface of the gut tissue, the resulting mucoadhesiveness of PEO and TMC is a more important indicator of the efficiency of the GEDD system than its gas formation as such figure 1 gives a schematic representation of the function of the GEDD system.

Figure 1. The schematic presentation of a GEDD system.

a) Enteric coated and PVP subcoated GEDD system in the stomach pH 1.2. b) The GEDD system reaching the small intestine at a pH of 6.8. The enteric coating starts to dissolve quickly at this pH. c) About 5 min later, the PVP subcoat is dissolving and the CO₂ gas formation starts to push the insulin drug and the excipients PEO and TMC to the absorbing surface of the intestinal wall (insulin, PEO and TMC). d) After about 10-15 min the tablet has completely dissolved and on the gut surface the mucoadhesive excipients together with insulin are attached. e) TMC induces the opening of the tight junctions and insulin is able to permeate into the underlying tissue by the paracellular route.
Table 2: Results of the factorial design experiment.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Response</th>
<th>C02 Production</th>
<th>Disintegration Time</th>
<th>Mucoadhesiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(+/++)</td>
<td>(min)</td>
<td>F(mN)</td>
</tr>
<tr>
<td>F1 (50% Acid-base, 2.5% Ac-Di-Sol, 5.0% PEO)</td>
<td>+</td>
<td>2.5'</td>
<td></td>
<td>2055±244</td>
</tr>
<tr>
<td>F2 (70% Acid-base, 2.5% Ac-Di-Sol, 5.0% PEO)</td>
<td>++</td>
<td>1.8'</td>
<td></td>
<td>2014±345</td>
</tr>
<tr>
<td>F3 (50% Acid-base, 5.0% Ac-Di-Sol, 5.0% PEO)</td>
<td>+</td>
<td>1.3'</td>
<td></td>
<td>2024±367</td>
</tr>
<tr>
<td>F4 (50% Acid-base, 2.5% Ac-Di-Sol, 10% PEO)</td>
<td>+</td>
<td>2.5'</td>
<td></td>
<td>2433±334</td>
</tr>
<tr>
<td>F5 (70% Acid-base, 5.0% Ac-Di-Sol, 5.0% PEO)</td>
<td>++</td>
<td>1.0'</td>
<td></td>
<td>1998±132</td>
</tr>
<tr>
<td>F6 (50% Acid-base, 5.0% Ac-Di-Sol, 10% PEO)</td>
<td>++</td>
<td>1.6'</td>
<td></td>
<td>2465±102</td>
</tr>
<tr>
<td>F7 (70% Acid-base, 2.5% Ac-Di-Sol, 10% PEO)</td>
<td>+</td>
<td>1.8'</td>
<td></td>
<td>2345±301</td>
</tr>
<tr>
<td>F8 (70% Acid-base, 5.0% Ac-Di-Sol, 10% PEO)</td>
<td>++</td>
<td>1.2'</td>
<td></td>
<td>2633±52.85</td>
</tr>
</tbody>
</table>

According to the results, the best gas production, tablet disintegration and mucoadhesion was obtained when the citric acid/sodium bicarbonate (CO₂ producing agents), Ac-Di-Sol (disintegrating agent) and PEO (mucoadhesive agent) contents were 70%, 5.0% and 10%, respectively. The disintegration time is slightly lower in the F8 formulation in comparison to the F5 formulation; however, the latter formula has a more suitable mucoadhesion than F5. Hence F8 formulation was chosen for further investigation as it had enough CO₂ power and Ac-Di-Sol for disintegration.
while the mucoadhesive property of the tablet was strong enough for attaching the system to the intestinal lumen.

### 3.2. In vitro insulin release from the GEDD system:

The results of insulin release from the uncoated tablets in PBS buffer pH 6.8 were as follows: First a burst release was observed in the first 5 minutes where about 85% of the insulin was released into the buffer solution. After 10 minutes all insulin has been released in the medium (data not presented). This test was done to detect and eventually eliminate a possible interaction of the PEO polymer with insulin. The results clearly indicate that no such interaction has taken place.

Insulin release from the enterically coated tablets was also investigated both in the acidic medium of stomach at 0.1N HCl followed by pH 6.8 buffer representing the small intestine. In the acidic medium, only about 3% insulin was released after 2 hours which suggests that the enteric coat was sufficiently protecting the tablets at low pH values (Fig 2). Figure 2 also presents the percentage of insulin released in PBS at pH 6.8. Accordingly, only a small amount of insulin was released in the first 10 minutes and after 15 minutes a burst release of approximately 80% of insulin was observed. This corresponds with the time required for the enteric coat to be dissolved at pH 6.8. In 20 minutes almost 100% of the insulin was released from the GEDD system.
Figure 2. Percent of insulin released from the enterically coated GEDD tablet 2hrs in 0.1N HCl followed by PBS buffer pH 6.8 (n = 3).

3.3. Mucoadhesion of PEO after its release from the GEDD system to the sheep’s intestine

The movement in the intestine is determined by 2 major forces: a) the horizontal force and b) the vertical force. The horizontal force is in the direction of the intestine to the rectum and pushes the food and the intestinal contents forward along the intestine. The vertical force is the contraction movements of the intestine [27]. For the drug delivery to be effectively attached to the intestine it preferentially should overcome the horizontal movements. Hence in this study a horizontal force was applied to evaluate the ability of PEO after its release from the GEDD tablets to attach to the intestinal wall. The results show that for the optimal formulation (formulation #8 in Table 1) a force of 2633±53 mN was needed to detach the tablets remnants from the mucous membrane which is sufficiently high to easily allow the delivery systems to stay long enough to the gut wall for the insulin release.
3.4. The CO₂ enhancing properties in rabbit’s intestine

Figure 3 presents the enhancing effect of the CO₂ on the insulin transport across the rabbit’s intestinal tissue. The apparent permeability values of insulin in the absence and presence of CO₂ were $2.5\pm1.2\times10^{-7}$ (cm/sec) and $7.5\pm1.6\times10^{-7}$ (cm/sec), respectively (n=3, p<0.05). Previous studies by Eichmann and Robinson [25] using hydrophilic drugs have shown similar results. As was indicated by their studies the increased drug transport is most probably due to the mechanical effect of CO₂ on opening of the tight junctions and increasing the paracellular pathway. Moreover, these authors have shown that CO₂ has the ability to decrease the transepithelial resistance (TEER) across the duodenal tissue with no apparent toxic effect indicated by the results of the MTT test. However it should be kept in mind that the CO₂ effect in increasing the paracellular transport of insulin across the intestine is a desirable side effect for our system. Primarily the CO₂ production is aimed to push the mucoadhesive tablet excipients to the absorbing surface of the gut wall.

![Figure 3. Insulin permeation across rabbit’s intestine in the presence and absence of CO₂ (n = 3).](image-url)
3.5. The effectiveness of the GEDD system for inducing insulin permeation across the sheep’s intestine (ex vivo studies)

The result of the ex vivo studies are presented in Figures 4 and 5. In this study, the effect of chitosan and TMC as permeation enhancers of insulin across the sheep’s intestine was investigated. According to these results, TMC with a higher positive surface charge shows a higher enhancing activity in comparison with chitosan. Without an enhancer only 1% of insulin was transported across the intestinal epithelium. With chitosan this amount was increased to about 4%, however, with TMC an increase of 7% in the insulin transport was observed. These data corresponds with our previous studies in Caco-2 cells [29] where it was shown that amongst different chitosan derivatives used, TMC showed the highest permeation enhancing effect. Moreover, the effect of the GEDD system in the transport of insulin in sheep’s intestine was investigated. Figure 5 presents the results obtained from the ex vivo tests done in sheep’s intestine using free insulin as the negative control (f1), free insulin in the presence of the TMC in the form of the solution (f2), the GEDD system with insulin in the absence and presence of the TMC (f3 and f4, respectively). According to figure 5 and in agreement with the ex vivo study, only about 1% of the free insulin was transported across the intestinal epithelium. The insulin containing GEDD tablet showed an increased permeation of about 3 times higher in comparison with the free insulin solution. This increase in transport can also be attributed to the enhancing effect of the CO2 in mechanically opening the tight junction and increasing the paracellular transport of the insulin across the sheep’s intestinal epithelium. The transport of insulin in the presence of the TMC in solution form resulted in a higher permeation in comparison to free insulin or the delivery system without the TMC. This indicates that the enhancing effect of TMC is more pronounced than that of the CO2 gas. The
best enhancing effect was observed with the insulin and TMC containing GEDD tablets. Insulin transport was significantly higher (p<0.05) than the other forms and was found to be about 7%. This increase in insulin permeation may be due to the synergistic effect of both the CO₂ and TMC in the form of mechanical and chemical enhancement, respectively. The apparent permeability for insulin using the GEDD tablet in the presence of TMC had the highest value of 2.7±0.18x10⁻⁶ (cm/sec) which is significantly higher than the P_app of free insulin 2.1±0.03 x 10⁻⁷ (cm/sec). The GEDD tablet containing only insulin had a P_app of 4.4±0.83 x 10⁻⁷ (cm/sec) and free insulin with soluble TMC had a P_app 4.1±0.65x 10⁻⁷ (cm/sec), respectively (n=3, p<0.05).

Figure 4. The effect of chitosan and TMC on insulin transport across the sheep’s intestine ex vivo (n= 3).
3.6. The in vivo studies

Table 1 contains all relevant data about the administration of the different insulin formulations used for the in vivo study. The mean plasma insulin concentration versus time profiles obtained after s.c and peroral administration of the different oral dosage forms are presented in Figure 6. S.c. injection showed a rapid increase in insulin concentration up to 6.34±1.7μIU within 30 minutes post administration followed by a steady state and finally a drop in insulin concentration. The reason for a rapid insulin increase in plasma could be due to the presence of numerous endothelial capillaries present at site of administration in the rabbit. The insulin molecules can quickly diffuse through the endothelial layer and result in an increase plasma concentration. In formulation F1, without TMC or PEO, as expected no significant increase in insulin plasma concentration was detected. This could be due to the presence of the proteolytic enzymes in the intestine that can inactivate the protein as soon as it is released in the intestine. Moreover, the large molecular size of the insulin and its hydrophilic properties are factors that limit the passage of insulin through the intestinal epithelium resulting in low plasma concentration. The low
amount of insulin detected in plasma could be due to the effect of CO₂ gas produced that may act as a mechanical enhancer to open the tight junctions. In formulation 2, containing PEO and no enhancer, a significant increase in insulin concentration is observed in plasma 30 minutes post administration as depicted in Figure 6. Accordingly, it can be concluded that the transit time of the dosage form in the stomach of the fasting rabbits was very brief and once in the intestine, the enteric coat was quickly dissolved. As was presented in Figure 2, the insulin was released from the enteric coated tablets in approximately 20 minutes at pH 6.8 which correlates very well with the in vivo results. Once the enteric coat has dissolved in the intestine, the PEO will interact with the mucus layer and prolong the insulin presence at the site of the absorption. The presence of PEO can only increase the residence time of the insulin at the site of the absorption and the large, hydrophilic insulin molecule still has to overcome epithelial barrier for transport across the membrane. Hence only a small increase in the amount of insulin plasma concentration is observed and a steady state is reached very quickly followed by a drop in the concentration of insulin. In formulation 3, (the complete GEDD tablet formulation) containing both the TMC and PEO, the initial part of the graph followed the same trend as in F2 due to the presence of the PEO. However, a lag time of approximately 60 minutes is needed to for the released TMC to completely dissolve and open the tight junctions for the paracellular transport of the insulin. Also a good correlation is observed between these results and the ex vivo studies, depicted in Figure 4, where it was shown that after 90 min the amount of insulin transported across the membrane was increased and reached its maximum at 240 min. Accordingly, one can conclude that the presence of both TMC and PEO is necessary for insulin transport across the intestinal membrane: PEO with its mucoadhesive characteristic can prolong the
insulin transit time at the site of absorption and TMC as permeation enhancer can act on opening the tight junctions and increasing the paracellular transport of the insulin along the enterocytes. The administration of oral insulin as negative control resulted in no significant insulin plasma concentration (data not shown). This is mainly due to degradation and inactivation of insulin in the stomach and intestine. The oral administration of placebo tablets to rabbits indicates no significant increase in insulin concentration in plasma concentration which indicates that the stress level had no effect on the results obtained in this study (data not presented). Mean insulin concentrations in plasma $C_{ins}$ after F2 and F3 administration were $2.3 \mu U/mL$ and $4.8 \mu U/mL$, respectively which are significantly higher than $C_{ins}$ after F1 administration ($1.3 \mu U/mL$). These results indicate that F2 and to a higher degree F3 formulations are able to increase the intestinal absorption of insulin. The relative bioavailabilities of insulin in rabbits were calculated to be $0.2\% \pm 0.1$, $0.6\% \pm 0.2\%$ and $1.1\% \pm 0.4$ using the F1, F2 and F3 formulations, respectively. These results indicate that F2 and F3 tablet (containing only PEO and PEO + TMC, respectively) show a 3 fold and 5.5 fold enhancements in comparison to the F1 tablet containing no PEO or TMC. The bioavailability of insulin in vivo is more pronounced when the formulations were compared to the oral insulin (data not presented). Moreover, these values are expected to be higher with diabetic induced rabbits. Hence it is recommended to either use diabetic induced animals or a non-endogenous peptide for a more precise evaluation of the potency of the delivery system.
Figure 6. In vivo studies of insulin absorption in rabbits using different GEDD formulations (n= 6).

Conclusions

In this study a novel Gas Empowered Drug Delivery (GEDD) system was designed for oral insulin delivery to the small intestine. The designed delivery system has shown to be able to push by the CO₂ formation of the mucoadhesive excipients PEO and TMC of the enteric coated tablet to the absorbing gut membrane and to have consecutively both mucoadhesion and permeation enhancing effects. While the permeation enhancing effect of the system is thought to be due to both mechanical enhancement resulting from the CO₂ gas and the chemical enhancement of the TMC, the mucoadhesion is a result of the PEO polymer used in the formulation. This bioactive polymer is used to deliver peptide drugs to the mucous membrane by attaching to the gut wall. The GEDD system developed so far was shown to
enable an insulin transport of about 7.0% of the applied dose ex-vivo. The bioavailabilities of insulin in vivo in rabbits were shown to be 0.2% ±0.1, 0.6±0.2% and 1.1±0.4% using the formulation containing a) no PEO or TMC (F1), b) formulation containing PEO alone (F2), and c) formulation containing both PEO and TMC (F3), respectively. Further animal studies using either big animals such as pigs of about 40kg body weight or humans are required for full assessment of the effect of the GEDD system in-vivo. Additionally the GEDD system developed so-far was made in lab-scale format. Industrial scale production (which is in progress) with optimized enteric coating and with other hydrophilic drugs like bisphosphonates or low molecular weight heparin (LMWH) will further improve and increase the versatility of the system.
References


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