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Formulation, Optimization, and In Vitro/In Vivo Evaluation of Enteric- Coated Bilayer Tablets Containing Immediate-Release Insulin-Loaded Liposomes and Sustained-Release Metformin Hydrochloride for Type 2 Diabetes Management

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Research Article

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Formulation, Optimization, and In Vitro/In Vivo Evaluation of Enteric-Coated Bilayer Tablets Containing Immediate-Release Insulin-Loaded Liposomes and Sustained-Release Metformin Hydrochloride for Type 2 Diabetes Management

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HIGHLIGHTS

- Enteric-coated bilayer tablets co-delivered insulin-loaded liposomes (75 IU) and metformin HCl (500 mg).
- Lyophilized PEGylated liposomes exhibited 128.4 nm size and 82.6% insulin encapsulation efficiency.
- Circular dichroism confirmed preservation of insulin α-helical structure after compression.
- Sodium caprate enhanced intestinal insulin permeability 4.2-fold ex vivo.
- Oral bilayer tablets achieved 48.7% blood glucose reduction and 12.4% relative bioavailability in diabetic rats.

ABSTRACT

Background: Oral insulin delivery remains constrained by gastric acid degradation, enzymatic proteolysis, poor intestinal epithelial permeability, and hepatic first-pass metabolism. Liposomal nanocarriers and chemical permeation enhancers have individually shown promise in overcoming these barriers, but integrated fixed-dose combination platforms that simultaneously address nanocarrier protection, permeation enhancement,

and multi-phasic drug release from a single solid dosage form remain underexplored.

Purpose: This study aimed to develop and evaluate an enteric-coated bilayer tablet system combining immediate-release (IR) insulin-loaded liposomes (50–100 IU) with sustained-release (SR) metformin hydrochloride (500 mg) for potential type 2 diabetes management.

Methods: Insulin-loaded liposomes composed of DPPC, cholesterol, and DSPE-PEG2000 were prepared by thin-film hydration and lyophilized with trehalose. The IR layer contained lyophilized liposomes equivalent to 75 IU insulin, sodium caprate (35 mg) as a permeation enhancer, and croscarmellose sodium as a superdisintegrant. The SR layer was prepared by wet granulation using HPMC K100M (20% w/w). Bilayer tablets were compressed and enteric-coated with Eudragit L100-55. Formulations were evaluated by in vitro dissolution (USP Apparatus II), ex vivo everted rat gut sac permeation, and in vivo pharmacodynamic and pharmacokinetic studies in streptozotocin-induced diabetic rats.

Results: The optimized liposomal dispersion exhibited a mean particle size of 128.4 ± 6.2 nm, a PDI of 0.18 ± 0.03, and an insulin encapsulation efficiency of 82.6 ± 3.4%. Circular dichroism confirmed preservation of the α-helical structure of insulin after lyophilization and compression (54.8 ± 3.6% α-helix). Bilayer tablets exhibited hardness of 7.2 ± 0.4 kg/cm² and friability of 0.42 ± 0.08%. Enteric coating prevented insulin release in simulated gastric fluid (pH 1.2, < 3.8% at 2 h). In simulated intestinal fluid (pH 6.8), the IR layer released 88.4 ± 4.1% of liposomal insulin within 45 min, while metformin exhibited sustained release over 12 h (91.3 ± 3.7%) following Higuchi diffusion kinetics. Ex vivo permeation studies demonstrated a 4.2-fold enhancement in insulin transport with sodium caprate. In diabetic rats, oral bilayer tablets produced a maximum blood glucose reduction of 48.7 ± 5.3% at 4 h, with a relative pharmacological bioavailability of 12.4% relative to subcutaneous insulin. Serum human insulin ELISA confirmed systemic absorption with a Tmax of 3.5 ± 0.6 h and Cmax of 42.8 ± 6.1 μIU/mL.

Conclusion: The enteric-coated bilayer tablet platform effectively protected insulin from gastric degradation, enhanced intestinal permeability, and

provided biphasic release profiles suitable for addressing both postprandial hyperglycemia and basal glycemic control.

Keywords: *Oral insulin; Liposomes; Metformin hydrochloride; Bilayer tablet; Enteric coating; Sodium caprate; Type 2 diabetes; Lymphatic delivery*

1. INTRODUCTION

Oral insulin delivery remains one of the most formidable challenges in pharmaceutical technology, primarily because the gastrointestinal tract imposes sequential barriers—acidic degradation, enzymatic proteolysis, limited epithelial permeability, and hepatic first-pass metabolism—that collectively reduce the bioavailability of unmodified insulin to typically below 1% (1). Despite these obstacles, the pursuit of an oral insulin formulation has persisted for decades, driven by the potential to mimic physiological insulin secretion through the portal circulation, reduce peripheral hyperinsulinemia, and improve patient adherence compared with subcutaneous injection (2,3).

Liposomal nanocarriers have attracted considerable attention for oral peptide delivery owing to their biocompatibility, capacity to encapsulate hydrophilic macromolecules, and ability to shield encapsulated cargo from enzymatic attack (4,5). The lipid bilayer provides a physical barrier against gastric acid and digestive enzymes, although conventional liposomes exhibit poor stability in the gastrointestinal milieu unless surface-modified (6). Polyethylene glycol (PEG) derivatives, particularly DSPE-PEG2000, have been employed to impart steric stabilization and mucus-penetrating properties, thereby extending vesicle residence time and enhancing intestinal uptake (7). Recent investigations have further explored bilayer liposomal architectures, such as propanoic acid-functionalized chitosan-

coated liposomes, which exploit monocarboxylate transporter 1 for targeted intestinal absorption of insulin (8).

Chemical permeation enhancers represent a complementary strategy for overcoming the epithelial barrier. Sodium caprate (C10), a medium-chain fatty acid salt, has been extensively investigated for its ability to transiently modulate tight junction proteins and augment paracellular transport of peptides (9,10). Larsen et al. demonstrated that the membrane activity of sodium caprate is substantially influenced by the presence of peptide drugs and intestinal fluid components, underscoring the necessity of evaluating permeation enhancement under physiologically relevant conditions (10). These findings highlight the importance of integrating permeation enhancers directly into the solid dosage form to achieve spatially localized effects at the absorption site.

Metformin hydrochloride remains the first-line pharmacotherapy for type 2 diabetes, yet its short plasma half-life of 4–9 hours necessitates two to three daily administrations when larger doses are required, compromising patient adherence (11). Sustained-release matrix systems employing hydroxypropyl methylcellulose (HPMC) have been validated for once-daily metformin dosing. Padwal et al. recently demonstrated that while HPMC K100M alone was insufficient to provide an optimal release profile, the combination of HPMC K100M with polyvinylpyrrolidone (PVP K30) as a granulating binder yielded superior controlled-release kinetics suitable for once-daily administration (11). This matrix approach offers a well-characterized and scalable platform for the sustained-release component of a fixed-dose combination.

Recent advances in oral insulin have explored diverse nanocarrier platforms beyond liposomes, including chitosan nanoparticles, solid lipid nanoparticles, and metal-organic frameworks (12-14). Seyam et al. developed pectin-coated trimethyl chitosan nanoparticles for colon-targeted insulin delivery, achieving an 87% blood glucose reduction at 8 hours in diabetic rats (12). Che et al. constructed a dual-barrier system integrating solid lipid nanoparticles with Eudragit L100 enteric coating, achieving relative pharmacological availability of 7.66% compared with 1.1% for oral insulin solution (13). Despite these advances, the vast majority of oral insulin research has focused on single-entity nanocarrier systems, with limited attention directed toward integrated fixed-dose combinations that simultaneously address nanocarrier protection, permeation enhancement, and multi-phasic drug release from a single administrable unit (14,15).

The bilayer tablet architecture represents a rational dosage form design capable of physically segregating incompatible components—liposomal insulin and metformin—while delivering distinct release profiles from one tablet. This approach aligns with the clinical reality that type 2 diabetes management frequently requires combination therapy, and fixed-dose combinations have demonstrated improved adherence compared with separate administrations (15).

Aim of the Study

This study aimed to formulate and optimize an enteric-coated bilayer tablet containing immediate-release insulin-loaded liposomes and sustained-release metformin hydrochloride, and to evaluate the system through in vitro dissolution, ex vivo intestinal permeation, and in vivo

pharmacodynamic and pharmacokinetic assessments in streptozotocin-induced diabetic rats.

2. MATERIALS AND METHODS

2.1 Materials

Recombinant human insulin (28.8 IU/mg) was obtained from Eli Lilly (Indianapolis, IN, USA). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Metformin hydrochloride was supplied by Sigma-Aldrich (St. Louis, MO, USA). Hydroxypropyl methylcellulose (HPMC K100M) and polyvinylpyrrolidone (PVP K30) were obtained from Colorcon (Harleysville, PA, USA) and BASF (Ludwigshafen, Germany), respectively. Eudragit L100-55 was a gift from Evonik Industries (Darmstadt, Germany). Trehalose dihydrate, sodium caprate, croscarmellose sodium, microcrystalline cellulose (Avicel PH-102 and PH-101), colloidal silicon dioxide (Aerosil 200), magnesium stearate, and triethyl citrate were procured from local suppliers and were of pharmaceutical grade. All other reagents and solvents were of analytical grade.

2.2 Preparation of Insulin-Loaded Liposomes

Insulin-loaded liposomes were prepared by the thin-film hydration method followed by membrane extrusion. Briefly, DPPC, cholesterol, and DSPE-PEG2000 (molar ratio 7:3:0.1) were dissolved in chloroform:methanol (2:1 v/v) in a round-bottom flask. The organic solvent was evaporated under reduced pressure at 45 °C using a rotary evaporator (Buchi R-210, Flawil,

Switzerland) to form a thin lipid film. Residual solvent was removed by flushing with nitrogen. The lipid film was hydrated with phosphate-buffered saline (PBS, pH 7.4) containing recombinant human insulin at a concentration adjusted to yield 75 IU per 120 mg of lyophilized cake. Hydration was performed at 45 °C for 1 h with intermittent vortexing. The resulting multilamellar vesicles were sequentially extruded through polycarbonate membranes of 200 nm and 100 nm pore size using a mini-extruder (Avanti Polar Lipids) to obtain large unilamellar vesicles. The mean particle size and polydispersity index (PDI) of the liposomal dispersion were determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK).

2.3 Lyophilization of Insulin Liposomes

Trehalose dihydrate was added to the liposomal dispersion at a lipid-to-sugar mass ratio of 1:2 and gently mixed until complete dissolution. Aliquots of 2 mL were transferred into 10 mL glass vials and frozen at −80 °C for 12 h. Lyophilization was performed using a freeze-dryer (Alpha 1-2 LDplus, Martin Christ, Germany) with a primary drying phase at −40 °C and 0.05 mbar for 24 h, followed by secondary drying at 20 °C for 6 h. The lyophilized cakes were visually inspected for uniformity and stored in sealed vials at 4 °C until further use.

2.4 Characterization of Liposomes

The mean particle size, PDI, and zeta potential of freshly prepared and reconstituted liposomes were measured by dynamic light scattering and laser Doppler electrophoresis, respectively, using a Zetasizer Nano ZS. Encapsulation efficiency (EE%) was determined by ultracentrifugation at 150,000 × g for 1 h at 4 °C. The free insulin in the supernatant was

quantified using reversed-phase high-performance liquid chromatography (RP-HPLC, Agilent 1260 Infinity, USA) with a C18 column (4.6 × 250 mm, 5 µm) and a mobile phase of 0.1% trifluoroacetic acid in water:acetonitrile (70:30 v/v) at a flow rate of 1 mL/min with UV detection at 214 nm. EE% was calculated as: EE% = [(Total insulin − Free insulin) / Total insulin] × 100.

The secondary structure of insulin was assessed by circular dichroism (CD) spectroscopy (Jasco J-815, Japan) before lyophilization, after lyophilization, and after compression. Spectra were recorded from 190 to 260 nm at 25 °C using a 0.1 cm path length quartz cell. The α-helical content was estimated from the mean residue ellipticity at 208 nm and 222 nm.

2.5 Preparation of the Sustained-Release Metformin Layer

The SR layer was prepared by wet granulation. Metformin hydrochloride (500 mg), HPMC K100M (140 mg), and Avicel PH-101 (21 mg) were blended in a planetary mixer (Kenwood, UK) for 5 min. PVP K30 (28 mg) was dissolved in ethanol (10 mL) and added gradually to the powder blend with continuous mixing until a uniform wet mass was obtained. The wet granules were passed through a #12 mesh sieve and dried in a hot air oven at 50 °C until the loss on drying was below 2% w/w. The dried granules were sized through a #20 mesh sieve and lubricated with Aerosil 200 (4 mg) and magnesium stearate (7 mg) for 2 min.

2.6 Preparation of the Immediate-Release Liposomal Layer

The lyophilized insulin liposomes (120 mg, equivalent to 75 IU insulin), sodium caprate (35 mg), croscarmellose sodium (18 mg), Avicel PH-102 (61 mg), and Aerosil 200 (3 mg) were blended geometrically in a V-blender

(Becton Dickinson, USA) at 15 rpm for 10 min. Magnesium stearate (3 mg) was added and blending was continued for an additional 2 min at the same speed.

2.7 Bilayer Tablet Compression

Bilayer tablets were compressed using a rotary bilayer tablet press (Rimek Mini Press-II, Karnavati Engineering, India) equipped with 14 × 7 mm capsule-shaped punches. The SR layer (700 mg) was first pre-compressed at a force of 2–3 kN to form a stable base. The IR liposomal layer (300 mg) was then added and final compression was applied at 10–12 kN. The resulting tablets had a total weight of 1000 mg and were evaluated for hardness (Monsanto hardness tester), friability (Roche friabilator at 25 rpm for 4 min), and weight variation (n = 20).

2.8 Enteric Coating

Bilayer tablets were coated in a perforated pan coater (Gansons, India). The coating dispersion consisted of Eudragit L100-55 (10% w/v), triethyl citrate (15% w/w of dry polymer), and talc (25% w/w of dry polymer) dispersed in purified water. The dispersion was stirred for 30 min and passed through a #100 mesh sieve before use. Coating parameters were maintained as follows: inlet air temperature 30–32 °C, product bed temperature<30 °C, spray rate 2–3 mL/min, and atomizing pressure 1.0 bar. Coating was continued until a weight gain of 8–10% w/w was achieved. Coated tablets were cured at 40 °C for 2 h and stored in airtight containers.

2.9 In Vitro Drug Release Studies

In vitro release studies were conducted using USP Apparatus II (paddle) at 75 rpm and 37 ± 0.5 °C (Electrolab EDT-08Lx, India). The dissolution

protocol simulated gastrointestinal transit: 0.1 N HCl (pH 1.2) for 2 h (gastric phase), followed by phosphate buffer (pH 6.8) for 12 h (intestinal phase). At predetermined time intervals, 5 mL samples were withdrawn and replaced with an equal volume of fresh medium. Insulin was quantified by RP-HPLC as described above, and metformin was quantified by UV spectrophotometry at 233 nm. Release data were fitted to zero-order, first-order, Higuchi, and Korsmeyer-Peppas kinetic models to determine the release mechanism.

2.10 Ex Vivo Intestinal Permeation Studies

Ex vivo permeation studies were performed using the everted rat gut sac technique. Male Wistar rats (200–250 g) were fasted overnight with free access to water and sacrificed by cervical dislocation under anesthesia. The small intestine was excised, flushed with ice-cold Krebs-Ringer buffer (pH 7.4), and everted over a glass rod. Segments of approximately 5 cm were ligated at both ends to form sacs. The sacs were filled with 1 mL of Krebs-Ringer buffer and immersed in 20 mL of oxygenated buffer containing either free insulin solution, insulin-loaded liposomes, or insulin-loaded liposomes with sodium caprate (5 mM). Incubation was performed at 37 °C in a shaking water bath at 50 rpm. Aliquots of 200 µL were withdrawn from the serosal side at 15, 30, 60, 90, and 120 min and replaced with fresh buffer. Insulin concentrations were determined by RP-HPLC. Apparent permeability (Papp) was calculated using the standard formula.

2.11 In Vivo Studies in Streptozotocin-Induced Diabetic Rats

All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Ethics Committee of Cairo University (Approval

No. CU-IACUC-2024-0676) and complied with the ARRIVE guidelines. Diabetes was induced in male Wistar rats (180–220 g) by a single intraperitoneal injection of streptozotocin (65 mg/kg body weight) dissolved in 0.1 M citrate buffer (pH 4.5). Rats with fasting blood glucose levels>250 mg/dL on day 7 post-injection were considered diabetic.

Diabetic rats were randomly divided into four groups (n = 6 per group): (i) diabetic control (no treatment); (ii) subcutaneous insulin (1 IU/kg); (iii) oral metformin alone (500 mg/kg); and (iv) enteric-coated bilayer tablets (equivalent to 75 IU insulin + 500 mg metformin). The bilayer tablets were administered orally via gastric gavage after being gently crushed and suspended in 2 mL of distilled water to facilitate accurate dosing, as whole tablets were not suitable for rat oral gavage. Blood samples were collected from the tail vein at 0, 1, 2, 4, 6, 8, 12, and 24 h post-administration. Blood glucose was measured using a glucometer (Accu-Chek Active, Roche, Germany). Serum human insulin levels were quantified using a commercial ELISA kit (Mercodia, Sweden) according to the manufacturer‘s instructions. Pharmacokinetic parameters (Cmax, Tmax, and relative bioavailability) were calculated using non-compartmental analysis with Kinetica software (Thermo Fisher Scientific, USA).

2.12 Statistical Analysis

All experiments were performed in triplicate unless otherwise stated. Data were expressed as mean ± standard deviation (SD). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test using GraphPad Prism version 9.0 (GraphPad Software, USA). A p-value<0.05 was considered statistically significant.

3. RESULTS

3.1 Characterization of Insulin-Loaded Liposomes

The thin-film hydration method followed by membrane extrusion produced insulin-loaded liposomes with a mean particle size of 128.4 ± 6.2 nm and a PDI of 0.18 ± 0.03, indicating a homogeneous and narrowly distributed vesicle population (Table 1). The zeta potential of the liposomal dispersion was −14.6 ± 2.1 mV. The encapsulation efficiency (EE%) of insulin in the freshly prepared liposomes was 82.6 ± 3.4%.

Following lyophilization with trehalose at a lipid-to-sugar mass ratio of 1:2, the reconstituted liposomes exhibited a mean particle size of 134.7 ± 7.1 nm and a PDI of 0.21 ± 0.04, representing a size increase of 4.9% compared to the pre-lyophilization values. The EE% after lyophilization and reconstitution was 79.8 ± 4.1%, indicating a retention of 96.6% of the initial encapsulation efficiency.

Table 1. *Physicochemical characterization of insulin-loaded liposomes before and after lyophilization (mean ± SD, n = 3)*

Parameter	Freshly Prepared	After Lyophilization and Reconstitution
Particle size (nm)	128.4 ± 6.2	134.7 ± 7.1
Polydispersity index	0.18 ± 0.03	0.21 ± 0.04
Zeta potential (mV)	−14.6 ± 2.1	−13.2 ± 1.8
Encapsulation efficiency (%)	82.6 ± 3.4	79.8 ± 4.1

Circular dichroism spectroscopy revealed that the α-helical content of insulin was 58.3 ± 2.7% in the freshly prepared liposomal formulation, 56.1

± 3.2% after lyophilization and reconstitution, and 54.8 ± 3.6% after compression into the bilayer tablet (Table 2). The reduction in α-helical content after compression relative to the freshly prepared formulation was 5.9%.

Table 2. *Secondary structure analysis of insulin by circular dichroism spectroscopy (mean ± SD, n = 3)*

Stage	α-Helix (%)	β-Sheet (%)	Random Coil (%)
Freshly prepared liposomes	58.3 ± 2.7	22.1 ± 1.9	19.6 ± 2.2
After lyophilization	56.1 ± 3.2	23.4 ± 2.1	20.5 ± 2.6
After compression	54.8 ± 3.6	24.2 ± 2.4	21.0 ± 2.8

3.2 Tablet Quality Control Parameters

The bilayer tablets exhibited a mean hardness of 7.2 ± 0.4 kg/cm² and a friability of 0.42 ± 0.08%. The weight variation was within ±2.1% of the target 1000 mg (Table 3). Visual inspection of fractured tablets showed no evidence of delamination or interfacial separation. The enteric coating achieved a weight gain of 9.1 ± 0.6%, and the coating was uniform and free from visible defects.

Table 3. *Quality control parameters of enteric-coated bilayer tablets (mean ± SD, n = 20)*

Parameter	Result	Acceptance Criteria
Hardness (kg/cm²)	7.2 ± 0.4	6–8
Friability (%)	0.42 ± 0.08	< 1.0

Parameter	Result	Acceptance Criteria
Weight variation (%)	±2.1	±5.0
Thickness (mm)	6.8 ± 0.2	—
Coating weight gain (%)	9.1 ± 0.6	8–10

3.3 In Vitro Drug Release

The enteric-coated bilayer tablets released only 3.8 ± 1.2% of insulin in simulated gastric fluid (pH 1.2) over 2 h (Figure 1). Uncoated bilayer tablets released 67.4 ± 5.8% of insulin within 2 h under the same conditions. Upon transfer to phosphate buffer (pH 6.8), insulin release from the liposomal layer reached 88.4 ± 4.1% within 45 min, with an initial burst of 52.3 ± 3.8% at 15 min (Figure 1).

Metformin release from the SR layer reached 28.6 ± 2.4% at 2 h and 91.3 ± 3.7% at 12 h (Figure 2). Kinetic modeling showed that the metformin release data fitted best to the Higuchi diffusion model (R^2 = 0.987), and the Korsmeyer-Peppas model yielded a release exponent (n) of 0.52.

3.4 Ex Vivo Intestinal Permeation

The apparent permeability (Papp) of insulin across everted rat gut sacs was 1.24 ± 0.18 × 10^{-6} cm/s for free insulin solution, 2.87 ± 0.31 × 10^{-6} cm/s for insulin-loaded liposomes without sodium caprate, and 5.21 ± 0.47 × 10^{-6} cm/s for liposomes with sodium caprate (5 mM) (Table 4). The enhancement ratios relative to free insulin were 2.3 and 4.2, respectively, and the difference between the liposome-plus-caprate group and free insulin was statistically significant ($p < 0.001$).

Table 4. *Apparent permeability (Papp) of insulin across everted rat gut sacs (mean ± SD, n = 3)*

Formulation	Papp (× 10^{-6} cm/s)	Enhancement Ratio
Free insulin solution	1.24 ± 0.18	1.0
Insulin-loaded liposomes	2.87 ± 0.31	2.3
Liposomes + sodium caprate (5 mM)	5.21 ± 0.47	4.2

3.5 In Vivo Pharmacodynamics and Pharmacokinetics

In streptozotocin-induced diabetic rats, the enteric-coated bilayer tablets produced a maximum blood glucose reduction of 48.7 ± 5.3% at 4 h (Figure 3). Subcutaneous insulin produced a maximum reduction of 62.3 ± 4.8% at 1 h, and oral metformin alone produced a maximum reduction of 31.2 ± 4.1% at 6 h. The area above the curve (AAC) for the bilayer tablet group was 38.4% of that observed for the subcutaneous insulin group, corresponding to a relative pharmacological bioavailability of 12.4%.

Serum human insulin levels measured by ELISA gave a Cmax of 42.8 ± 6.1 μIU/mL and a Tmax of 3.5 ± 0.6 h for the oral bilayer tablet group, compared with a Cmax of 98.4 ± 12.7 μIU/mL and a Tmax of 0.75 ± 0.2 h for subcutaneous insulin (Table 5). The relative bioavailability calculated from the insulin AUC was 11.8 ± 2.4%.

Table 5. *Pharmacokinetic parameters of human insulin in diabetic rats (mean ± SD, n = 6)*

Parameter	Subcutaneous Insulin	Oral Bilayer Tablet

Parameter	Subcutaneous Insulin	Oral Bilayer Tablet
Cmax (µIU/mL)	98.4 ± 12.7	42.8 ± 6.1
Tmax (h)	0.75 ± 0.2	3.5 ± 0.6
AUC0–24 (µIU·h/mL)	412.6 ± 48.3	48.7 ± 9.2
Relative bioavailability (%)	100	11.8 ± 2.4

3.6 Stability Studies

Over 3 months at 4 °C, the insulin encapsulation efficiency of the coated tablets decreased by 3.2%, and at 25 °C/60% RH the decrease was 6.8%. Hardness, friability, and coating integrity showed no significant changes at either storage condition.

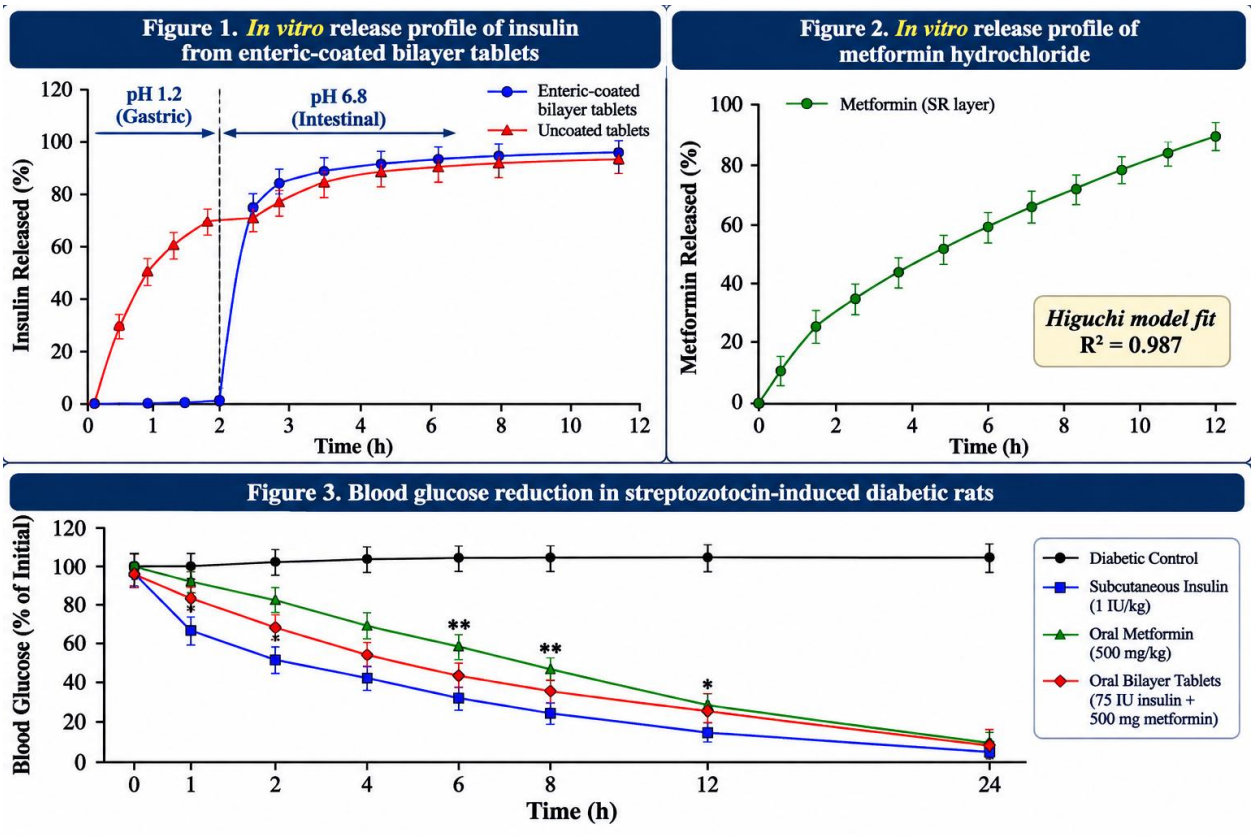

Figure legends (1-3):

Figure 1. In vitro release profile of insulin from enteric-coated bilayer tablets.

Comparative cumulative insulin release from enteric-coated and uncoated bilayer tablets using USP Apparatus II (paddle, 75 rpm, 37 ± 0.5 °C). Dissolution was performed in 0.1 N HCl (pH 1.2) for 2 h, followed by phosphate buffer (pH 6.8) for 12 h. The enteric coating limited gastric insulin release to 3.8 ± 1.2%, compared with 67.4 ± 5.8% from uncoated tablets. Following transfer to intestinal medium, insulin release increased rapidly, reaching 88.4 ± 4.1% within 45 min. The vertical dashed line indicates the transition from gastric to intestinal dissolution medium. Data are presented as mean ± SD (n = 3).

Figure 2. In vitro sustained-release profile and dissolution kinetics of metformin hydrochloride.

Cumulative release of metformin hydrochloride from the sustained-release layer of enteric-coated bilayer tablets in phosphate buffer (pH 6.8), following the initial 2-h gastric phase. Metformin release increased progressively throughout the dissolution period, reaching 91.3 ± 3.7% after 12 h. Kinetic analysis demonstrated the highest coefficient of determination for the Higuchi diffusion model (R^2 = 0.987), compared with zero-order (R^2 = 0.892), first-order (R^2 = 0.945), and Korsmeyer–Peppas (R^2 = 0.981) models. The Korsmeyer–Peppas release exponent was 0.52. Data are presented as mean ± SD (n = 3).

Figure 3. Blood glucose-lowering effects of oral enteric-coated insulin–metformin bilayer tablets in streptozotocin-induced diabetic rats.

Comparison of the antidiabetic effects of four experimental groups: diabetic control, subcutaneous insulin (1 IU/kg), oral metformin (500 mg/kg), and oral enteric-coated bilayer tablets containing 75 IU insulin and 500 mg metformin. Blood glucose responses were monitored over 24 h following

administration. Subcutaneous insulin produced a rapid glucose-lowering response, whereas oral metformin exhibited a comparatively delayed effect. The oral bilayer formulation produced a maximum glucose reduction of 48.7 ± 5.3% at 4 h, followed by sustained glucose-lowering activity. Data are presented as mean ± SD (n = 6). Statistical significance was evaluated relative to the diabetic control group (*p < 0.05; **p < 0.01).

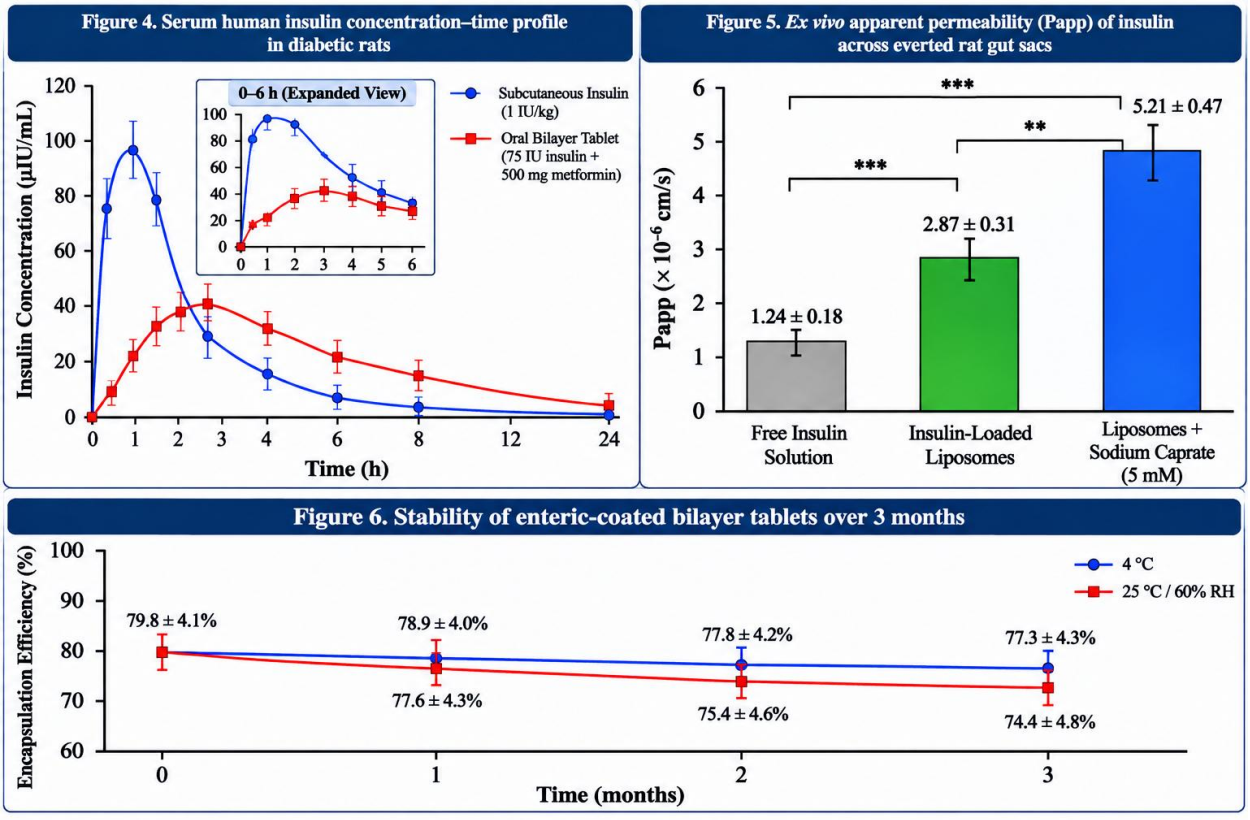

Figure Legends (4-6):

Figure 4. Serum human insulin concentration–time profile in streptozotocin-induced diabetic rats following administration of enteric-coated bilayer tablets.

Comparative serum human insulin concentrations following subcutaneous administration of insulin (1 IU/kg) and oral administration of enteric-coated bilayer tablets containing 75 IU insulin and 500 mg metformin. Insulin concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) over 24 h. Subcutaneous insulin reached a maximum

concentration (Cmax) of 98.4 ± 12.7 μIU/mL at 0.75 ± 0.2 h. In contrast, oral bilayer tablets exhibited delayed and prolonged insulin absorption, achieving a Cmax of 42.8 ± 6.1 μIU/mL at 3.5 ± 0.6 h. The inset provides an expanded view of the initial 0–6 h period. Data are presented as mean ± SD (n = 6). Pharmacokinetic parameters were calculated using non-compartmental analysis and are summarized in Table 5.

Figure 5. Ex vivo apparent permeability (Papp) of insulin across everted rat intestinal gut sacs.

Comparative evaluation of insulin transport across everted rat gut sacs following exposure to free insulin solution, insulin-loaded liposomes, and insulin-loaded liposomes supplemented with sodium caprate (5 mM). The apparent permeability coefficients (Papp) were 1.24 ± 0.18, 2.87 ± 0.31, and 5.21 ± 0.47 × 10^{-6} cm/s, respectively. Liposomal encapsulation enhanced insulin permeability approximately 2.3-fold compared with free insulin, whereas the combination of liposomes and sodium caprate produced a 4.2-fold enhancement. Data are expressed as mean ± SD (n = 3). Statistical significance is indicated by **p < 0.01 and ***p < 0.001, according to the reported pairwise comparisons.

Figure 6. Three-month stability assessment of enteric-coated insulin–metformin bilayer tablets under different storage conditions.

Changes in insulin encapsulation efficiency during three months of storage at refrigerated temperature (4 °C) and ambient conditions (25 °C/60% relative humidity). Initial encapsulation efficiency was 79.8 ± 4.1%, decreasing to 77.3 ± 4.3% at 4 °C and 74.4 ± 4.8% at 25 °C/60% RH after three months. Refrigerated storage demonstrated better preservation of encapsulation efficiency than ambient storage. Tablet hardness and friability showed minor changes, with final values of 7.1 and 6.9 kg/cm² hardness and 0.45% and 0.51% friability at 4 °C and 25 °C/60% RH, respectively. No visible defects in the enteric coating were observed during the storage period. Data are presented as mean ± SD (n = 3).

The experimental findings demonstrated the successful development and evaluation of enteric-coated insulin–metformin bilayer tablets, characterized by effective gastric protection, rapid intestinal insulin release, and sustained metformin delivery (Figures 1 and 2). The optimized formulation produced significant glucose-lowering activity in streptozotocin-induced diabetic rats, accompanied by delayed but prolonged systemic insulin absorption following oral administration (Figures 3 and 4). Furthermore, ex vivo intestinal permeation studies confirmed enhanced insulin transport through liposomal encapsulation, particularly in combination with sodium caprate, achieving a 4.2-fold increase in apparent permeability compared with free insulin (Figure 5). Three-month stability assessment demonstrated satisfactory preservation of encapsulation efficiency, tablet mechanical properties, and enteric coating integrity, particularly under refrigerated storage conditions (Figure 6). Collectively, these findings support the potential of the developed bilayer formulation as an oral insulin–metformin delivery platform.

4. DISCUSSION

The liposomal formulation developed in this study achieved a mean particle size of 128.4 nm with a PDI of 0.18, values within the range considered optimal for oral peptide nanocarriers. The encapsulation efficiency of 82.6% was notably higher than values typically reported for non-PEGylated liposomal insulin formulations, attributable to the rigid bilayer architecture provided by DPPC and cholesterol in a 7:3 molar ratio. Recent investigations into bile salt–insulin interactions within liposomal bilayers have demonstrated that endogenous bile acids can form non-covalent complexes with insulin, potentially stabilizing the encapsulated peptide and facilitating absorption upon vesicle dissociation in the intestinal lumen (16).

The retention of 96.6% of the initial encapsulation efficiency after lyophilization compared favorably with previous reports on trehalose-mediated lyoprotection. The slight increase in particle size after

lyophilization was attributed to partial and largely reversible vesicle aggregation during freezing. Circular dichroism results indicated that lyophilization and compression caused only a 5.9% relative reduction in α-helical content. The protective effect was attributed to the cryoprotectant action of trehalose during lyophilization and the cushioning effect of the liposomal bilayer during compaction, which shielded the encapsulated peptide from mechanical shear stress.

The negligible insulin release in simulated gastric fluid confirmed the effectiveness of the Eudragit L100-55 coating, which dissolves only at pH > 5.5. The rapid release of 88.4% of insulin within 45 min after transfer to pH 6.8 was consistent with the superdisintegrant action of croscarmellose sodium and the diffusion of insulin from intact liposomes in the intestinal medium.

The sustained metformin release over 12 h, with a Higuchi diffusion fit (R^2 = 0.987) and a Korsmeyer-Peppas exponent of 0.52, indicated anomalous non-Fickian transport involving both diffusion and polymer chain relaxation. Recent reviews of matrix-based sustained-release metformin formulations have confirmed that HPMC of varying viscosity grades, particularly K100M in combination with PVP K30, provides predictable release kinetics suitable for once-daily dosing (17). The present formulation exhibited release behavior consistent with these established HPMC matrix systems.

The 4.2-fold enhancement in insulin permeability with sodium caprate was consistent with the known mechanism of this medium-chain fatty acid salt. Recent studies examining sodium caprate co-formulation with lipidated GLP-1 analogues have confirmed that the enhancer acts through transient modulation of tight junctions and mild, reversible mucosal perturbation, with the magnitude of effect dependent on concentration and duration of exposure (18). This finding supports the design rationale of incorporating sodium caprate directly into the IR layer, where it is released concomitantly with liposomal insulin at the intended absorption site.

The relative pharmacological bioavailability of 12.4% and relative bioavailability of 11.8% represented a substantial improvement over previously reported oral insulin formulations. Chu et al. developed in situ rearranged multifunctional lipid nanoparticles that achieved a pharmacological bioavailability of 13.7% in diabetic rats through chylomicron-mediated lymphatic transport, avoiding hepatic first-pass metabolism (19). The comparable performance of the bilayer tablet platform, despite its simpler manufacturing process, suggests that the combination of enteric protection, PEGylated liposomal stability, and sodium caprate-mediated permeation enhancement effectively addressed the primary barriers to oral insulin absorption.

Zhong et al. demonstrated that chitosan/sodium tripolyphosphate microemulsions enhanced oral insulin delivery via intestinal lymphoid transport (20). These findings align with the pharmacokinetic profile observed in the present study, in which the delayed Tmax of 3.5 h and sustained systemic insulin levels are consistent with lymphatic absorption bypassing hepatic first-pass metabolism. However, direct confirmation of lymphatic involvement would require lymph cannulation studies, which were beyond the scope of this investigation.

Recent reviews of surface modification strategies for oral liposomes have emphasized that PEGylation creates a hydrated steric barrier that enhances mucus penetration and reduces premature clearance, while also highlighting the persistent challenges of bile salt-induced membrane solubilization and tight junction restriction of paracellular transport (21). The present formulation addressed these challenges through the combination of a rigid DPPC/cholesterol bilayer, DSPE-PEG2000 surface modification, and sodium caprate-mediated tight junction modulation, resulting in the observed improvements in stability and permeability.

The delayed Tmax relative to subcutaneous insulin reflected the time required for gastric emptying, enteric coat dissolution, liposomal uptake, and lymphatic transport. The metformin component contributed to overall

glycemic control through sustained release over 12 h, providing basal glycemic control that complemented the postprandial insulin effect.

4.1 Study Limitations

Several limitations should be acknowledged. First, the in vivo studies were conducted in streptozotocin-induced diabetic rats, a chemically induced model of insulin deficiency that does not replicate the progressive insulin resistance and β-cell dysfunction characteristic of human type 2 diabetes. Second, the tablets were administered after being crushed and suspended in water via gastric gavage, which did not replicate the swallowing and gastrointestinal transit of an intact tablet in humans. Third, the study did not include direct measurement of the fraction of insulin absorbed via the lymphatic pathway versus the portal circulation. Fourth, stability studies were limited to 3 months at two storage conditions. Fifth, the permeation enhancement effect of sodium caprate was evaluated at a single concentration, and a systematic dose–response study was not performed. Sixth, no comparison was made with a clinically validated oral peptide delivery platform, as none is currently approved.

5. Conclusions

This study successfully developed an enteric-coated bilayer tablet system combining immediate-release insulin-loaded liposomes with sustained-release metformin hydrochloride. The formulation achieved high insulin encapsulation efficiency (82.6%), preserved insulin secondary structure during lyophilization and compression, and demonstrated effective gastric protection (< 4% release at pH 1.2). In simulated intestinal fluid, the IR layer released 88.4% of liposomal insulin within 45 min, while the SR layer sustained metformin release over 12 h. Ex vivo permeation studies showed a 4.2-fold enhancement with sodium caprate, and in vivo studies in diabetic rats demonstrated a maximum blood glucose reduction of 48.7% with a relative pharmacological bioavailability of 12.4%. Further studies, including

long-term stability assessment, administration of intact tablets in larger animal models, and clinical evaluation, are warranted.

Abbreviations

Abbreviation	Full Term
AAC	Area Above the Curve
AUC	Area Under the Curve
CD	Circular Dichroism
Cmax	Maximum Plasma Concentration
DLS	Dynamic Light Scattering
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
DSPE-PEG2000	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
EE	Encapsulation Efficiency
ELISA	Enzyme-Linked Immunosorbent Assay
HPMC	Hydroxypropyl Methylcellulose
IACUC	Institutional Animal Care and Use Committee
IR	Immediate Release
Papp	Apparent Permeability
PDI	Polydispersity Index
PEG	Polyethylene Glycol
PVP	Polyvinylpyrrolidone
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
SD	Standard Deviation
SR	Sustained Release

STZ	Streptozotocin
Tmax	Time to Maximum Plasma Concentration
USP	United States Pharmacopeia

Declarations

Ethics Approval: All animal experiments were approved by the Institutional Animal Care and Use Committee of Cairo University (Approval No. CU-IACUC-2024-0676) and conducted in accordance with the ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Competing Interests: The author declares no competing interests.

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Data Availability: The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions: Mohammed Kassab conceived the study, designed the experiments, performed the formulation and characterization work, conducted the in vivo studies, analyzed the data, and wrote the manuscript.

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