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ENGINEERING CHITOSAN–EXENATIDE NANOCOMPLEXES WITH FATTY ACIDS FOR IMPROVED PEPTIDE DELIVERY

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Diabetes Mellitus (DM) is a common endocrine disorder responsible for high morbidity and mortality worldwide. The standard medical treatment for DM is oral hypoglycemic agents and/or insulin. Exenatide, a glucagon-like peptide, has been used to reduce blood sugar and treat DM in the last 20 years. Exenatide administration is limited to parenteral routes. The development of an orally administered Exenatide represents a worthy study that provides significant benefits to patients with diabetes by improving compliance and adherence to the treatment and reduce the burden of frequent injections and enhance treatment outcomes.

***The aim of the study** is to prepare Exenatide as an oral drug delivery system by combining the advantages of nanoencapsulation with the use of an oily vehicle using fatty acids.*

***Method**: The polyelectrolyte complexation method was used to prepare Exenatide-chitosan complexes (PEC) as an aqueous environment in order to create orally administered Exenatide. The potential of PEC-fatty acids nanoparticles as oral delivery carriers of Exenatide was studied.*

***Results**: The sizes of the formed nanodispersed particles were different when loaded with diluted chitosan or PEC. The vortex mechanical mixing method produced superior results and provided about 20% greater Exenatide gastrointestinal protection than the stirring mechanical method. The results indicated that hydroxypropyl-β-cyclodextrin (HP-β-CD) had a more promising effect on oleic acid formula (F4), providing 87.1% Exenatide gastrointestinal protection but with a larger nanodispersed particle size of 200 nm. However, it did not produce significantly better results for linoleic acid (F8), which provided 81.6% gastrointestinal protection and a nanodispersed particle size of 210 nm. An in vivo study showed that formula F4 has the C_{max} of Exenatide with T_{max} of 3 h. Blood glucose was effectively reduced to a level of 91 mg/dl level within 3 h, with a sustained reduction up to 8 h.*

***Conclusion**: Exenatide could be protected from gastrointestinal enzymes by incorporation into chitosan lipid-based formulation. The vortexing mechanical mixing method is preferred method for the preparation. The use of the HP-β-CD improved gastrointestinal protection. The formula F4 is a promising oral alternative to the paraenteral Exenatide*

***Keywords**: Exenatide, chitosan, fatty acids, polyelectrolyte complex (PEC)*

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1. Introduction

Diabetes mellitus (DM) is a prevalent endocrine disease that is associated with high rates of morbidity and mortality on a global scale. The etiology of this endocrine disorder may be attributed to a deficiency in insulin production or resistance to insulin action. The deficiency in insulin production is attributed to the destruction of the pancreatic tissues responsible for insulin production, a condition known as type I DM. The resistance of insulin action is Type II DM which may be influenced by underlying genetics, obesity, or ethnicity. The prevailing medical approach to DM involves the administration of oral hypoglycemic agents and/or insulin. Exenatide, a recently developed pharmaceutical agent for the treatment of DM. It is a glucagon-like peptide that has been utilized in the management of DM for the past two decades. Its FDA approved was in 2005. It is a peptide comprised of 39 amino acids, has a molecular weight of 4,186 Da, and an isoelectric point of pH 4.86. Exenatide is glucagon-like peptide-1 (GLP-1) with respect to its glucoregulatory actions, and it is employed in the treatment of type 2 diabetes [1]. Exenatide has been shown to effectively induce satiety, reduce body weight, and may possess additional cardiovascular protective effects [2]. The administration of Exenatide is restricted to parenteral routes. The experiment to develop an orally administered Exenatide is a study that merits serious consideration. The potential benefits of this oral Exenatide include improved compliance and adherence to treatment, a reduced burden of frequent parenteral injections, and enhanced treatment outcomes. The development of oral delivery systems for proteins is underway, with nanoparticle drug delivery systems emerging as a particularly promising approach [3].

2. Planning (methodology) of research

The methodology includes the following in experiments to prepare and evaluate Exenatide nano-oily base system:

I. The preparation of low-molecular-weight (LMW) chitosan.

II. The preparation of the aqueous phase chitosan-Exenatide polyelectrolyte complex (PEC).

III. The preparation of an oily phase (Fatty acid-surfactant mixture).

IV. The preparation of nanoparticle oil-based systems.

V. The assessment of the protective effect against GIT degradation.

VI. An in vivo investigation to assess the effectiveness of the experimental approach.

3. Materials and methods

3. 1. Materials

Exenatide was purchased from Shanghai GL Biochem Co., Ltd. (China), High Molecular Weight Chitosan > 250 KDa, DDA 95% was purchased from Xiamen Xing Co., Ltd. (China), Sodium Sulfate was purchased from ACROS Co., Ltd. (Belgium), Potassium Dihydrogen phosphate was purchased from Merck Co., Ltd. (Germany), Hydrochloric Acid was purchased from Merck Co. Ltd. (Germany), Acetonitrile was purchased from Merck Co., Ltd. (Germany), Methanol was purchased from Fisher Co., Ltd. (UK), Acetic acid was purchased from ACROS Co., Ltd. (Belgium), Sodium Chloride was purchased from ACROS Co., Ltd. (Belgium), Sodium Hydroxide was purchased from Acros Co., Ltd. (Belgium), Ortho-Phosphoric acid was purchased from GFS chemicals Co., Ltd. (Germany), Oleic Acid was purchased from Merck KGaA Co., Ltd. (Germany), Linoleic Acid was purchased from ACROS Co., Ltd. (Belgium), Plurol Oleique® (Polyglyceryl-6-Dioleate) was purchased from Gattefosse Co., Ltd. (France), Labrasol® (Caprylocaproyl macrogol-8 Glyceride) was purchased from Gattefosse Co., Ltd. (France), Pepsin was purchased from ACROS Co., Ltd. (Belgium), Pancreatin was purchased from ACROS Co., Ltd. (Belgium), D-Trehalose was purchased from Thermo Scientific Chemicals Co., Ltd. (China).

3. 2. Instruments

UV/Vis spectrophotometer (Du 640i spectrophotometer, Beckman Coulter, USA), lyophilization (Heto Power Dry PL 9000 freeze dryer, Thermo Fisher Scientifica-Inc, Waltham-MA, USA), Zetasizer Nano-ZS (Malvern Instruments, UK), Viscometer (SV-10, A&D Company, Japan), water bath shaker (GFL, GmbH, Germany), centrifuge (Hermle Z233M Centrifuge, UK) reversed phase high pressure liquid chromatography (RP-HPLC) which consist from TSP 1000 pump, TSP 1000 UV-VIS detector and a TSP AS 3000 autosampler (Spectra System, USA), Vortex Mixer-ZX3 (Velp Scintefica, Italy), Abbe refractometer.

3. 3. Method

3. 3. 1. Preparation of LMW chitosan

The depolymerization of high molecular weight (HMW) chitosan was achieved through the synthesis of 13kDa LMW chitosan by means of HCl hydrolysis of HMW chitosan. Subsequently, the mixture was stirred (1000 rpm) and reacted under reflux at 200°C for a period of 3 h. The viscosity of the chitosan hydrochloride was measured by a viscometer to determine the 13 kDa LMW. The viscosity average molecular weight was calculated using the Mark-Houwink's equation, $[\eta] = K$. In this equation, [η] denotes the intrinsic viscosity and M represents the viscosity-average molecular weight. The values for K and a were determined to be 0.00058 and 0.69, respectively [4, 5].

3. 3. 2. Preparation of an aqueous phase chitosan-Exenatide complex (PEC)

Preparation:

a) preparation of chitosan solution. A 125 mg of 13-kDa LMW chitosan was dissolved in 3 ml of deionized water. The pH was adjusted to 5.5 using 0.2-M NaOH, and the final volume was brought to 5 ml using deionized water;

b) preparation of Exenatide solution. 5 mg of Exenatide powder were dissolved in 1 ml of 0.01 M HCl. Then, 3 ml of trehalose buffer solution (prepared by dissolving 5% trehalose in 0.05 M acetate buffer solution at pH 5.5) were added;

c) preparation of aqueous phase of PEC. The PEC was prepared by adding 1 ml of the chitosan solution to 1 ml of the Exenatide solution. The solution was stirred gently for 30 minutes. When cyclodextrin (CD) was added to the formulas, 0.1 g of CD was dissolved in 10 ml of trehalose buffer solution, this solution was then used to prepare the PEC.

3. 3. 3. The preparation of an oily phase (fatty acid-surfactant mixture):

Preparation:

a) viscosity measurement of fatty acids. In this study, 2 types of fatty acids were selected: oleic acid and linoleic acid. The viscosity and the refractive index of each fatty acid were measured at 25°C, with each fatty acid analyzed in triplicate;

b) preparation of an oily phase. For each formula, the oily phase was prepared by placing a 1:1 the surfactant mixture (S_{mix}) of Labrasol® and the Plurol Oleique® with the specific fatty acid in Fatty acid:S_{mix} ratio 20%:80% of each prepared formula in a tube. The mixture was then vortexed vigorously for 3 min.

3. 3. 4. Preparation of nanoparticle oily-based systems

Preparation:

a) construction of the pseudo-ternary phase diagram. The diagram consisted of the surfactant:co-surfactant mixture Labrasol®:Plurol Oleique®, fatty acids (the oleic acid and linoleic acid) as oil phases and the deionized water as aqueous phase. The oleic acid and linoleic acid (C18) were used without water pre-saturation as their aqueous miscibility are very poor [6]. The pseudo-ternary phase diagram was constructed by titrating a homogeneous liquid mixture of each oil and surfactant mixture with deionized water separately at room temperature. A total of 3 g of fatty acid:S_{mix} was weighed accurately. The weight ratios of fatty acid:S_{mix} were set at 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9. The samples were vortexed for 5 min, then loaded with water and

mixed with a vortexer for 2 min to accelerate equilibrium. After adding an aliquot of water, the mixture was visually examined for transparency using a UV/Vis spectrophotometer at wavelengths ranging from 600 to 700 nm. The blanks used had the same fatty acid:S_{mix} ratio, but without water loading. Then, the phase diagram results were used to select suitable percentages of the aqueous phases for the nanoparticle dispersion system;

b) particle sizes measurement. They were carried out with a Zetasizer Nano-ZS at 25°C. The scattering light was detected at a 173° angle. Data analysis required the viscosity and refractive index of each measurement. The particle size of each oily phase containing the oleic acid and the linoleic acid, loaded with 0.5%, 1%, 1.5%, or 2% diluted chitosan (with a 1:1 ratio of chitosan solution to water) or PEC, was measured;

c) the nanoparticle oily-based systems. The nanoparticle oily-based systems were prepared for each formula by adding the PEC solution dropwise to the oily phase. The mixture was then stirred or vortexed for 30 seconds at room temperature. Tabю 1 shows composition and method of preparation of each prepared formula.

To ensure reproducibility, all formulations were prepared under controlled and standardized conditions, with all preparation parameters maintained constant throughout the experiments.

Table 1

Different formulas (nanoparticle oily-based systems): composition and methods of preparation

Formula No.	Fatty acid used	Fatty acid: S_{mix}	PEC composition	% PEC loaded	External additions
F1*	Oleic acid	80:20	25 mg Chitosan solu. : 1.25 mg/ml Exenatide	2%	/
F2	Oleic acid	80:20	25 mg Chitosan solu. : 1.25 mg/ml Exenatide	2%	/
F3	Oleic acid	80:20	25 mg Chitosan solu. : 1.25 mg/ml Exenatide	2%	α-CD
F4	Oleic acid	80:20	25 mg Chitosan solu. : 1.25 mg/ml Exenatide	2%	HP-β-CD
F5	Oleic acid	80:20	25 mg Chitosan solu. : 1.25 mg/ml Exenatide	2%	γ-CD
F6*	Linoleic acid	80:20	25 mg Chitosan solu. : 1.25 mg/ml Exenatide	2%	/
F7	Linoleic acid	80:20	25 mg Chitosan solu. : 1.25 mg/ml Exenatide	2%	/
F8	Linoleic acid	80:20	25 mg Chitosan solu. : 1.25 mg/ml Exenatide	2%	HP-β-CD

*Note: all formulas were prepared by vortexing, except F*1 *and F*6*, which were prepared by stirring, (* prepared by stirring*).

3. 3. 5. The assessment of the protective effect against GIT degradation

Preparation of stimulated gastric fluid (SGF) and stimulated intestinal fluid (SIF):

a) preparation of stimulated gastric fluid (SGF). A 0.35-ml solution of concentrated HCl was diluted in 40 ml of deionized water. Next, 0.1 g of sodium chloride (NaCl) and 0.16 g of pepsin were dissolved in the solution. The volume was made up to 50 ml with deionized water. The pH of the resulting solution is expected to be 1.2;

b) preparation of stimulated intestinal fluid (SIF). A total of 6.8 g of monobasic potassium phosphate (KH_2PO4) was dissolved in 25 mL of deionized water. Then, 7.7 mL of 0.2 N NaOH was added, followed by 50 mL of deionized water. This solution was subsequently employed to dissolve 1 g of pancreatin, and the pH was adjusted to 6.8 ± 0.1 with 0.2 N NaOH. The solution was subsequently diluted to a volume of 100 ml with water;

c) assessment of the protective effect against gastric and intestinal degradation. To evaluate the formula's ability to resist gastric degradation, 2 ml of each prepared formula was incubated at 37°C and shaken with 5 ml of simulated gastric fluid pH 1.2 (SGF) for 15 minutes in a water bath shaker at 100 strokes per minute. Enzymatic degradation was achieved by the addition of pepsin. Then the oily based system was separated from the SGF and samples were taken for Exenatide analysis using reversed-phase high-performance liquid chromatography (RP-HPLC). Also 1.5 ml of the gastric incubated oily based system were taken and incubated with 5 ml of simulated intestinal fluid pH 6.8 (SIF) at 37°C and shaken for 45 minutes in a water bath shaker at 100 strokes per minute. Enzymatic degradation was achieved by adding pancreatin. Then, the oily-based system was separated from the SIF, and samples were withdrawn for Exenatide analysis by RP-HPLC.

Quantitative analysis of exenatide. In this study, the analytical assay procedure method used for quantitative determination of Exenatide was reversed-phase high performance liquid chromatography (RP-HPLC). The conditions employed were: A BioBasic-C18 column with the following specifications: ACE 5 µm, 250 × 4.6 mm inner diameter, and 300 Å pore size; detection at 210 nm; and the mobile phases are A: 0.1% trifluoroacetic acid (TFA) in deionized water and B: 0.1% trifluoroacetic acid (TFA) in 80% acetonitrile (gradient: 15–45% B over 30 minutes). The flow rate was 1 ml/min and the sample size was 20 µl. All experiments were carried out at 40°C.

Samples preparation (extraction method). Extraction solvent was prepared from mixing of methanol and acetonitrile at a ratio of 2:3 (v/v). An extraction solvent was used to extract Exenatide for analytical determinations. 1 ml of the oily preparation was mixed with 5 ml of extraction solvent. The mixture was vortexed vigorously for 2 min and then immediately centrifuged at 3,000 rpm for 45 min. An aliquot of the separated aqueous phase was diluted with extraction solvent at a 1:1 ratio, then analyzed by HPLC.

3. 3. 6. *In vivo* assessment:

Male Sprague–Dawley rats (250 g) were fasted overnight and divided into two groups (n = 6). The reference group received subcutaneous Byetta® (10 µg/kg), while the test group received oral oleic acid nano-oily formula, F4 (100 µg/kg), which showed enhanced gastrointestinal protection in vitro. Blood samples were collect-

ed for plasma Exenatide (ELISA) and glucose measurements to compare PK and PD profiles.

3. 3. 7. Statistical analysis:

The Statistical analysis was performed using Student's t-test, a one-way ANOVA followed by a Dunnett's post hoc test, or a two-way ANOVA followed by a Tukey's post hoc test, as appropriate. Data are expressed as the mean ± SD, and differences were considered statistically significant at $p < 0.05$.

4. Results

1. The preparation of low-molecular-weight (LMW) chitosan.

The depolymerization of HMW chitosan using HCl acid yielded LMW chitosan 13-kDa molecular weight.

2. The preparation of the aqueous phase chitosan-Exenatide polyelectrolyte complex (PEC).

PEC is a term used to describe a stable complex formed through the electrostatic interaction between cationic chitosan and anionic Exenatide at a 1:1 ratio and a pH of 5.5. PEC for each formula was prepared as listed in Table 1.

3. The preparation of an oily phase (Fatty acid-surfactant mixture).

Viscosity and refractive index (RI) values measurement of fatty acids: The physicochemical properties of oleic acid and linoleic acid were evaluated based on the viscosity and the refractive index. The results, presented in Table 2, confirmed the acceptability of these fatty acids for oral formulations.

Table 2

The viscosities and the refractive index values of oleic and linoleic acids.

Fatty acid type	Viscosity (centipoise, cP)	Refractive Index
Oleic acid	27.64	1.45
Linoleic acid	24.03	1.46

The tested oils showed viscosities of 24.03–27.64 CP and refractive indices of 1.45–1.46, all within acceptable pharmaceutical ranges, confirming stability and purity.

An oily phase preparation: Transparent and stable oily systems were achieved by combining the fatty acids as an oily carrier with a 1:1 surfactant mixture of Labrasol® and Plurol oleique®.

4. The preparation of nanoparticle oil-based systems.

The construction of pseudo-ternary phase diagrams was undertaken to ascertain the optimal concentration range of components for a transparent system. The shaded regions in Fig. 1, 2 denote the concentration range of transparent water-in-oil (w/o) microemulsions. The optimal w/o microemulsion was selected from the transparent area and it was composed of 20% surfactants mixture, 78% oily phase and 2% aqueous phase for oleic acid and linoleic acid.

Particle size measurement: Dynamic light scattering of the nanoparticles formulated with 13 KDa chitosan is presented in Fig. 3, 4. In the present study we used oleic and linoleic acids loaded with either diluted chitosan or PEC. Initially, the sizes of the dispersed phase particles loaded with diluted chitosan solution were slightly smaller than those loaded with PEC (Fig. 3, 4). As the percentage of the chitosan solution increased, the size of the dispersed phase particles progressively and significantly increased ($p < 0.05$). In contrast, when PEC was loaded into the oily phase, the dispersed phase particle size first increased up to a certain limit and then decreased. This indicates that there is a significant difference between diluted chitosan and PEC formulations.

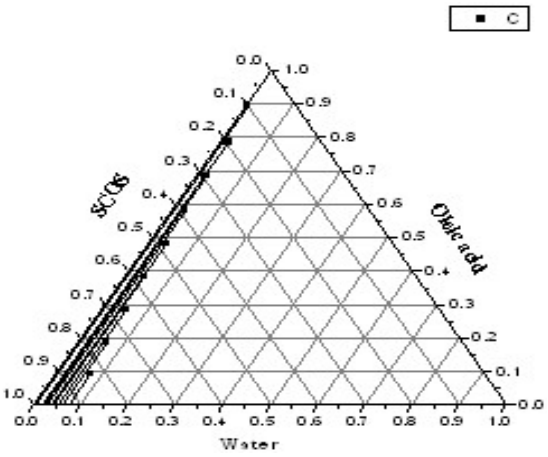

Fig. 1. Pseudo-ternary phase diagram of microemulsion composed of oleic acid (oil), surfactant (Labrasol®), co-surfactant (Plurol-oleique®), and water, ($n = 3$)

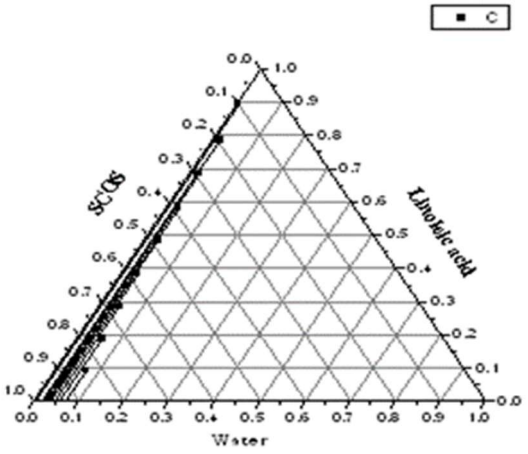

Fig. 2. Pseudo-ternary phase diagram of microemulsion composed of linoleic acid (oil), surfactant (Labrasol®), co-surfactant (Plurol-oleique®), and water, ($n = 3$)

Nanoparticle oily-based systems preparation: the prepared nanoparticle oily-based formulas were transparent with acceptable particle size and stability (Table 1).

5. The assessment of the protective effect against GIT degradation:

– different formulas (nanoparticle oily-based systems) were prepared to study the influence of many factors on the in vitro gastrointestinal protection and particle size and these include:

1) influence of mechanical mixing method used to prepare the formulas;

2) the influence of the number of the double bonds in the fatty acid;

3) the influence of adding different types of cyclodextrin (CD) (α-CD, HP-β-CD, and γ-CD) to the PEC of the nanoparticle oily-based system.

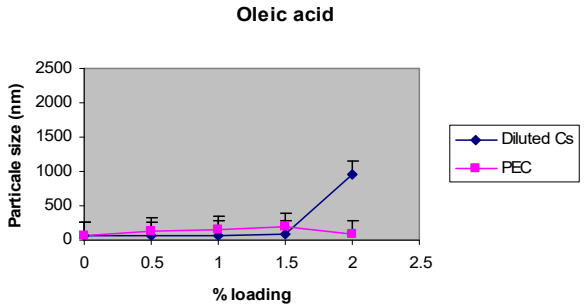

Fig. 3. Nanodispersed particle sizes of oleic acid oily phase loaded by different percentages of diluted chitosan solution and PEC are presented as mean ± SD (n = 6). Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test ($p < 0.05$)

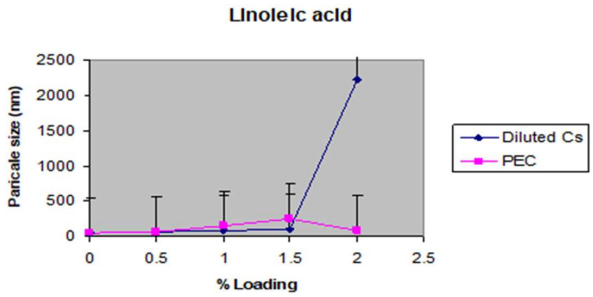

Fig. 4. Nanodispersed particle sizes of the linoleic acid oily phase loaded by different percentages of diluted chitosan solution and PEC are presented as mean ± SD (n = 6). Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test ($p < 0.05$)

Influence of mechanical mixing method used to prepare the formulas.

Two methods of mechanical mixing were used – stirring with a magnetic stirrer and vortexing – to determine which method produced better results. Formulas F1 and F6, which were prepared by stirring, were compared with formulas F2 and F7, which were prepared by vortexing. The size of the nanodispersed particles and the gastric and intestinal protection were measured.

The results showed that, (Fig. 5, 6), formulas prepared by stirring (F1 and F6) gave 63.7–54.7 and 55.6–48.4% for the gastric and intestinal protection with nanodispersed particle sizes 116 and 73.4 nm, for F1 and F6 respectively, compared with formulas prepared by vortexing (F2 and F7) which gave 82.1–72.6 and 90.9–83.2% gastric and intestinal protection with nanodispersed particle sizes 86.1 and 88.6 nm, for F2 and F7 respectively. An unpaired, two-tailed Student's t-test revealed significant differences in particle size and gastrointestinal protection for oleic and linoleic acid formulations prepared using stirring and vortexing methods ($p < 0.05$).

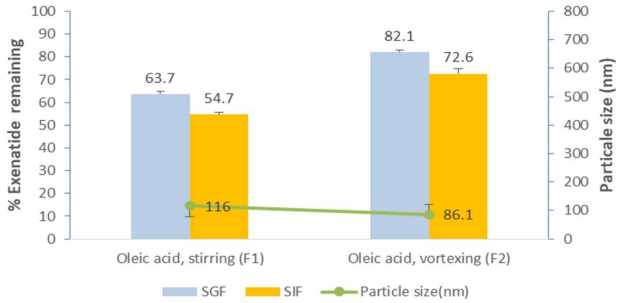

Fig. 5. Influence of the mechanical mixing method on the nanodispersed particle size and gastric and intestinal protection on the oleic acid formula are presented as mean ± SD (n = 4). Statistical analysis was performed using an unpaired two-tailed Student's t-test ($p < 0.05$)

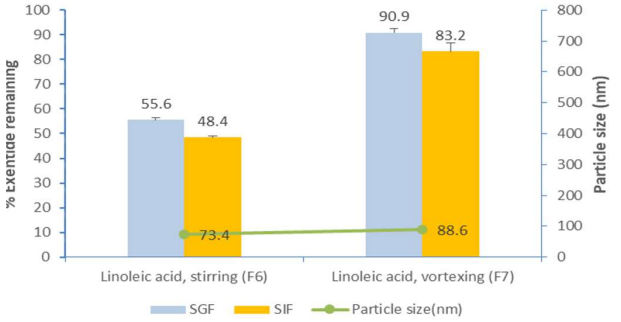

Fig. 6. Influence of the mechanical mixing method on the nanodispersed particle size and gastric and intestinal protection on the linoleic acid formula are presented as mean ± SD (n = 4). Statistical analysis was performed using an unpaired two-tailed Student's t-test ($p < 0.05$)

The influence of the number of the double bonds in the fatty acid.

The fatty acids under scrutiny encompass oleic acid (C18:1), distinguished by a single double bond and a single bend in its molecular structure, and linoleic acid (C18:2), which is endowed with two double bonds and two bends in its molecular structure. The nanoparticles oily based formulas derived from oleic acid (F2) and linoleic acid (F7) exhibited satisfactory outcomes, providing 82.1–72.6% and 90.9–83.2% gastric and intestinal protection with the nanodispersed particle sizes 86.1 nm and 88.9 nm, respectively (Fig. 7). An unpaired two-tailed Student's t-test demonstrated a significant difference between oleic acid and linoleic acid formulations in gastric and intestinal protection ($p < 0.05$), while no statistically significant difference was observed in particle size ($p > 0.05$).

The influence of adding different types of cyclodextrin (CD) (α-CD, HP-β-CD, and γ-CD) to the PEC of the nanoparticle oily-based system.

Formulas F3, F4, and F5 were prepared by adding different types of cyclodextrin (α-CD, HP-β-CD, and γ-CD) to an oily base containing oleic acid formula (F2). One-way ANOVA followed by Dunnett's post hoc test demonstrated statistically significant differences between the F2 and F3, F4, F6 in gastrointestinal protection, and particle size ($p < 0.05$). Formulas F3, F4, and F5 showed increased Exenatide gastric and intestinal protection, with values of 91.8–84.3%, 94–87.1%, and 87.4–79.7% for α-,

HP-, and γ-CDs, respectively. This is compared to formula F2, which had values of 82.1–72.6% (Fig. 8). However, they exhibited higher nanodispersed particle sizes of 153 nm, 200 nm, and 282 nm, respectively, compared to the 86.1 nm nanodispersed particle size of formula F2 (Fig. 8). Formula F4 provided the best gastrointestinal protection for Exenatide and had an acceptable particle size. Therefore, HP-β-CD was tested with a basic linoleic acid formula F7 to create formula F8. Fig. 9 shows that formula F8 provided comparable with no statistically significant difference ($p > 0.05$) of total Exenatide gastrointestinal protection (81.6%) to that of formula F7, which provided 83.2%, but with a significant ($p < 0.001$) larger nanodispersed particle size (210 nm).

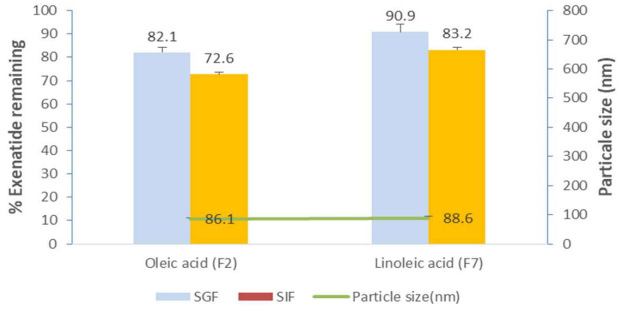

Fig. 7. Influence of using the fatty acid types on the nanodispersed particle size and gastric and intestinal protection are presented as mean ± SD ($n = 4$). Statistical analysis was performed using an unpaired two-tailed Student's t-test ($p < 0.05$)

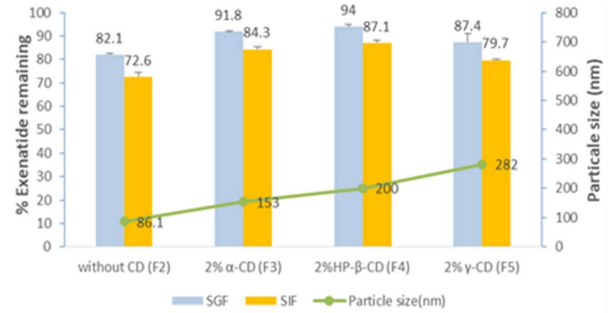

Fig. 8. Influence of addition of different CD types on the nanodispersed particle size and gastric and intestinal protection of the formula of oleic acid are presented as mean ± SD ($n = 4$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test ($p < 0.05$)

6. An in vivo investigation to assess the effectiveness of the experimental approach.

As Formula F4 provided the best gastrointestinal protection for Exenatide with an acceptable particle size, tested in vivo in compared to the marketed SC Byetta®. An unpaired, two-tailed Student's t-test revealed significant differences in plasma Exenatide concentrations and blood glucose levels between SC Byetta® and oral F4 at most evaluated time points ($p < 0.05$). These results confirm the presence of significant pharmacokinetic (PK) and pharmacodynamics (PD) differences between the two formulations. SC Byetta® (10 µg/kg) exhibited rapid absorption, reaching a maximum plasma concentration (C_{max}) of 400 ng/ml at a time (T_{max}) of 1 h, followed by a steep decline. In contrast, oral F4 (100 µg/kg) reached a lower C_{max} of 190 ng/ml at a delayed T_{max} of 3 h, resulting in prolonged plasma exposure (Fig. 10). As shown in Fig. 11, blood glucose mirrored these pharmacokinetic (PK) patterns. Byetta® produced a rapid decline to 90 mg/dl at 1 h, but glucose rebounded by 4–6 h. Oral F4 achieved a similar nadir (91 mg/dl) at 3 h, with sustained lowering up to 8 h. Overall, SC Byetta® provides a fast and high peak exposure, as well as rapid glucose reduction. On the other hand, Oral F4 offers a delayed, yet more sustained, level of drug exposure and glycemic control.

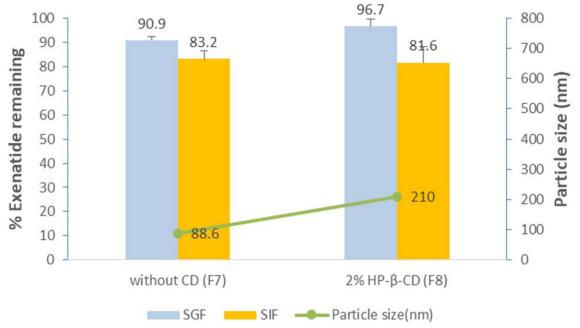

Fig. 9. Influence of addition of HP-β-CD types on the nanodispersed particle size and gastric and intestinal protection of the formula of linoleic acid are presented as mean ± SD ($n = 4$). Statistical analysis was performed using an unpaired two-tailed Student's t-test ($p < 0.05$)

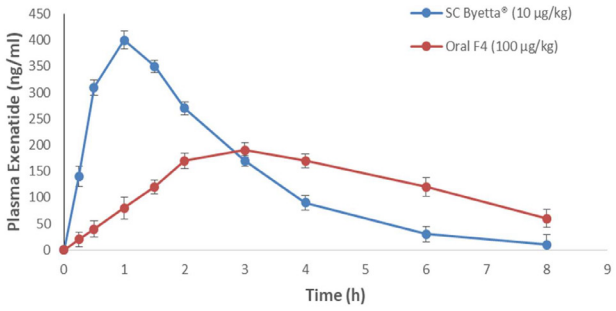

Fig. 10. Simulated Pharmacokinetic (PK) Data (Plasma Exenatide, ng/ml). Data are presented as mean ± SD ($n = 6$). Statistical analysis was performed using an unpaired two-tailed Student's t-test ($p < 0.05$)

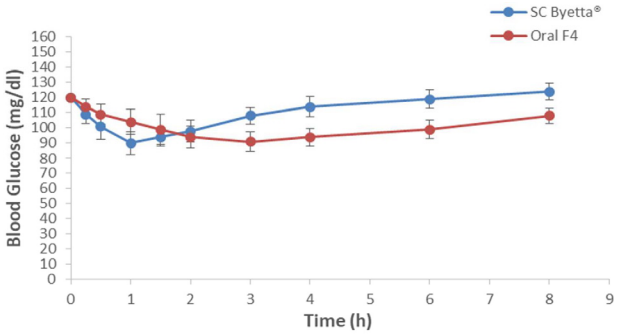

Fig. 11. Simulated Pharmacodynamic (PD) Data (Blood Glucose, mg/dl). Data are presented as mean ± SD ($n = 6$). Statistical analysis was performed using an unpaired two-tailed Student's t-test ($p < 0.05$)

5. Discussion

This study focuses on the development of an oral delivery system for Exenatide. The study proposes the production of an Exenatide–Chitosan complex that encapsulate Exenatide and provide a sustained release of Exenatide over an extended period. The complex will be combined with lipid-based carriers to provide a protective barrier against enzymatic degradation and the acidic conditions of the stomach. The incorporation of Exenatide-chitosan complexes into fatty acids is expected to facilitate the formation of stable nanoparticles. This, in turn, is expected to enable the bypassing of gastric degradation and the improvement of Exenatide small intestinal absorption. This dual protection and absorption-enhancement strategy aim to improve Exenatide oral bioavailability.

Stable PEC is obtained through the electrostatic interaction between cationic chitosan and anionic Exenatide at a 1:1 ratio and a pH of 5.5. The mechanism of PEC formation involves charge neutralization between the cationic chitosan and the anionic Exenatide. The primary amine of chitosan has a pK_a value of ~6.5. Thus, at pH 5.5, most of the amino groups will be protonate, and chitosan will have a positive charge. Exenatide, on the other hand, has an isoelectric point (pI) of 4.86 [7, 8], so it becomes negatively charged at pH 5.5. This ratio was used because at this ratio, the chitosan has sufficiently protonated NH_3^+ groups (cationic), which are essential for electrostatic interaction with Exenatide and subsequent incorporation into the fatty acid oily phase. Additionally, chitosan typically forms an extended random coil in solution [9]. It is expected that the chitosan molecular chain will be more extended at pH 5.5 due to the repulsion of highly protonated amino groups. Consequently, Exenatide has greater accessibility to amine groups without significant steric hindrance. LMW chitosan was used to prepare the PEC_s because LMW chitosan possess high reactivity due to a greater number of amino groups available for interactions with the anionic actives [10].

Selecting suitable fatty acids is critical to developing nano-oily base systems for oral peptide delivery. The oleic acid exhibited higher viscosity (27.64 cP) with a RI of 1.45 (Table 2), favorable for stabilizing nano-emulsions and controlling Exenatide release. The Linoleic acid (24.03 cP, RI 1.46) (Table 2) balanced stability and fluidity, with higher unsaturation enhancing membrane permeability, consistent with improved oral absorption of Exenatide via nanocapsules [11]. These outcomes align with prior studies on oleic acid in self-nanoemulsifying Exendin-4 formulations and the recognized role of medium-chain fatty acids in enhancing solubility and absorption [12, 13]. Collectively, the 2 selected oils are suitable for nano-oily base systems in oral Exenatide delivery, with the choice depending on the desired balance of stability, release, and absorption.

Transparent and stable oily systems (Fig. 1, 2) were achieved could be due to the presence of a cosurfactant, which reduces the bending stress of the interface, allowing the interfacial film to be flexible enough to form the different curvatures required for microemulsion formation [14], or because a system composed of Labrasol® and Plurol oleique® in a 1:1 ratio significantly enhanced the efficiency of Labrasol® and optimized microemulsion formation [15], as well as lower particle size, as reported by [7]. Fatty acids were chosen because they are beneficial in pharmaceutical preparations as penetration enhancers [16].

The spontaneous formation of microemulsions in pseudo-ternary phase diagrams (Fig. 1, 2) is attributed to the presence of surfactants, which are composed of a relatively long hydrophobic organic chain that functions as a “tail” to a polar or ionic head [17].

The size of the dispersed phase particles progressively increased as the percentage of the chitosan solution increased, (Fig. 3,) is aligns with the works [18, 19], who demonstrated that nanoparticle size is significantly impacted by chitosan concentration. They showed a linear relationship, indicating that higher loading concentrations result in larger particle sizes. At low volumes, the flexible polymer chains of the chitosan molecules tend to localize at the interface and intercalate between non-ionic surfactants [20]. As the loading concentration increases, intermolecular hydrogen bonding and electrostatic repulsion promote particle growth. At even higher concentrations, more chitosan molecules entangle with each other, forming larger aggregates [19]. Additionally, fatty acids can self-aggregate or adsorb chitosan chains, resulting in larger particles, as described by [21]. Several additional factors influence this behavior. [22] reported that lower chitosan molar mass allows for better control of particle size and distribution. This is likely due to the reduced viscosity of the internal aqueous phase and the greater disentanglement of polymer chains during processing. The micelle size itself is influenced by the aqueous phase/surfactant weight ratio W [23]. Thus, higher chitosan concentrations demand larger amounts of surfactant to maintain emulsion stability. Both the size and surface charge of emulsions are significantly affected by chitosan concentration, primarily through electrostatic interactions between the positively charged chitosan and the negatively charged fatty acids [24]. In contrast, when PEC was loaded into the oily phase, the dispersed phase particle size first increased up to a certain limit and then decreased. At low to moderate loadings, the increase may be explained by aggregation or partial fusion of PEC particles at the oil–water interface. At higher concentrations, interfacial saturation and stabilization dominate, sufficient NH_3^+ groups in chitosan interact electrostatically with COO^- groups of fatty acids, and the compact PEC nanoparticles act as stabilizers, limiting further aggregation. The number of protonatable amine groups determines polymer solubility, hydrophobicity, and polyelectrolyte complexation ability [25]. The differences in particle size observed among fatty acids can be attributed to their chemical structures, specifically the presence of double bonds. These double bonds introduce bends that affect interfacial packing. The statistically significant differences observed between the diluted chitosan and PEC formulations confirm that PEC systems are su-

perior at maintaining nanoscale particle size at higher levels of fatty acid loading.

The oral Exenatide delivery system exhibited a dual advantage, combining the benefits of nanoencapsulation and the utilization of an oily vehicle. The employment of surfactants proved to be a pivotal element in the development of a transparent system. The system, composed of a mixture of surfactants in a 1:1 ratio, exhibited transparency and underwent rapid separation of the aqueous phase from the oily system. Consequently, a postulation can be formulated that this surfactant mixture may enhance Exenatide stability across diverse gastrointestinal segments [26]. Furthermore, the occurrence of a hydrophobic interaction between the polyelectrolyte complex (PEC) and fatty acids is contingent upon the length of the fatty acid chains, thereby rendering the particles' surface more hydrophobic and increasing the stability of the prepared formula [27]. The interaction between PEC and fatty acids was also found to be governed by an electrostatic mechanism. This mechanism was found to be significantly influenced by the pH level [28], which affected the amalgamation of fatty acids with PEC. At a pH of 5.5, the amino group of chitosan (pK_a = 6.5) is positively charged, and it can interact with the negatively charged carboxyl group of the fatty acid (pK_a = 4.8) [29]. The combined effects of the use of surfactant mixture and the hydrophobic and electrostatic interactions result in a stable and acceptable particle size for the prepared formulas.

The results of the study show a significant difference ($p < 0.05$) between the stirring and vortexing mixing methods and indicate that the vortexing mechanical mixing method employed for the formulation preparation yielded enhanced gastric and intestinal protection and yielding a smaller particle size and is regarded as the optimal approach for the preparation (Fig. 5, 6). The improved gastrointestinal protection observed with vortex-prepared formulations may be due to the enhanced mixing intensity leading to enhanced dispersion capacity and improved structural integrity. Our results are consistent with those reported by Boughanmi *et al.*, who demonstrated that mixing speed and mixing efficiency play an essential role in controlling nanoparticle size, structure, and stability, which directly influence formulation performance [30].

The propensity of Exenatide to aggregate when a magnetic stirrer is employed may be attributable to the exposure of proteins or peptides to stresses such as air contact or interactions with metal surfaces. Such exposure has been shown to induce surface denaturation and subsequent aggregation [31, 32].

Despite a significant difference ($p < 0.05$) in gastrointestinal protection between oleic acid and linoleic acid formulations, they observed good gastric and intestinal protection phenomenon (F2 and F7) (Fig. 7), this may be attributed to the presence of a protracted carbon chain in both entities. However, the minor discrepancy in their resistance to gastrointestinal fluids and nanodispersed particle sizes may be ascribed to the distinct configuration of PEC with each distinctive fatty acid. This variation could be ascribed to the differing number of double bonds present in each entity. The results demonstrate that there was no significant difference between their particle size ($p > 0.05$), which may be due to the interaction and the arrangement between the fatty acid and PEC within the formula.

The α-, HP-β-, and γ-cyclodextrin (CD) types are the most studied and used in industry because they can selectively encapsulate molecules, thereby altering the solubility, stability, and bioavailability of guest compounds [33]. Several researchers have reported that HP-β-CD produces superior results compared to β-CD. Since the aqueous solubility of β-CD is too low for use in formulations, HP-β-CD was used instead [34]. Formulas containing the three types of CD showed a significant increase in gastrointestinal Exenatide. The difference in the total Exenatide gastrointestinal protection values and the nanodispersed particle sizes of formulas F3, F4, and F5, were prepared by adding different types of cyclodextrin (α-CD, HP-β-CD, and γ-CD) to an oily base containing oleic acid formula (F2) (Fig. 8), may be due to variations in the structures and properties of the different types of cyclodextrin. Our results are consistent with study [35], which reported that the incorporation of CD significantly improved gastrointestinal protection and altered particle size, this confirms its role in increasing formulation stability and performance.

Formula F4 provided the best gastrointestinal protection for Exenatide and had an acceptable particle size. This may be due to its optimal cavity diameter of HP-β-CD for guest molecules [34]. Therefore, HP-β-CD was tested with a basic linoleic acid formula F7 to create formula F8. Formula F8 provided comparable total Exenatide gastrointestinal protection to that of formula F7 but with larger nanodispersed particle size (Fig. 9), this may be due to the structure of linoleic acid, which has two double bonds that do not blend well with the structure of HP-β-CD, as the formation and stability of inclusion complexes depend on hydrophobic interactions between the cyclodextrin and gust molecule [36]. Further chemical engineering investigations are required to determine the exact arrangement. The in vivo PK and PD results revealed statistically significant differences between the subcutaneous (SC) Byetta® and oral F4 profiles. These results confirm that the oral F4 formulation produced a decline in blood glucose levels comparable to that of the SC Byetta® formulation, with sustained glucose-lowering effects that lasted up to 8 h. This may be due to the formula's components (oleic acid, chitosan and HP-β-CD). The protective nano-dispersion system (F4) appears to enhance the gastrointestinal stability of Exenatide and increase its bioavailability by improving the PK and PD results. This result is consistent with study [37], who demonstrated that lipid-associated chitosan nanoparticles enhance the absorption and pharmacological activity of oral peptides. Finally, these results suggest that F4 is a promising oral alternative to injectable Exenatide.

Practical relevance. The study results provide a basis for manufacturing an oral Exenatide pharmaceutical dosage form.

Research limitations. The main limitation of the study is the difficulty in handling Exenatide because it is a peptide and highly susceptible to degradation.

Prospects for further research. Further studies on bioavailability, stability and long-term efficacy are necessary.

Recommendation: Further studies on the bioavailability and long-term efficacy of Exenatide are recommended. A further toxicological evaluation is preferred to be conducted in the future studies to confirm the safety of the developed system.

6. Conclusions

Exenatide could be protected from gastric and intestinal enzymes by incorporation into lipid-based formulation. The results indicate that the nano-oily base system of Exenatide was able to withstand the preparation procedure. The vortexing mechanical mixing method is a better method for preparation of the formulas without resulting in destruction of Exenatide. The results indicate that oleic acid and linoleic acid nanodispersed oily based systems showed good gastric and intestinal enzymes protection with suitable nanodispersed particle sizes results. The use of the HP-β-CD improved the gastrointestinal protection. The results suggest that the formula F4 is a promising oral alternative to the paraenteral Exenatide.

Conflict of interest

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

Manuscript has no associated data.

Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies in creating the submitted work.

Authors' contributions

Rana Al-Shaikh Hamed: Formulations preparation, Laboratory experiments, Animal studies, Investigation, Methodology, Validation, Writing – original draft; **Muhammed Hameed Al-Jumaily:** Writing – review & editing, Manuscript organization, Supervision, Funding support.

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