International Journal of Pharmaceutics 536 (2018) 63–72

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Sustained-release multiparticulates for oral delivery of a novel peptidic ghrelin agonist: Formulation design and *in vitro* characterization

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ARTICLE INFO

Keywords:
Pellet
Film coating
Sustained-release
Bioactive peptide
Ghrelin

ABSTRACT

There is an impetus to provide appropriate sustained release oral delivery vehicles to protect biofunctional peptide loads from gastric degradation *in vivo*. This study describes the generation of a high load capacity pellet formulation for sustained release of a freely water-soluble dairy-derived hydrolysate, FHI-2571. The activity of this novel peptidic ghrelin receptor agonist is reported using *in vitro* calcium mobilization assays. Conventional extrusion spheronization was then used to prepare peptide-loaded pellets which were subsequently coated with ethylcellulose (EC) film coats using a fluid bed coating system in bottom spray (Wurster) mode. Aqueous-based EC coating dispersions produced mechanically brittle coats which fractured due to osmotic pressure build-up within pellets in simulated media. In contrast, an ethanolic-based EC coating solution provided robust, near zero-order release in both USP Type 1 and Type 4 dissolution studies. Interestingly, the functionality of aqueous-based EC film coats was restored by first layering pellets with a methacrylic acid copolymer (MA) subcoat, thereby hindering pellet core swelling in acidic media. Broadband Acoustic Resonance Dissolution Spectroscopy (BARDS) was utilised as a complementary technique to confirm the results seen in USP dissolution studies. Retention of activity of the ghrelinergic peptide hydrolysate in the final encapsulated product was confirmed as being greater than 80%. The described pellet formulation is amenable to oral dosing in small animal studies in order to assess *in vivo* efficacy of the whey-derived ghrelinergic hydrolysate. In more general terms, it is also suitable as a delivery vehicle for peptide-based bioactives to special population groups *e.g* paediatric and geriatric.

1. Introduction

Oral peptide delivery remains a bottle-neck in the transition of potentially effective therapeutics from bench to bedside (Brayden and Alonso, 2016). Bioavailability of peptides is consistently poor due to the acidic and enzyme-mediated degradation in gut lumen, leading to loss of efficacy. The rapid degradation of bioactive peptide structures *in vivo* necessitates drug delivery technologies which protect the payload in the gastric compartment and allow for site specific delivery to the small and large intestine (Malik et al., 2007). Various formulation approaches have been adopted to protect peptides from degradation within the gastrointestinal tract and increase oral bioavailability, ranging from standard formulations containing functional excipients, to micro- and nano- based (colloidal) delivery systems (Lakkireddy et al., 2016). However, commercial success in terms of an orally active peptide formulation has been limited to a few niche, high potency peptides which can achieve therapeutic efficacy with limited bioavailability (*i.e.* < 1%) (Aguirre et al., 2016). Micro- and nano-based delivery systems encompass a large proportion of the efforts to translate peptide functionality *in vitro* to the clinical setting. However, various limitations exist to these respective approaches: the former typically involves complex processing steps leading to peptide degradation (Witschi and Doelker, 1998; Yin et al., 2008), while the latter displays poor loading capacity (1–5%), variable release characteristics and limited scalability (Jain et al., 2008; Redhead et al., 2001). Furthermore, stresses during processing, including shear forces, exposure to organic solvents and

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https://doi.org/10.1016/j.ijpharm.2017.11.051
Received 25 August 2017; Received in revised form 29 October 2017; Accepted 22 November 2017
Available online 23 November 2017
0378-5173/

excessive drying time will adversely impact on peptide stability, as well as interactions with hydrophilic/hydrophobic interfaces. There is thus an impetus to develop more suitable oral delivery platforms to enable assessment of *in vivo* efficacy for peptidic compounds showing promise in the *in vitro* setting.

The main aim of this study is the encapsulation of a novel bioactive peptide using a traditional multiparticulate formulation approach. These coated pellets are intended for use pre-clinically to investigate bioactive functionality in rodents. In addition, from a clinical utility perspective, pelletised dosage forms offer numerous advantages such as flexible dosing and ease of administration in special population groups. Conventional formulations such as coated pellets are widely used in the pharmaceutical industry to obtain suitable release profiles for a variety of active pharmaceutical ingredients (API) (Lecomte et al., 2004; McGinity and Felton, 2008) . Millimetre size-range pellets have notable advantages compared to sub-micron and colloidal delivery approaches. A narrower particle size distribution allows for homogeneous film formation and more reproducible release profiles. Higher peptide loading can typically be achieved by inclusion of a pelletisation aid such as microcrystalline cellulose. The process is readily scalable to industrial size, while critically this represents a flexible dosing platform ranging from pre-clinical proof of concept studies, to clinical dosing in special population groups, *i.e.* paediatric and geriatric patients. Fluid bed coating technology holds many advantages for coating peptide-loaded matrix pellets. This is a well-established process that allows for simple and efficient polymer layer deposition and subsequent reliable delayed/sustained drug release, depending on the nature of the functional polymeric coat applied. Furthermore, the processing conditions are mild relative to other methods such as pan coating, while low weight gains achieve reliable, uniform coating. Typically, pellets are fluidized by high flow air, while an atomized coating solution or suspension is pulsed onto the pellets. As liquid coating material is deposited and simultaneously dried, the latent heat of evaporation of solvent means that the microenvironment of each individual pellet is considerably lower than the pre-heated inlet air (El Mafadi et al., 2005; Poncelet et al., 2009).

The therapeutic potential of bioactive peptides for treating many health problems, including appetite-related disorders, is becoming increasingly apparent (Torres-Fuentes et al., 2015). Recent work in rodents has demonstrated the ability of whey protein isolate to reduce the expression of satiating genes in the hypothalamus, thereby increasing energy intake (Nilaweera et al., 2017). This study describes a novel peptidic dairy hydrolysate, FHI-2571, with ghrelin receptor agonist activity. Ghrelin, a 28-amino acid containing peptide, is produced in the stomach and functions as an endogenous appetite-stimulant (Howick et al., 2017; Kojima et al., 1999; Nakazato et al., 2001). The ghrelin receptor has thus been a pharmacological target to reduce appetite in obesity as well as to stimulate food intake in conditions of malnutrition and cachexia (wasting syndrome) (Howick et al., 2017; Schellekens et al., 2010). While the precise site of action of ghrelin is still open to some debate (Howick et al., 2017), the high prevalence of the ghrelin receptor throughout the small and large intestinal mucosa is thought to facilitate communication with appetite centres in the brain via the vagus nerve (Date, 2012), and thus may hold potential as a local therapeutic target (Lakkireddy et al., 2016).

Overall, this study aims to first assess the *in vitro* efficacy of a novel ghrelin receptor agonist, FHI-2571, and investigate a formulation approach to progress this bioactive to *in vivo* studies. To overcome the acidic and proteolytic degradation of this whey-derived hydrolysate in the stomach and upper intestine, we have developed a sustained-release oral delivery system to minimise exposure to gastric acid and intestinal peptidases. *In vitro* release profiles of FHI-2571 in traditional USP dissolution tests, confirmed using BARDS, demonstrate the capability of our formulation approach in achieving prolonged, elevated levels of bioactive throughout the small intestine *in vivo*. Activity assays confirm that the peptide retains good bioactive functionality post-encapsulation.

2. Materials and methods

2.1. Materials

Dairy-derived peptide hydrolysate (FHI-2571) was provided by Food for Health Ireland (see Section 2.2). Methacrylic acid and ethyl acrylate copolymer type C (MA, Acryl-EZE® II) and ethylcellulose (EC) (Ethocel™ Standard 20 Premium) were both purchased from Colorcon Corp., Dartford, Kent, UK, while aqueous pseudo-latex of EC (Surelease® Type B NF) was sourced from Colorcon Corp., Indianapolis, IN, USA. Microcrystalline cellulose (MCC, Avicel® PH-101 NF Ph. Eur.) was purchased from FMC Corp., Little Island, Cork, Ireland. Pharmaceutical grade ethanol 96% (v/v) was procured from Carbon Chemicals Group Ltd., Ringaskiddy, Cork, Ireland. Unless otherwise stated, only deionised water was used in this study. For the Ca^{2+} mobilisation assays, fetal bovine serum (3.3%) was obtained from Sigma-Aldrich, Arklow, Wicklow, F7524. Assay buffer was composed of 1 × Hanks balanced salt solution, HBSS, Gibco™ 14,065,049 (Thermo Fisher Scientific™), containing 20 mM HEPES (Sigma-Aldrich, Arklow, Wicklow, H0887). The endogenous agonist, ghrelin (rat), was supplied by Tocris Bioscience, Avonmouth, Bristol, UK (Cat. No. 1465).

2.2. Generation of FHI-2571

A dairy-peptide hydrolysate was prepared by a method similar to a previously published method (Mukhopadhya et al., 2015). Briefly, bovine milk derived whey protein (80% w/w protein, Carberry Group, Ballineen, Cork, Ireland) was suspended at 10% protein (w/w) in reverse osmosis-treated water and agitated continuously at 50 °C for 1 h in a jacketed tank. The pH was adjusted using a NaOH 4.0 N solution (VWR, Dublin, Ireland). A bacterial food-grade enzyme preparation, was added to the protein solution until 7–12% degree of hydrolysis was achieved. The enzyme was then inactivated by heat-treatment and the resultant hydrolysate solution was dried in a Niro TFD 20 Tall-Form Dryer (GEA, Düsseldorf, Germany).

2.3. Ca^{2+} Mobilisation assay for peptide ghrelin receptor activity pre- and post-encapsulation

Ghrelin receptor mediated changes in intracellular Ca^{2+} mobilisation were monitored on a FLIPR Tetra High-Throughput Cellular Screening System (Molecular Devices Corporation, Sunnyvale, California, USA). Ca^{2+} mobilisation assays were performed according to the manufacturer's instructions and modified from a previously described method (Pastor-Cavada et al., 2016). Human Embryonic Kidney (HEK293A) cells stably transfected with the ghrelin receptor were seeded in black 96-well microtiter plates at a density of 3×10^4 cells/well and maintained overnight at 37 °C in a humidified atmosphere containing 5% CO_2. Twenty-four hours before the experiment, media was replaced with serum-free media containing 1% non-essential amino acids (NEAA). On experimental day cells were incubated with 80 µL of 1 × Ca5 dye in assay buffer (1 × Hanks balanced salt solution – HBSS, supplemented with 20 mM HEPES buffer) according to the manufacturer's protocol (R8141, Molecular Devices Corporation, Sunnyvale, CA). Addition of compound (40 µL/well) was performed by the FLIPR Tetra, and fluorescent readings were taken for 120 s at excitation wavelength of 485 nm and emission wavelength of 525 nm. The relative increase in cytosolic Ca^{2+} was calculated as the difference between maximum and baseline fluorescence and depicted as percentage relative fluorescent units (RFU) normalized to maximum response (100% signal) obtained with 3.3% fetal bovine serum (FBS). Background fluorescence was recorded in cells in assay buffer alone and subtracted from RFUs. All compounds and hydrolysates used in experiments were prepared in assay buffer. FBS (3.3%) and the endogenous agonist