European Journal of Pharmaceutics and Biopharmaceutics 86 (2014) 544–551

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

The role of citric acid in oral peptide and protein formulations: Relationship between calcium chelation and proteolysis inhibition

Søren H. Welling [a,b], František Hubálek [a], Jette Jacobsen [b], David J. Brayden [c], Ulrik L. Rahbek [a], Stephen T. Buckley [a,*]

[a] Diabetes Research Unit, Novo Nordisk A/S, Måløv, Denmark
[b] Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[c] UCD School of Veterinary Medicine and UCD Conway Institute, University College Dublin, Dublin, Ireland

ARTICLE INFO

Article history:
Received 27 August 2013
Accepted in revised form 23 December 2013
Available online 31 December 2013

Keywords:
Oral peptide delivery
Citric acid
Proteolysis inhibition
Chelation
Intestinal drug permeability
Insulin

ABSTRACT

The excipient citric acid (CA) has been reported to improve oral absorption of peptides by different mechanisms. The balance between its related properties of calcium chelation and permeation enhancement compared to a proteolysis inhibition was examined. A predictive model of CA's calcium chelation activity was developed and verified experimentally using an ion-selective electrode. The effects of CA, its salt (citrate, Cit) and the established permeation enhancer, lauroyl carnitine chloride (LCC) were compared by measuring transepithelial electrical resistance (TEER) and permeability of insulin and FD4 across Caco-2 monolayers and rat small intestinal mucosae mounted in Ussing chambers. Proteolytic degradation of insulin was determined in rat luminal extracts across a range of pH values in the presence of CA. CA's capacity to chelate calcium decreased ~10-fold for each pH unit moving from pH 6 to pH 3. CA was an inferior weak permeation enhancer compared to LCC in both *in vitro* models using physiological buffers. At pH 4.5 however, degradation of insulin in rat luminal extracts was significantly inhibited in the presence of 10 mM CA. The capacity of CA to chelate luminal calcium does not occur significantly at the acidic pH values where it effectively inhibits proteolysis, which is its dominant action in oral peptide formulations. On account of insulin's low basal permeability, inclusion of alternative permeation enhancers is likely to be necessary to achieve sufficient oral bioavailability since this is a weak property of CA.

1. Introduction

Development of oral delivery systems for proteins and peptides offers the promise of improved patient compliance compared to conventional parenteral administration. Moreover, in the case of certain protein therapeutics (e.g., insulin), the physiological response elicited may exhibit a pharmacodynamic profile which more closely resembles the natural physiological response. However, delivery of protein therapeutics is severely hindered by poor absorption across the intestinal barrier and extensive degradation by proteolytic enzymes. Thus, to effectively overcome these impediments, a formulation strategy which can modulate both of these processes is necessary to achieve acceptable oral bioavailability with low intra-subject variation.

Although degradation of proteins by gastric enzymes and low pH may be overcome via inclusion of an enteric coating, the approach to minimise proteolytic activity in the small intestine, while simultaneously ensuring efficient release and permeation represents a more significant challenge. In this regard, one such concept extensively explored is that of acidic inhibition of proteolysis. Luminal proteases, such as trypsin and chymotrypsin, exhibit maximum activity at pH $\geqslant$ 6.5 [1,2] i.e., that typically observed in the pH microenvironment of the jejunum and ileum. Via adjustment of the local pH to values corresponding to pH < 6.5, proteolytic activity of enzymes such as chymotrypsin [1], the primary luminal degrading enzyme for insulin [2], can be significantly diminished.

Indeed, acidic inhibition of proteolysis as a strategy for the oral delivery of therapeutic peptides recently gained attention following Tarsa Therapeutics (Philadelphia, PA) successful completion of a phase III trial ('ORACAL') for orally delivered salmon calcitonin (sCT) [3]. Such technology typically comprises of an enteric coated capsule or tablet, which bypasses the stomach unchanged, along

Abbreviations: CA, citric acid; Cit, citrate; DOC, sodium deoxycholate; EDTA, ethylenediaminetetraacetic acid; ISE, ion selective electrode; KH, Krebs–Henseleit; LCC, lauroyl carnitine chloride; sCT, salmon calcitonin; TDC, taurodeoxycholate; TEER, transepithelial electrical resistance.

* Corresponding author. ADME Department, Novo Nordisk A/S, Novo Nordisk Park, 2760 Måløv, Denmark. Tel.: +45 3079 4609.
E-mail address: spby@novonordisk.com (S.T. Buckley).

0939-6411/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejpb.2013.12.017

with a pH-lowering excipient contained in vesicles (e.g., an organic acid such as citric acid). Upon entry into the duodenum with its luminal pH range of between 5 and 6, pH-dependent disintegration of the polymer coating of the dosage form commences, followed by release from the vesicle of both co-localised API and citric acid (CA). Concomitant association of CA maintains a decrease in local pH, thus stabilising the co-released peptide. In this way, it facilitates a reduction in the luminal enzymatic activity, providing a higher concentration gradient of the API over time, which in turn promotes improved absorption and bioavailability [4,5].

Alongside pH-lowering agents, co-administration of an absorption enhancer(s) has generally been regarded as indispensable due to the inherently poor epithelial permeability properties of proteins and peptides [5,6]. Indeed, previous publications exploring this technology have employed LCC, an amphiphilic surfactant [5–7]. However, based upon the recent ORACAL sCT study, where an absorption enhancer was omitted, one may speculate that either the need for co-administration is diminished on account of the proposed permeation enhancing properties of citric acid or citrate (Cit) [3], or that enhancers might not be required for oral sCT where bioavailability of 1–3% is typical for marketed nasal versions of this particular potent molecule [8]. CA and Cit are GRAS excipients and have been widely employed in oral formulations of small molecules. Thus, despite this formulation strategy being comparatively new, a body of literature exists examining the multiple mechanisms by which CA, in its salt form (i.e., tri-sodium citrate) may promote oral absorption. Cit exhibits calcium chelating properties and evidence exists to suggest that it may increase paracellular absorption, by triggering disruption of tight junction complexes via depletion of intracellular calcium [9–11].

In this report, the potential mechanism of action of CA as both an acidic proteolysis inhibitor and calcium chelator/permeation enhancer was addressed and conclusions made as to which might be its dominant action at relevant pH values in the upper small intestine. *In silico* and *in vitro* determination of CA's calcium chelation activity and its capacity to prevent insulin degradation by peptidases across a broad range of pH values were obtained. From this data we assessed whether or not a common pH range existed over which *both* proteolysis inhibition and calcium chelation occurred. Finally, the capacity of CA/Cit to enhance permeability was investigated in Caco-2 monolayers and rat intestinal tissue and compared to that of lauroyl carnitine chloride (LCC), an established amphiphilic permeation enhancer [5–7] previously employed as an additional agent in pH-lowering oral peptide formulations.

2. Materials and methods

2.1. Materials

Caco-2 cells (ATCC-HTB-37) were obtained from American Type Culture Collection (ManassasVA). Cell culture media (Dulbecco's modified essential media (DMEM)) and penicillin/streptomycin were purchased from Lonza (Verviers, Belgium). All other supplements i.e., foetal bovine serum (FBS), HEPES buffer and non-essential amino acids (NEAA) as well as Hanks' balanced salt solution (HBSS) and trypsin were purchased from Gibco (Naerum, Denmark). Corning Transwell® filter inserts (1.12 cm^2 surface area, 0.4 μm pore diameter) were purchased from Fisher Scientific (Slangerup, Denmark). FITC-dextran 4 kDa (FD4) and D-glucose were purchased from Sigma Aldrich (Dublin, Ireland). Bovine serum albumin (BSA) was purchased from Sigma Aldrich (Copenhagen, Denmark). All other reagents were of the highest analytical grade.

The Iso-Insulin ELISA assay kit was purchased from Mercodia (Uppsala, Sweden). Lauroyl-DL-carnitine (LCC) was purchased from Chemos (Regenstauf, Germany). [^{3}H]-mannitol, [^{14}C]-mannitol and Ultima Gold® scintillation fluid were purchased from Perkin Elmer (Waltham, MA). Liquid scintillation counting was carried out using a TopCount C990201 or a TriCarb 2900TR liquid scintillation counter (both Perkin Elmer). Luminescent measurements were performed using a Spectramax® 250 or Gemini® microplate reader (both Molecular Devices, Sunnyvale, CA). Fluorescent measurements were performed on a Tecan® GENios fluorescent microplate reader (Tecan, Durham, NC).

2.2. Cell culture

Caco-2 cells (passage numbers 40–60) were seeded at a density of 2.5×10^5 cells/flask and grown to 70–90% confluence in DMEM (supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and 1% (v/v) NEAA). For transport studies, Caco-2 monolayers were cultured on permeable Transwell® 12 mm diameter inserts with pore sizes of 0.4 μm at a density of 10^5 cells/cm^2 and used after 14–17 days in culture. Cells were cultured at 37 °C and 5% CO_2 atmosphere and the medium was changed every other day.

2.3. Modelling chelation activity of citric acid (CA)

A model to predict free calcium fraction was constructed as described in Supplementary materials. The conditional pKa and citrate (Cit) calcium chelation constant, K, corresponded to previously published values in which similar ionic strengths were applied [12–14]. The model was not corrected retrospectively to take account of the experimentally determined calcium electrode measurements.

2.4. Calcium electrode measurements

A pH-meter (744; Metrohm, Herisau, Switzerland) was fitted with a micro pH-electrode (6.0224.100; Metrohm), a calcium selective electrode (6.0508.110; Metrohm) and an AgCl reference electrode (Dri-Ref-L; World Precision Instruments, Sarasota, FL). All titrations were performed in calcium-free transport media at room temperature (RT). To 20 ml of calcium-free HBSS 300–500 μl CA or Cit (1.5–2 M) was added to yield a final solution of CA/Cit (30 mM) and pH values of 4, 5, 6, and 7.4. The solution was titrated with 40 mM $CaCl_2$ from 0.5 μl to 1310 μl [5×10^{-3}–2.5×10^0] mM $CaCl_2$. Electrical motive force (EMF, mV) and pH were concomitantly monitored during titration. Double standard curves of calcium added to transport media (without CA/Cit), assuming that free calcium concentration was equivalent to total calcium. Titrations were performed at room temperature to improve reproducibility. All solutions were maintained at room temperature for 4 h prior to titration, as the ISE was sensitive to temperature changes. Activity of the ISE was assessed within the pH range of 3–7.4.

2.5. In vitro inhibition of proteolysis

Cit and CA were added to zinc-free transport medium (see "Transepithelial transport studies in Caco-2″; zinc-free) to give Ca/Cit stock solutions a total concentration of 12.5 mM of CA species and a range of pH values (3.5–7.4). Subsequently, enzyme-rich washes were extracted from fasted rat duodenal lumens by rinsing 10 cm fresh duodenum with 10 ml water and instantly freezing the eluate at −80 °C until use. At time point zero, 100 mM recombinant human insulin (Novo Nordisk A/S, Copenhagen, Denmark) was mixed with duodenal extracts and CA stock solutions in a ratio of 1:1:8 respectively, yielding 10 mM insulin and 10 mM CA species. The kinetic study was performed using an autosampler robot