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Research paper

Feasibility, stability and release performance of a time-dependent insulin delivery system intended for oral colon release

Alessandra Maroni, Maria Dorly Del Curto, Mauro Serratoni [1], Lucia Zema, Anastasia Foppoli, Andrea Gazzaniga *, Maria Edvige Sangalli

Istituto di Chimica Farmaceutica e Tossicologica "P. Pratesi", University of Milan, Milan, Italy

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ABSTRACT

The aim of the present work was to evaluate the viability of a time-dependent delivery platform (Chronotopic™) in preparing an insulin-based system intended for oral colon delivery. The main objectives were to assess the influence of the manufacturing process and storage conditions on the protein stability. Insulin-loaded cores were manufactured by direct compression and were subsequently coated with hydroxypropyl methylcellulose (HPMC) in a top-spray fluid bed up to increasing weight gains, namely 20%, 60% and 100%. In order to evaluate the impact the operating conditions may have on the protein integrity, insulin and its main degradation products (*A21*-desamido insulin – *A21*, Other Insulin-Related Compounds – *OIRCs*, and High-Molecular Weight Proteins – *HMWPs*) were assayed on samples collected after each process step by chromatographic methods. Furthermore, long-term (4 °C) and accelerated (25 °C–60% RH) stability studies were carried out on tablet cores and coated systems by assessing insulin, A21, OIRC and HMWP percentages throughout a one-year storage period. In addition, the *in vitro* release behaviour was investigated during the same study period. The overall results indicated that the manufacturing process is not detrimental for insulin integrity and that 4 °C storage temperature alters neither the protein content nor the release performances of the device.

It was therefore concluded that insulin-containing systems intended for oral colon delivery can be obtained by the Chronotopic™ technology.

1. Introduction

Protein and peptide drugs are gaining more and more interest due to the recent advances in molecular biology and DNA-recombinant technology. Although numerous proteins have been approved or are in advanced clinical studies, the development of effective and convenient delivery systems represents a major current challenge. Indeed, due to the intrinsic instability and poor permeation through biological membranes, injectable formulations are by far the most common dosage forms for peptides and proteins. However, their parenteral administration is associated with poor patient compliance especially in those cases of chronic diseases when frequent dosing is required [1]. In this respect, many efforts have been made to devise innovative delivery systems intended for non-parenteral administration routes [2–4]. The oral route is generally considered particularly convenient due to the non-invasive nature and generally high compliance. Still, the gastrointestinal absorption of therapeutic biomacromolecules is hindered by their high-molecular weight, hydrophilic properties and susceptibility to enzymatic inactivation. Among the different strategies proposed to overcome these limitations, colon delivery is attracting increased attention because of the lower presence of digestive enzymes compared to other gastrointestinal districts, long residence time and lower fluid volume that avoids a high dilution of important adjuvants such as enzyme inhibitors and absorption enhancers [5]. In the case of insulin, oral delivery could be of remarkable therapeutic interest as it might mimic the physiological pathway of the hormone thereby reducing the risks of hypoglycaemic events and artificially elevated peripheral blood levels of the protein that may occur when it is injected [3,6]. A recent clinical study in Type II diabetes patients has demonstrated that an enteric coated capsule containing insulin combined with an absorption enhancer was able to elicit measurable protein plasma concentrations thus showing a potential for oral application [7]. Moreover, novel oral insulin-loaded systems intended for colon delivery have yielded promising results in terms of ameliorated pharmacological responses in animal models [8–10].

* Corresponding author. Istituto di Chimica Farmaceutica e Tossicologica "P. Pratesi", University of Milan, via G. Colombo 71, 20133 Milan, Italy. Tel.: +39 02 50324654; fax: +39 02 50324658.
E-mail address: andrea.gazzaniga@unimi.it (A. Gazzaniga).
[1] Present address: Eurand Int., Milan, Italy.

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Hence, a previously developed time-dependent colon delivery system (Chronotopic™) is proposed here in pursuit of oral insulin administration. This system is composed of a drug-loaded core (tablet, capsule, or multiple units), an intermediate hydroxypropyl methylcellulose (HPMC) layer and an outer gastro-resistant film that dissolves after reaching the small intestine region [11–16]. Upon interaction with biological fluids, the hydrophilic polymer starts to swell and erode/dissolve thereby allowing drug release to occur after a programmable lag phase, the duration of which depends on the polymer characteristics and coating level. The external gastro-resistant film prevents the functional HPMC layer from being activated in the stomach, thus overcoming the highly variable inherent residence time. For the application of the hydrophilic polymeric layer, an aqueous spray-coating procedure was set up [12–14]. Methocel® E50, a low-viscosity HPMC grade, was selected as the coating material because of a favourable balance among various key aspects, such as process feasibility, effectiveness in delaying drug liberation, fine modulation of lag time and lack of impact on the release rate. The ability of Methocel® E50-coated systems to selectively deliver small bioactive molecules to the large bowel was demonstrated by pharmacokinetic and γ-scintigraphic human investigations [15,16]. The manufacturing procedure, however, involves various steps that may impair the protein stability, such as, in particular, the aqueous spray-coating process aimed at the application of the HPMC layer. Indeed, the degradation kinetics of insulin is well known to be enhanced by heat and humidity [17–19].

Based on these premises, the aim of the present work was to explore the viability of the Chronotopic™ technology in the preparation of an insulin delivery system and to evaluate its one-year stability under differing storage conditions.

2. Materials and methods

2.1. Materials

Bovine insulin (d_v = 38.2 μm, 28 IU/mg, Sigma–Aldrich, St. Louis, MO, USA), microcrystalline cellulose (Avicel®PH200, FMC Europe, Brussels, Belgium), sodium starch glycolate (Explotab®CLV, Mendell, Patterson, NY, USA), magnesium stearate (Carlo Erba Reagenti, Milan, Italy), hydroxypropyl methylcellulose – HPMC (Methocel®E50, Colorcon, Dartford, UK), and polyethylene glycol (PEG 400, A.C.E.F., Piacenza, Italy).

2.2. Methods

2.2.1. Preparation and characterization of the systems

An exactly weighed amount of bovine insulin was mixed in a mortar with the core excipients. The final w/w % composition of the powder mixture was 2.5 bovine insulin, 92.5 Avicel PH®200, 4.5 Explotab®CLV, and 0.5 Mg stearate. Tableting was carried out at 570 Kgp in a rotary machine (AM-8S, Officine Ronchi, Milano, Italy) with concave punches (diameter 5 mm, curvature radius 5 mm). Tablet cores were checked for weight, height and diameter (digital micrometer calibrated at 0.01 mm, CD-15D Mitutoyo Corporation, Kawasaki, Japan), crushing strength (crushing tester TBH28, Erweka, Heusenstamm, Germany), friability (friabilometer TA3R, Erweka, Heusenstamm, Germany) and disintegration time (USP 25 disintegration apparatus DT3, Sotax AG, Basel, CH). All measurements were performed in 20 replicates except for the disintegration test (n = 6).

Tablets were coated up to increasing weight gains in a top-spray fluid bed (Uniglatt®, Glatt GmbH, Binzen, Germany) equipped with a peristaltic pump (VRX 88, Verder GmbH, Düsseldorf, Germany) connected with a two-fluid atomizer through a neoprene tubing (4 mm diameter). The coating formulation consisting in an aqueous Methocel®E50 (8.0% w/v) and PEG 400 (0.8% w/v) solution was prepared by mixing the latter component with distilled water and subsequently with HPMC powder under magnetic stirring at 80 °C. The dispersion was then stored at 4 °C for 24 h to enable dissolution of the polymer and was heated up to 40 °C before use. The coating process was performed under the following operating conditions: 600 g batch size, 58–60 °C inlet air temperature, 36–38 °C outlet air temperature, 36–38 °C product temperature, 40–45 inlet air flap position, 1.2 mm nozzle port size, 3.0–3.5 bar atomizing pressure, and 5.5–6.0 g/min spray rate.

Samples were collected in process at differing times (6, 15 and 20 h) to obtain systems with approximately 20%, 60% and 100% weight gains with respect to the starting cores. The coated units (n = 20) were characterized in terms of weight, height and diameter. Weight gain was determined by subtracting the mean weight of the cores from that of the coated systems. The mean diameter and height increases were in turn calculated by subtracting the mean diameter and height of cores from those of the final systems. The HPMC coating thickness was determined by halving the mean increases in diameter and height of coated units and by averaging the resulting values according to the equation:

$$CT = (a/2 + b/2)/2 \quad (1)$$

where CT is the coating thickness, a is the increase in diameter and b is the increase in height.

The temperature and humidity conditions under which the formulations were prepared and characterized ranged between 20–25 °C and 40–60% RH, respectively.

2.2.2. Assay of insulin and degradation products

Insulin and its main degradation products, i.e. A21-desamido insulin (A21), High-Molecular Weight Proteins (HMWPs) and Other Insulin-Related Compounds (OIRCs), were assayed in drug powder, powder mixture, tablet core and coated system samples as described below.

An appropriate volume of HCl, 0.01 N, was added to each sample. In the case of coated systems, the units were cut before HCl addition and then shacked to enable a fast core disintegration. The obtained suspensions were filtered through a 0.45 μm GHP membrane (Acrodisc®, Pall Life Sciences, Ann Harbour, MI, USA) prior to high performance liquid chromatography (HPLC) analysis. The HPLC system (Waters, Milford, MA, USA) was composed of a binary pump (Waters®1525), dual lambda UV detector (Waters®2487), autosampler (Waters®717plus) and column oven. Analyses were performed according to the methods reported in the Eur. Ph. 6th Ed. bovine insulin monograph employing the drug powder stored at −20 °C as the reference. The method was validated for precision, limit of detection (LOD), limit of quantification (LOQ), linearity and accuracy. In particular, linearity test was performed on four concentrations using three determinations per concentration, whereas precision and accuracy were measured using at least three determinations on six concentrations.

Insulin, A21 and OIRCs were assayed by Reverse Phase-HPLC using a Discovery Bio Wide Pore column C18, 4.6 × 240 mm, 5 μm, 300 Å (Supelco, Bellefonte, PA, USA) thermostated at 40 °C. Mobile phase A was a sodium sulfate solution, pH = 2.3 and mobile phase B was a mixture of solution A and acetonitrile (55:45). The flow rate was 1.0 mL/min. UV detector was set at 214 nm. In the case of insulin assay, elution was isocratical with 42% phase A and 58% phase B, while for A21 and OIRC the following gradient was applied: 0–30 min 42% A, 58% B; 30–44 min 42% A to 11% A, 58% B to 89% B; 44–50 min 11% A, 89% B.

A21 and OIRC percentages were calculated by applying Eqs. (2) and (3), respectively:

$$\%\text{A21} = \frac{\text{A21 peak area}}{\sum \text{total peak areas}} \times 100 \quad (2)$$

$$\%\text{OIRC} = \frac{\text{OIRC peak area}}{\sum \text{total peak areas}} \times 100 \quad (3)$$

Covalent insulin dimers (CIDs) and polymer insulin complexes (PICs) were assayed by Size Exclusion-HPLC on an insulin HMWP Waters® column, 7.8 × 300 mm (Waters®, Milford, MA, USA). Mobile phase was a mixture of 1.0 g/L L-arginine solution, acetonitrile and glacial acetic acid (65:20:15). The flow rate was 0.5 mL/min. UV detector was set at 276 nm.

The sum of CID and PIC percentages, defined as High-Molecular Weight Proteins (HMWPs), was calculated by applying

$$\%\text{HMWP} = \frac{\text{CID peak area} + \text{PIC peak area}}{\sum \text{total peak areas}} \times 100 \quad (4)$$

2.2.3. Stability studies

Stability studies were carried out according to the EMEA ICH Q1A (R2) 2003 guideline for new drug products intended to be stored in a refrigerator [20]. Insulin drug powder and physical mixture, placed in glass vials and polyethylene/aluminum (PE/Al) blisters, were stored at 4 °C for one year. Moreover, tablet cores and coated systems with 100% weight gain, packaged in PE/Al blisters using a MiniPak blister machine (Minipack-Torre, Bergamo, Italy), were stored at 4 °C and 25 °C–60% RH for one year. At predefined time points (0, 1, 2, 3, 6, 9 and 12 months), insulin, A21, OIRCs and HMWPs were assayed by HPLC as previously described. Each analysis was performed in triplicate.

2.2.4. In vitro release tests

Release tests were performed in a three-position USP 30 disintegration apparatus. This equipment was used to prevent the adhesion of hydrated units to the vessels, as encountered when employing a conventional dissolution apparatus [12]. Each tablet was inserted in a basket-rack assembly so that only one of the six available tubes was occupied. Every basket-rack assembly moved at a rate of 31 cycles/min in a separate vessel containing 900 mL of phosphate buffer, pH = 6.8. Fluid samples were withdrawn at fixed time points and the concentration of insulin was determined by RP-HPLC as previously described.

3. Results and discussion

3.1. Impact of manufacturing on insulin stability

As most therapeutic proteins, insulin is poorly stable under room conditions [17–19]. Both in the solid state and in water solution it is indeed subject to non-enzymatic degradation reactions finally leading to the formation of an A21-desamido derivative (A21) and of High-Molecular Weight Proteins (HMWPs) [18,21]. A21 is still able to elicit a pharmacological response as Asn^{21} residue seems not to be involved in the receptor binding [22]. On the other hand, HMWPs are generally endowed with a low activity, poor solubility and high immunogenicity as compared with the parent drug.

Therefore, it seemed worthy to explore the impact of the main manufacturing steps involved in the preparation of the delivery system proposed to convey the protein to the colon on insulin stability. For this purpose, bovine insulin powder (complying with Eur. Ph. 6th Ed. requirements) was blended with the core formulation excipients, and the resulting mixture underwent compaction. The obtained tablets were subjected to aqueous spray-coating in a top-spray fluid bed under the operating conditions reported in Section 2. Throughout increasing process times, system batches with differing coating levels (approximately 20%, 60%, 100% weight gains) were prepared. All batches exhibited satisfactory physico-technological characteristics (Table 1).

The influence of the aforementioned manufacturing phases on insulin integrity was evaluated by assaying the protein and its main degradation products in samples of powder mixture (I_{mix}), tablet cores (I_{tab}) and systems with increasing HPMC weight gains ($I_{sst}20$, $I_{sst}60$ and $I_{sst}100$). Although several degradation products are described in the literature [21], only A21, Other Insulin-Related Compounds (OIRCs) and HMWPs were considered in the present investigation in accordance with Eur. Ph. 6th Ed. monograph on bovine insulin. The relevant results are reported in Table 2.

Neither mixing nor tableting seems to influence insulin stability. In fact, A21 percentage was not significantly increased ($p < 0.05$) in I_{mix} and I_{tab} samples as compared with insulin bulk powder. Therefore, the drug powder was proven to withstand ambient temperature and humidity conditions as well as mechanical stresses involved in the mixing and tableting steps.

On the other hand, all coated systems showed fluctuations in insulin content and a significant A21 increase ($p < 0.05$) with respect to tablet cores. It can be noticed that although the various systems were exposed to the aqueous spray-coating process for differing time lapses depending on weight gain, the extent of relevant degradation did not grow significantly from $I_{sst}20$ to $I_{sst}100$. The initial film-coating phase, i.e. when insulin-containing cores come into contact with the aqueous polymeric solution, could thus be considered as the one that may impact on the protein stability. Subsequently, while increasing amounts of hydrophilic polymer are deposited on the substrate surface, insulin would not be subject to any further significant degradation. These results suggest that the formation of a HPMC layer might be effective in protecting the protein-containing cores from heat and humidity.

Table 1
Physico-technological characteristics of tablet cores (I_{tab}) and coated systems with 20%, 60% and 100% weight gains ($I_{sst}20$, $I_{sst}60$ and $I_{sst}100$).

	I_{tab}	$I_{sst}20$	$I_{sst}60$	$I_{sst}100$
Height (mm) ± std	4.27 ± 0.02	4.82 ± 0.06	5.19 ± 0.03	5.86 ± 0.04
Diameter (mm) ± std	5.02 ± 0.00	5.53 ± 0.06	5.90 ± 0.05	6.61 ± 0.06
Weight (mg) ± std	83.34 ± 2.55	101.23 ± 1.69	132.08 ± 2.53	163.78 ± 1.84
Weight gain (%)	–	21.47	58.48	96.52
Coating thickness (μm)	–	265.50	451.50	794.25
Friability (%)	<0.1%	–	–	–
Crushing strength (N)	57.6 ± 7.6	–	–	–
Disintegration time (min)	<1	–	–	–

Table 2
Insulin and degradation product percentages in drug powder (I), powder mixture (I_{mix}), tablet cores (I_{tab}) and coated systems with 20%, 60% and 100% weight gains ($I_{sst}20$, $I_{sst}60$ and $I_{sst}100$). The relevant coating process times are also reported.

Sample	Process time (h)	%Insulin ± std	%A21 ± std	%OIRC ± std	%HMWP ± std
I	–	100.10 ± 0.55	0.93 ± 0.01	n.d.	0.17 ± 0.10
I_{mix}	–	99.67 ± 1.22	0.94 ± 0.16	n.d.	0.20 ± 0.02
I_{tab}	–	100.47 ± 2.77	0.92 ± 0.06	n.d.	0.16 ± 0.02
$I_{sst}20$	6	99.10 ± 2.41	1.25 ± 0.05	n.d.	0.30 ± 0.01
$I_{sst}60$	15	98.76 ± 1.46	1.28 ± 0.07	0.03 ± 0.01	0.34 ± 0.07
$I_{sst}100$	20	99.68 ± 1.65	1.29 ± 0.03	0.01 ± 0.02	0.31 ± 0.02

n.d., non-detectable.

3.2. Stability profile

Insulin powder, powder mixture, tablet cores and systems collected at the end of the film-coating process (100% weight gain) were subjected to long-term and accelerated stability studies. These were carried out at 2–8 °C and 25 °C/60% RH, respectively [20].

On account of a possible influence of the primary packaging on protein stability, *I* and I_{mix} samples were packaged in glass vials and PE/Al blisters and were stored at 4 °C. I_{tab} and $I_{sst}100$ were packaged in blisters only, because these would most likely represent their final packaging. Blistered I_{tab} and $I_{sst}100$ were stored at 4 °C and 25 °C/60% RH.

At 0, 1, 2, 3, 6, 9 and 12 months, insulin and its degradation products were assayed in samples collected from *I*, I_{mix}, I_{tab} and $I_{sst}100$. The percentages of insulin and degradation products in *I* and I_{mix} samples stored in glass vials at 4 °C are reported in Tables 3 and 4. Insulin content underwent an approximately 3% decrease after 12 months in the bulk powder. This result is consistent with literature data concerning bovine insulin stability under the same storage conditions [23]. The percentage of degradation products increased slightly over time without exceeding the specified limits. Insulin degradation rate constant (K_d) relevant to *I* and I_{mix} samples stored at 4 °C in glass vials was estimated from the slope of the respective linear regression curves ($R^2 = 0.92$ for *I* and $R^2 = 0.94$ for I_{mix}) obtained by plotting Ln insulin concentration versus time. No significant difference ($p < 0.05$) was highlighted between the degradation rate constants of *I* ($K_d = -0.00176$ months^{-1}, confidence limits = $-0.00229 \div -0.00120$) and I_{mix} ($K_d = -0.00281$ months^{-1}, confidence limits = $-0.00360 \div -0.00203$). It can thus be concluded that mixing with the excipients does not affect the long-term integrity of the protein.

A different stability profile was found for *I* and I_{mix} stored in blisters (Table 5). The protein content in the bulk powder fluctuated around 100% for the first 2 months and decreased to approximately 97% at the end of the study period. On the other hand, a rapid insulin degradation of approximately 20% was observed in the physical mixture after 2 months, followed by a 10-month plateau without major variations. A21, OIRC and HMWP percentages remained within the Eur. Ph. 6th Ed. limits (A21 and OIRC: 3.0%, HMWP: 1.0%) in both sample types (Table 6). Therefore, it was hypothesized that the protein loss in I_{mix} could be related to the formation of degradation compounds other than those mentioned by the Pharmacopoeia, which were consequently not assayed in the present study [21,24].

Table 3
Insulin content in drug powder (*I*) and powder mixture (I_{mix}) stored at 4 °C in glass vials.

Time (months)	*I* %insulin ± std	I_{mix} %insulin ± std
0	100.10 ± 0.55	99.67 ± 1.22
1	99.74 ± 1.03	100.20 ± 3.00
2	99.70 ± 1.32	99.76 ± 2.74
3	99.51 ± 2.56	99.75 ± 4.11
6	99.05 ± 2.10	98.48 ± 3.39
9	98.86 ± 0.45	97.82 ± 1.85
12	97.65 ± 1.23	96.75 ± 2.33

Table 5
Insulin content in drug powder (*I*) and powder mixture (I_{mix}) stored at 4 °C in PE/Al blisters.

Time (months)	*I* %insulin ± std	I_{mix} %insulin ± std
0	100.24 ± 2.80	99.67 ± 1.22
1	99.50 ± 0.71	92.77 ± 1.71
2	99.17 ± 0.77	77.20 ± 2.70
3	98.15 ± 0.79	80.21 ± 1.10
6	97.99 ± 0.70	80.79 ± 1.18
12	97.30 ± 0.51	77.75 ± 2.11

The percentages of insulin and its degradation products found in blistered tablets and coated systems stored at 4 °C are shown in Tables 7 and 8, respectively. Approximately 5% of insulin was degraded within one year in both I_{tab} and $I_{sst}100$. Slightly higher percentages of degradation compounds were observed in $I_{sst}100$. However, they remained below the specified limits. Furthermore, the degradation rate constant of insulin obtained by linear regression fitting ($R^2 = 0.93$ for I_{tab} and $R^2 = 0.95$ for $I_{sst}100$) was not significantly different ($p < 0.05$) in the two samples ($K_d = -0.0048$ months^{-1}, confidence limits = $-0.0064 \div -0.0031$ for I_{tab} and $K_d = -0.0034$ months^{-1}, confidence limits = $-0.0058 \div -0.0010$ for $I_{sst}100$).

Unexpectedly, the amount of insulin in tablet samples after one year was by far less diminished than in the case of the powder mixture, the degradation data of which were also confirmed by additional experiments. In this respect, a possible influence of PE/Al packaging was hypothesized to occur when insulin is in the form of particles thus exposing a large surface area. However, as a comparable decrease in the protein content was not observed in the bulk drug, the degradation phenomena might reasonably be ascribed to insulin interactions with the employed packaging material which would take place when other synergic factors are operating, e.g. in the presence of particular excipients within the formulation.

Stability results obtained from I_{tab} and $I_{sst}100$ stored under 25 °C–60%RH conditions were less satisfactory (Tables 9 and 10). Degradation to a large extent occurred indeed in both tablets and HPMC-coated systems. Within one year, insulin content decreased by 70% and 50% in I_{tab} and $I_{sst}100$, respectively. A corresponding increase in the percentages of all the considered degradation products above the acceptance limits was observed. Surprisingly, the protein degradation rate constant of tablet cores was significantly higher ($p < 0.05$) than that of coated systems ($K_d = -0.10152$ months^{-1}, confidence limits = $-0.11675 \div -0.08629$ for I_{tab}

Table 4
Degradation product percentages in drug powder (*I*) and powder mixture (I_{mix}) stored at 4 °C in glass vials.

Time (months)	*I*			I_{mix}		
	%A21 ± std	%OIRC ± std	%HMWP ± std	%A21 ± std	%OIRC ± std	%HMWP ± std
0	0.93 ± 0.01	n.d.	0.17 ± 0.01	0.94 ± 0.16	n.d.	0.20 ± 0.02
1	0.92 ± 0.02	0.02 ± 0.01	–	0.98 ± 0.03	0.01 ± 0.02	–
2	0.99 ± 0.01	0.05 ± 0.02	–	1.10 ± 0.14	0.09 ± 0.02	–
3	0.99 ± 0.03	0.11 ± 0.04	0.23 ± 0.01	0.95 ± 0.18	0.12 ± 0.01	0.21 ± 0.02
6	1.10 ± 0.05	0.14 ± 0.01	0.24 ± 0.00	1.09 ± 0.04	0.22 ± 0.02	0.25 ± 0.03
9	1.17 ± 0.05	0.33 ± 0.01	0.26 ± 0.01	1.10 ± 0.07	0.36 ± 0.04	0.24 ± 0.02
12	1.19 ± 0.02	0.40 ± 0.00	0.30 ± 0.03	1.11 ± 0.05	0.47 ± 0.01	0.22 ± 0.01

n.d., non-detectable.

Table 6
Degradation product percentages in drug powder (I) and powder mixture (I_{mix}) stored at 4 °C in PE/Al blisters.

Time (months)	I			I_{mix}		
	%A21 ± std	%OIRC ± std	%HMWP ± std	%A21 ± std	%OIRC ± std	%HMWP ± std
0	0.93 ± 0.01	n.d.	0.17 ± 0.01	0.94 ± 0.16	n.d.	0.20 ± 0.02
1	0.95 ± 0.01	1.00 ± 0.73	0.42 ± 0.03	1.05 ± 0.50	0.71 ± 0.80	0.38 ± 0.01
2	1.07 ± 0.15	0.87 ± 0.14	0.47 ± 0.18	1.22 ± 0.02	0.60 ± 0.09	0.43 ± 0.03
3	1.15 ± 0.03	0.59 ± 0.05	0.50 ± 0.04	1.42 ± 0.19	0.58 ± 0.04	0.54 ± 0.06
6	1.48 ± 0.04	0.64 ± 0.09	0.54 ± 0.06	1.83 ± 0.04	0.87 ± 0.03	0.64 ± 0.04
12	1.59 ± 0.20	0.47 ± 0.18	0.44 ± 0.10	1.84 ± 0.14	0.73 ± 0.11	0.53 ± 0.15

n.d., non-detectable.

Table 7
Insulin content in tablet cores (I_{tab}) and coated systems with 100% weight gain ($I_{sst}100$) stored at 4 °C in PE/Al blisters.

Time (months)	I_{tab} % insulin ± std	$I_{sst}100$ % insulin ± std
0	100.47 ± 2.77	98.76 ± 1.46
1	99.38 ± 1.03	98.39 ± 0.73
2	99.20 ± 0.32	98.79 ± 1.70
3	99.07 ± 0.94	98.55 ± 1.55
6	96.25 ± 0.97	96.79 ± 5.40
9	96.43 ± 1.08	96.15 ± 3.00
12	94.79 ± 1.63	95.07 ± 2.10

Table 9
Insulin content in tablet cores (I_{tab}) and coated systems with 100% weight gain ($I_{sst}100$) stored at 25 °C–60% RH in PE/Al blisters.

Time (months)	I_{tab} % insulin ± std	$I_{sst}100$ % insulin ± std
0	100.47 ± 2.77	98.76 ± 1.46
1	95.22 ± 2.71	83.33 ± 0.73
2	71.93 ± 4.59	78.20 ± 0.56
3	69.77 ± 2.37	75.48 ± 1.73
6	50.30 ± 4.53	66.02 ± 6.40
9	39.48 ± 0.48	63.06 ± 1.60
12	29.36 ± 3.74	45.19 ± 4.64

and $K_d = -0.05368$ months^{-1}, confidence limits = $-0.07094 \div -0.03641$ for $I_{sst}100$). It was therefore inferred that the hydrophilic layer might actually be working as an effective barrier protecting the core from heat and humidity, as it was previously hypothesized on the basis of the protein degradation profile observed throughout the spray-coating process.

3.3. In vitro release evaluation

In vitro release profiles of insulin from uncoated tablets and coated systems are provided in Fig. 1. As previously observed with low-molecular weight drugs, the devices showed a prompt and quantitative release of the protein after a programmable lag phase. A linear relationship ($R^2 = 0.999$, $y = 0.2226x - 35.81$) was found between lag time, expressed as the time needed for 10% release ($t_{10\%}$), and the HPMC layer thickness, thus confirming the possibility of modulating the onset of release by varying the amount of hydrophilic polymer applied on the drug-containing core [11,12,15].

After the appropriate storage conditions were assessed by studying the chemical stability of the protein, the overall physical stability of delivery systems ($I_{sst}100$) kept at 4 °C was investigated throughout one year. For this purpose, *in vitro* release tests were repeated after 3, 6 and 12 months. The obtained lag times were shown not to vary significantly ($p < 0.05$) within the period considered. $t_{10\%}$ values at 0, 3, 6, 12 months were 140.3 ± 15.3, 138.7 ± 17.6, 147.0 ± 26.5, 135.3 ± 12.6 min, respectively. Moreover, the *in vitro* release behaviour at each time point was unchanged with respect to both phase duration and release kinetics. The maintenance of the typical release pattern over time pointed out the physical stability of the formulation under investigation.

Based on the outcome of all the stability studies in terms of insulin content, degradation product percentages and release performances, it was possible to confirm that 4 °C storage would be appropriate for insulin delivery systems prepared by the Chronotopic™ technology.

4. Conclusions

An oral insulin delivery system intended for colonic release was prepared by the Chronotopic™ technology. The influence of the involved manufacturing steps on protein integrity and the stability profile under differing storage (4 °C and 25 °C–60% RH) and packaging (glass vials and PE/Al blisters) conditions were evaluated.

None of the manufacturing operations, namely mixing, tableting and aqueous spray-coating, was shown to have a major impact on insulin content and the formation of the main protein degradation compounds. In particular, no significant increase in deamidated insulin percentage was observed in physical mixture and tablet core samples with respect to the drug bulk powder. On the other hand, the percentage of A21 augmented significantly ($p < 0.05$) in

Table 8
Degradation product percentages in tablet cores (I_{tab}) and coated systems with 100% weight gain ($I_{sst}100$) stored at 4 °C in PE/Al blisters.

Time (months)	I_{tab}			$I_{sst}100$		
	%A21 ± std	%OIRC ± std	%HMWP ± std	%A21 ± std	%OIRC ± std	%HMWP ± std
0	0.92 ± 0.06	n.d.	0.16 ± 0.02	1.28 ± 0.07	0.03 ± 0.01	0.34 ± 0.07
1	0.96 ± 0.08	0.02 ± 0.00	–	1.29 ± 0.11	0.06 ± 0.02	–
2	1.00 ± 0.12	0.09 ± 0.03	–	1.46 ± 0.18	0.13 ± 0.00	–
3	1.10 ± 0.06	0.15 ± 0.02	0.20 ± 0.03	1.55 ± 0.13	0.26 ± 0.01	0.51 ± 0.01
6	1.12 ± 0.12	0.26 ± 0.03	0.25 ± 0.01	1.69 ± 0.52	0.41 ± 0.02	0.69 ± 0.03
9	1.15 ± 0.20	0.37 ± 0.01	0.28 ± 0.09	1.69 ± 0.10	0.59 ± 0.03	0.79 ± 0.04
12	1.23 ± 0.11	0.46 ± 0.02	0.33 ± 0.05	1.71 ± 0.02	0.71 ± 0.01	0.92 ± 0.03

n.d., non-detectable.

Table 10
Degradation product percentages in tablet cores (I_{tab}) and coated systems with 100% weight gain ($I_{sst}100$) stored at 25 °C–60% RH in PE/Al blisters.

Time (months)	I_{tab}			$I_{sst}100$		
	%A21 ± std	%OIRC ± std	%HMWP ± std	%A21 ± std	%OIRCstd	%HMWP ± std
0	0.92 ± 0.06	n.d.	0.16 ± 0.02	1.28 ± 0.07	0.03 ± 0.01	0.34 ± 0.07
1	1.51 ± 0.11	0.06 ± 0.02	–	1.84 ± 0.16	0.05 ± 0.03	–
2	1.54 ± 0.03	0.12 ± 0.03	–	–	–	–
3	1.59 ± 0.16	0.29 ± 0.05	1.87 ± 0.03	2.07 ± 0.24	0.33 ± 0.06	1.99 ± 0.05
6	2.00 ± 0.10	0.39 ± 0.01	2.45 ± 0.03	2.43 ± 0.34	0.44 ± 0.05	3.79 ± 0.10
9	2.06 ± 0.12	0.51 ± 0.06	3.01 ± 0.05	2.45 ± 0.14	0.60 ± 0.06	6.41 ± 0.06
12	2.22 ± 0.18	0.74 ± 0.08	3.34 ± 0.04	2.90 ± 0.42	0.81 ± 0.02	8.68 ± 1.01

n.d., non-detectable.

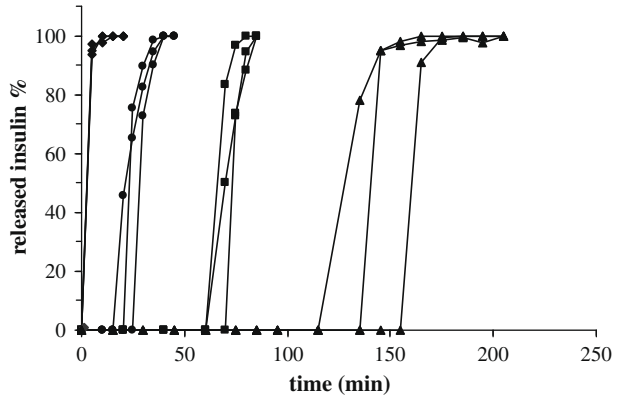

Fig. 1. *In vitro* individual release profiles of I_{tab} (♦), $I_{sst}20$ (●), $I_{sst}60$ (■) and $I_{sst}100$ (▲).

coated systems, even though the relevant pharmacopoeial limits were never exceeded. When comparing units collected at the beginning of the coating process ($I_{sst}20$) and units provided with higher coating levels ($I_{sst}60$ and $I_{sst}100$), however, no further increase was found in the percentages of A21, OIRCs and HMWPs, thus suggesting that only the starting phases of the film-coating process, when insulin cores are in direct contact with the aqueous polymeric solution, may affect the protein integrity. These results may indicate that any further deposition of hydrophilic polymer possibly needed to achieve the desired lag phase would not cause protein damage.

Long-term and accelerated stability studies showed that the temperature of 4 °C would represent the most suitable storage condition for the proposed insulin-containing devices. Indeed, the percentages of the protein and its degradation products thoroughly complied with Eur. Ph. 6th Ed. requirements, and the typical *in vitro* pulsatile release performances of coated systems were unchanged after one-year storage. By using differing packaging configurations, it turned out that under particular circumstances, the primary container may influence insulin integrity, as already highlighted in the case of liquid formulations. The described insulin delivery devices were demonstrated to possess feasibility, stability, physico-technological and *in vitro* release characteristics in full accordance with those of previously developed systems containing low-molecular weight active ingredients, thus indicating that the Chronotopic™ technology might advantageously be exploited to pursue an oral colon delivery of this protein, with possible broader applications to other peptidic molecules.

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