In Vitro and *In Vivo Evaluation* of an Oral Multiple-Unit Formulation for Colonic Delivery of Insulin

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Abstract

A multiple-unit formulation for time-dependent colonic release of insulin was obtained by coating insulin and sodium glycocholate immediate-release minitablets with: i) Methocel® E50, a low-viscosity hydroxypropyl methylcellulose (inner coating); ii) 5:1 w/w Eudragit® NE/Explotab® V17, a mixture of a neutral polymethacrylate with a pore-forming superdisintegrant (intermediate coating); iii) Aqoat® AS, enteric-soluble hydroxypropyl methylcellulose acetate succinate (outer coating). Sodium glycocholate was added as a permeation enhancer while the inner, intermediate and outer coatings were aimed, respectively, at delaying the onset of release through swelling/erosion processes, extending the duration of the lag phase by slowing down water penetration into the underlying functional layer, and overcoming variable gastric residence time. *In vitro* studies showed that neither insulin nor sodium glycocholate were released from the three-layer system during 2 h of testing in 0.1 N HCl, while complete release of the protein and of the enhancer occurred in phosphate buffer, pH 6.8, after consistent lag phases. No significant changes were noticed in the release profiles following twelve-month storage at 4 °C. Oral administration of the novel formulation to diabetic rats elicited a peak in the plasma insulin concentrations after 6 h, which was associated with a sharp decrease in the glycemic levels. The relative bioavailability and pharmacological availability of such a formulation, as determined *vs* the uncoated tablets, were 2.2 and 10.3, respectively. Based on these results, the three-layer system presented was considered a potentially interesting tool for oral colonic delivery of insulin and adjuvant compounds.

Keywords

Oral drug delivery; colon delivery; pulsatile release; oral peptide delivery; insulin; swellable/erodible coating.

Introduction

Colon delivery is under extensive investigation as a promising approach to improve the oral bioavailability of peptide and protein drugs [1-3]. Although the large bowel fails to be ideally suited for absorption, it may indeed offer a number of advantages over the small intestine, including prolonged transit time, low levels of peptidases and good responsiveness to permeation enhancers [4-6]. A swellable/erodible time-dependent colon delivery platform (Chronotopic™) based on low-viscosity hydroxypropyl methylcellulose (HPMC) coating was demonstrated to provide the pursued *in vitro* and *in vivo* release of low molecular weight drugs [7,8]. Accordingly, this system was proposed for colonic release of insulin combined with selected adjuvants, such as a protease inhibitor and an absorption enhancer [9-11]. For this purpose, the influence of all the involved manufacturing steps on the protein integrity was explored, thereby ruling out the occurrence of any significant degradation during the preparation of the delivery system. More recently, the design of such a system, originally presented in single-unit configurations, was modified to give multiple-unit dosage forms on account of the improved consistency of gastrointestinal transit and drug absorption profiles [12]. The thickness of the HPMC layer required to provide *in vivo* lag phases of suitable duration would indeed clash with size requirements of multiple units [13]. Thus, in order to improve the relevant efficiency in delaying the drug release, HPMC-coated minitablet cores were further coated with a mixture of neutral polymethacrylate Eudragit® NE and superdisintegrant sodium starch glycolate, the latter acting as a pore former [14-16]. Such a film was intended to slow down the penetration of water into the underlying HPMC coating. This would extend the delay time prior to drug release while preserving the typical pulsatile release pattern of the original delivery system. Two-layer formulations having inner 250 µm HPMC layer and outer 20-30 µm Eudragit® NE film containing 20% (on the dry polymethacrylate) of sodium starch glycolate were shown *in vitro* to yield programmable lag phases followed by prompt release, which resulted in the desired *in vivo* behavior. Moreover, they were physically stable over a three-year period when stored under ambient conditions.

On the basis of such premises, the present work was undertaken to evaluate this novel multiple-unit formulation as a possible time-dependent colon delivery system for insulin and permeation enhancer sodium glycocholate.

Materials and Methods

Bovine insulin and streptozotocin were obtained from Sigma-Aldrich (St. Louis, Missouri, US). Hydroxypropyl methylcellulose (HPMC, Methocel® E50) was obtained from Colorcon (Gallarate, Italy) and microcrystalline cellulose (Avicel® PH200) from FMC (Brussels, Belgium). Hydroxypropyl methyl cellulose acetate succinate (HPMCAS, Aqoat® AS LG, Shin-Etsu, Tokyo, Japan) and poly(ethylacrylate, methylmethacrylate) (2:1 monomer molar ratio) 30% aqueous dispersion (Eudragit® NE 30 D, Evonik Röhm, Darmstadt, Germany) were kind gifts of Seppic (Milan, Italy) and Rofarma (Gaggiano, Italy), respectively. Magnesium stearate was purchased from Carlo Erba Reagenti (Milan, Italy), microcrystalline cellulose (Avicel® PH200) from FMC (Brussels, Belgium), polyethylene glycol (PEG 400) from ACEF (Fiorenzuola D'Arda, Italy), sodium glycocholate (NaGly) from Tokyo Chemical Industry (Tokyo, Japan) and sodium starch glycolate (Explotab® V17 and Explotab® CLV) from JRS Italia (Castenedolo, Italy).

Preparation of minitablet cores

Convex minitablet cores were prepared by processing 4.0% bovine insulin, 51.0% microcrystalline cellulose, 40% sodium glycocholate, 4.5% sodium starch glycolate and 0.5% magnesium stearate powder mixture by a rotary machine (AM-8S, Officine Ronchi, Milan, Italy; die 2.5 mm diameter, 3 mm curvature radius). The cores were checked for weight (n=20), height (digital micrometer Absolute, Mitutoyo, Kawasaki, Japan; n=20), crushing strength (crushing tester TBH28, Erweka, Heusenstamm, Germany; n=10) and disintegration time (USP 38 disintegration apparatus DT3, Sotax, Basel, Switzerland; n=6). The weight, height, crushing strength and the disintegration time of minitablet cores were 12.0±0.4 mg, 2.2±0.1 mm, 42.3±9.9 N and <1 min, respectively.

Coating of minitablet cores

Minitablets were first coated by a rotary fluid bed (GPCG 1.1, Glatt, Binzen, Germany) with an aqueous solution of Methocel® E50 (8.0% w/V) and PEG 400 (0.8% w/V). The resulting HPMC-coated insulin minitablets (one-layer) were then coated by a bottom-spray fluid bed with diluted (1:1) Eudragit® NE 30 D containing 20% w/w (on dry polymer) of Explotab® V17 as previously described [14,15]. The two-layer formulation was finally enteric coated with a hydro-alcoholic solution of Aqoat® AS (6% w/w in 3:1 w/w ethanol:water) by a ventilated coating pan (GS, Osteria Grande, Italy) under the following operating conditions: 150 g batch size; 60 °C inlet air temperature; 32–34 °C product temperature; 0.75 and 0.50 bar nebulizing and pattern pressure, respectively; 8 g/min spray rate; 30 rpm rotating speed; 11-12 cm nozzle – product distance. The three-layer formulation was finally cured at 40°C in an oven for 30 min. The Methocel® E50 /PEG 400, Eudragit® NE/Explotab® V17 and Aqoat® AS coating levels were determined by subtracting the weight of units obtained at each coating step from that of the starting units (n=20), i.e. minitablets, one-layer systems or two-layer systems, and expressed as mg of solid applied per cm^2. The surface and cross-section of the coated units were analyzed by scanning electron microscopy (SEM, Sigma, Zeiss, Oberkochen, Germany; gold sputtering 10 nm).

***In vitro* release studies**

In vitro release studies (n=6) were carried out by means of an adapted three-position disintegration testing apparatus. This was selected in order to overcome adhesion of the hydrated HPMC coating to the vessels of a paddle dissolution apparatus [17]. Each unit, either a minitablet or a coated system, was inserted into a basket-rack assembly so that only one of the 6 available tubes was filled. During the test, every basket-rack assembly moved at a rate of 31 cycles/min in a separate vessel containing 160 mL of appropriate medium. A reduced volume of fluid was used so that insulin and sodium glycocholate released from each single uncoated or coated minitablet could be assayed.

Sink conditions were maintained throughout the whole test. Non-gastroresistant units were tested in phosphate buffer, pH 6.8, while enteric-coated ones were tested in 0.1 N HCl for 2 h and then in the phosphate buffer. The medium temperature of was set at 37±1 °C. Fluid samples of 0.5 mL were withdrawn at scheduled time points, and bovine insulin and sodium glycocholate were assayed by RP-HPLC as previously described [10]**.** Quantitation of insulin and sodium glycocholate was carried out using four-point calibration curves (R^2 >0.99). Asn^{21} desamido insulin (A21) was not detected throughout the test. The release study (n=3) of final three-layer systems stored in closed glass vials at 4 °C was repeated after 3 and 12 months.

In vitro lag time was expressed as the time required for 10% drug release in phosphate buffer, pH 6.8 ($t_{10\%}$). $t_{10\%}$ was calculated by linear interpolation of the experimental data immediately before and after this release percentage.

***In vivo* studies**

Male Sprague Dowley rats, fed *ad libitum* and handled in accordance with the provisions of the European Economic Community Council Directive 86/209 (recognized and adopted by the Italian Government with the approval decree D.M. No. 230/95-B) and the NIH publication No. 85-23, revised in 1985, were subcutaneously treated with 65 mg/kg of streptozotocin. Animals with glucose levels of 400-500 mg/dL were divided into 3 groups (6 animals/group) and each received, via oral administration, 1 uncoated minitablet (Group 1), 1 three-layer coated system (Group 2) or 0.4 mg insulin in solution (Group 3**).** At scheduled time points, 100 µL of blood was collected from the tail vein and centrifuged. Plasma samples (10 µL) were diluted with 10 mM phosphate buffer, 0.15 M NaCl, pH 7.2, and glucose concentration was estimated by Trinder Kit (Sigma-Aldrich, St. Louis, Missouri, US) using a calibration curve obtained from standard glucose solutions. Insulin was assayed in plasma by ELISA using a human insulin-specific ELISA kit (Sigma-Aldrich, St. Louis, Missouri, US). Plasma samples of 10 µL were diluted to 100 µL with phosphate buffered saline before analysis.

Glucose and insulin concentration data were processed by using Kinetica Software (ThermoScientific, Rodano, Italy). Pharmacological availability was calculated as the area above the glucose concentration *vs* time curve considering the initial glucose level as the baseline point ($AAC_{0\rightarrow 50\ h}$). The protein bioavailability was calculated as the area under the insulin concentration *vs* time curve ($AUC_{0\rightarrow 50\ h}$). The relative pharmacological availability (PA_{rel}) and relative bioavailability (BA_{rel}) of the three-layer system (S) *vs* the minitablet (M) were also calculated as follows [18]:

$PA_{rel} = [AAC_S] / [AAC_M]$

$BA_{rel} = [AUC_S] / [AUC_M]$

In vivo lag time (t_{lag}) was calculated from the insulin concentration *vs* time curve as the time to 5% of $AUC_{0\rightarrow 50\ h}$.

Statistical analysis

Statistical analysis of *in vitro* and *in vivo* data was performed by two-group two-tail unpaired t-Student test accounting for heteroscedasticity. The differences were considered significant with $p < 0.05$.

Results and Discussion

The three-layer system depicted in Figure 1 was designed to yield release of insulin into the colon according to a time-dependent approach [1]. Such an approach relies on the relative consistency of small intestinal transit time (SITT) of dosage forms, which was shown to poorly be affected by the characteristics of the administered units and by the fasted/fed state of the subjects [19,20]. Hence, time-dependent colon delivery systems are generally devised so as to maintain integrity during unpredictable gastric residence and then start a lag phase intended to cover the entire SITT before releasing their drug load.

According to this formulation strategy, minitablet cores containing the protein along with sodium glycocholate were coated with low-viscosity HPMC (inner erodible layer), Eudragit® NE/Explotab® V17 (intermediate permeable layer) and HPMCAS (outer gastroresistant layer).The intermediate layer was intended to prolong the duration of the lag phase prior to the protein release as imparted by the underlying erodible layer. The outer film was meant to overcome unpredictable gastric residence that would hinder timely release of insulin into the colon.

Sodium glycocholate was incorporated into the core as an absorption enhancer. According to previous studies, this "active" excipient was selected because of its compatibility with the protein in the solid state and was demonstrated to effectively promote insulin permeation through the intestinal epithelium [10,21]. Sodium glycocholate was also found to inhibit proteases, which would be beneficial to insulin stability [22-25]. The gastrointestinal tolerability was another important feature in the permeation enhancer selection, as it is a requisite for the development of safe oral pharmaceutical products [21,26]. In the case of insulin delivery, this would especially be critical in view of the chronic use of this drug.

All spray-coating steps were accomplished without major technical problems. Coated units obtained at each step met pre-set requirements for weight variability (RSD <6%). The HPMC, Eudragit® NE/Explotab® V17 and HPMCAS coatings resulted in 30.6, 1.9 and 7.8 mg/cm^2 of each polymer applied, respectively, which corresponded to nominal layer thicknesses of 250, 20 and 100 µm. SEM analysis performed on the cross-sectioned three-layer systems showed continuous overlapping layers also at the tablet edges (Figure 2).

In vitro studies were performed to evaluate the effect of the HPMC and Eudragit® NE/Explotab® V17 coats on the release of insulin and sodium glycocholate.

The profiles reported in Figures 3a and 4a show that the protein and the adjuvant were rapidly released from the minitablet cores. The HPMC layer markedly delayed the onset of insulin and sodium glycocholate release, as pointed out by Figures 3b and 4b. The Eudragit® NE/Explotab® V17 coating enhanced the delaying effect of the HPMC layer, resulting in almost two-fold duration

of the lag phase in the case of the protein (Figures 3c and 4c). The release of sodium glycocholate started generally earlier than that of insulin. This behavior could be ascribed to the greater solubility of sodium glycocholate, at least of 2 orders of magnitude, and its lower molecular weight, which may favor the relevant diffusion through the coatings during swelling. However, either a concurrent or slightly earlier release of the adjuvant might be suitable *in vivo* for enhancing permeation of the protein through the enteric mucosa [27-29]. The release rate of both compounds, and particularly of insulin, was decreased in the presence of the Eudragit® NE/Explotab® V17 film.

The lag time values ($t_{10\%}$) of insulin and sodium glycocholate from each formulation, collected in Table I, were fairly reproducible.

After 2 h of testing in the acidic fluid, HPMCAS-coated units did not release either the protein or the permeation enhancer (Figure 5). This result confirmed the proper deposition of the enteric-soluble polymer onto the substrate and, according to compendial requirements, would reflect in the needed protection of the delivery system during gastric residence. On the other hand, the gastroresistant coating was proved not to significantly delay the onset or affect the rate of release of insulin and sodium glycocholate at pH 6.8, thus pointing out rapid dissolution of HPMCAS under intestinal testing conditions and no alteration in the performance of the functional underlying layers (Figure 5, Table I).

The release profiles obtained after 3 and 12 months of storage of the three-layer system at 4 °C showed minor, non-significant differences in terms of release onset as compared with the formulations tested at t=0 (Figure 6, Table I). However, a decreased release rate was observed after 12 months. In this respect, a more extensive investigation into the curing conditions could help understand and overcome possible ageing phenomena.

Overall, the *in vitro* performance, and in particular the duration of the lag phase observed under simulated intestinal pH conditions, was deemed potentially suitable for colonic release according to *in vivo* results previously collected from analogous delivery systems [16].

In vivo studies were comparatively performed by oral administration of the three-layer system, uncoated minitablet and insulin in solution to diabetic rats. Figures 7 and 8 report the insulin and glucose concentration profiles in the blood over time.

When dosed as a solution, insulin did not elicit any decrease in glycemia and, in the bloodstream, only fluctuations in a relatively narrow range around its initial concentration were observed. The uncoated minitablets yielded a slight increase in the insulin blood levels. A negligible decrease in the glucose concentration was seen within 3 h from administration but the hyperglycemic levels were rapidly restored. In contrast, the three-layer system brought about a sharp rise in the insulin concentration and decrease in the glucose levels. Statistical analysis of insulin concentration vs. time profiles pointed out significant differences in the $AUC_{0\rightarrow 50\ h}$ obtained from the three-layer formulation against both the uncoated minitablet and the solution. The bioavailability of insulin from the three-layer system, the uncoated minitablet and the solution was 98.1, 44.5 and 17.7 $\mu g_{*}mL^{-1}{}_{*}h$, respectively. Analogously, the $AAC_{0\rightarrow 50\ h}$ from the glucose concentration vs. time profiles of the three-layer system was significantly greater as compared with the uncoated minitablet and the solution. The relative insulin bioavailability (BA_{rel}) of the three-layer system, obtained as the $AUC_{0\rightarrow 50\ h}$ ratio *vs* the uncoated minitablet, was 2.2, while the relative pharmacological availability (PA_{rel}), calculated as the $AAC_{0\rightarrow 50\ h}$ ratio between the two formulations, was 10.3.

The maximal protein concentration (C_{max} 3.9 ng/mL) and glycemia reduction (about 70%) were obtained after 6 h from administration. Between 5 h and 50 h post-dose, the concentrations of insulin provided by the three-layer system were markedly higher than those detected with the uncoated minitablet and the solution. Lag time, expressed as the time to 5% of $AUC_{0\rightarrow 50h}$, was of approximately 5 h. As expected based on previous data, the system was proved to delay the appearance of insulin in the bloodstream for a considerably more extended period of time with respect to the *in vitro* lag phase [15,16]. Besides, the rate of protein release into the gastrointestinal tract was such as to yield a prompt pharmacodynamic response at the end of lag time.

Overall, the above-discussed results indicate that, when administering the proposed three-layer system, insulin would rapidly and effectively be absorbed after a lag phase. This delay may have allowed distal intestinal release and permeation of the protein. From literature data on gastrointestinal transit in rats, it could be inferred that insulin might have been absorbed while the delivery system was located in the ileo-colonic region [30-32]. Such findings seem to confirm that the terminal small bowel and large intestine would represent a viable peptide absorption site, at least with reference to the animal model in use.

Conclusions

An oral multiple-unit formulation for time-dependent colonic release of insulin was proposed. The system comprises an immediate-release minitablet core containing the protein and sodium glycocholate as a permeation enhancer, a swellable/erodible internal layer, a flexible and increasingly permeable intermediate film and an outermost enteric coating.

The desired performance was obtained *in vitro*. The drug and the enhancer were not released during the acid stage of the test, and a prompt as well as complete liberation of both compounds occurred, after reproducible delay phases, under simulated intestinal pH conditions. Twelve-month storage at 4 °C did not result in significant changes in the duration of lag phases.

Administered to diabetic rats, the three-layer system described gave rise to a peak in plasma insulin concentration and a fall in the glucose level approximately 6 h after dosing. The bioavailability of the protein from the three-layer system was more than two-fold as compared with the relevant minitablet core, and pharmacological availability was ten-fold higher.

Based on the outcome of this study, and on the potential scalability of the proposed delivery technology, the latter may represent a viable strategy to improve the oral bioavailability of a peptide drug.

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Figure Captions

Figure 1. Outline of the three-layer system: (a) drug-containing minitablet core; (b) HMPC layer; (c) Eudragit® NE/Explotab® V17 film; (d) enteric coating.

Figure 2. SEM photomicrographs of a cross-sectioned three-layer system: (I) edge (magnification 500x) and (II) side (magnification 1000x) views. (a) minitablet core; (b) HMPC layer; (c) Eudragit® NE/Explotab® V17 film; (d) enteric coating.

Figure 3. Individual release profiles of insulin from (a) uncoated minitablets, (b) HPMC-coated (one-layer) systems and (c) HPMC and Eudragit® NE/Explotab® V17-coated (two-layer) systems.

Figure 4. Individual release profiles of sodium glycocholate from (a) uncoated minitablets, (b) HPMC-coated (one-layer) systems and (c) HPMC and Eudragit® NE/Explotab® V17-coated (two layer) systems.

Figure 5. Individual release profiles of insulin and sodium glycocholate from three-layer systems in HCl 0.1 M and phosphate buffer, pH 6.8.

Figure 6. Individual release profiles of insulin and sodium glycocholate from three-layer systems after (a) 3 months and (b) 12 months of storage at 4 °C.

375

Figure 7. Plasma insulin concentration vs. time profiles in diabetic rats following oral administration of three-layer systems, uncoated minitablets or insulin in solution (bars indicate standard deviation).

380 Figure 8. Plasma glucose concentration vs. time profiles in diabetic rats following oral administration of three-layer systems, uncoated minitablets or insulin in solution (bars indicate standard deviation).

385 Table I. *In vitro* lag time of insulin and sodium glycocholate from the formulations under investigation.

	$t_{10\%}$ ± STD (min)	
Formulation	***Insulin***	***Sodium glycocholate***
One-layer system	35.0 ± 1.9	28.7 ± 0.5
Two-layer system	61.2 ± 2.7	45.4 ± 2.8
Three-layer system	64.4 ± 6.9	52.0 ± 6.3
Three-layer system (3 months)	73.7 ± 3.8	60.1 ± 2.5
Three-layer system (12 months)	70.6 ± 7.0	57.3 ± 2.8

Figure(s)

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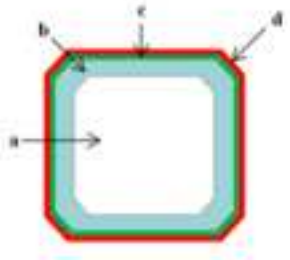

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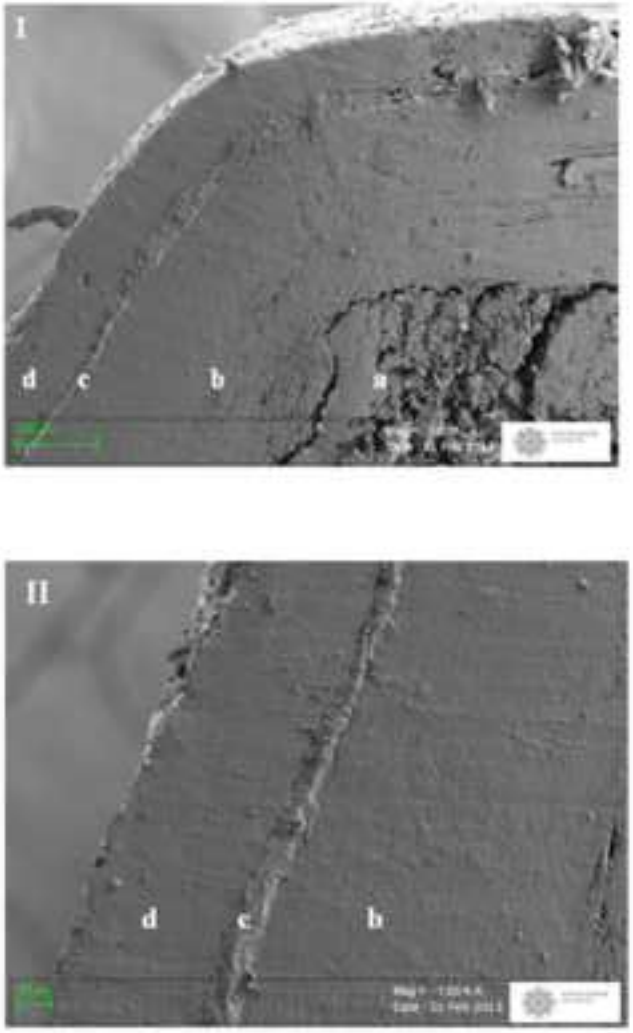

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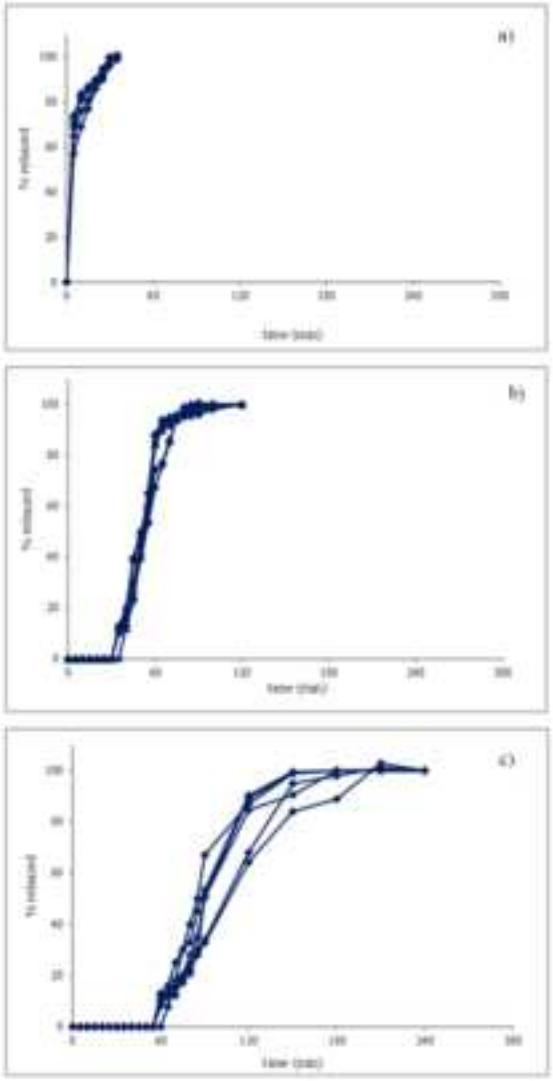

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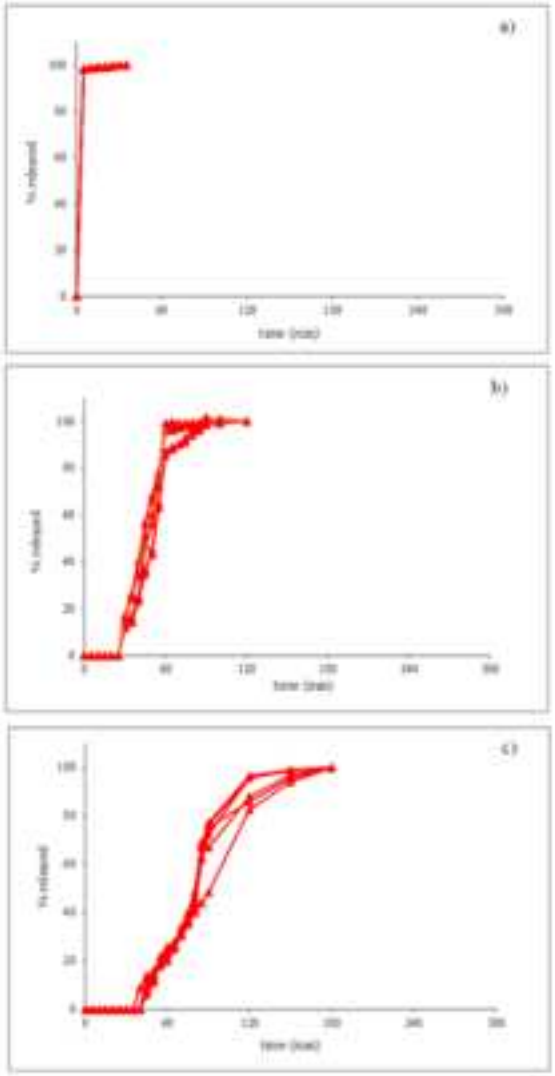

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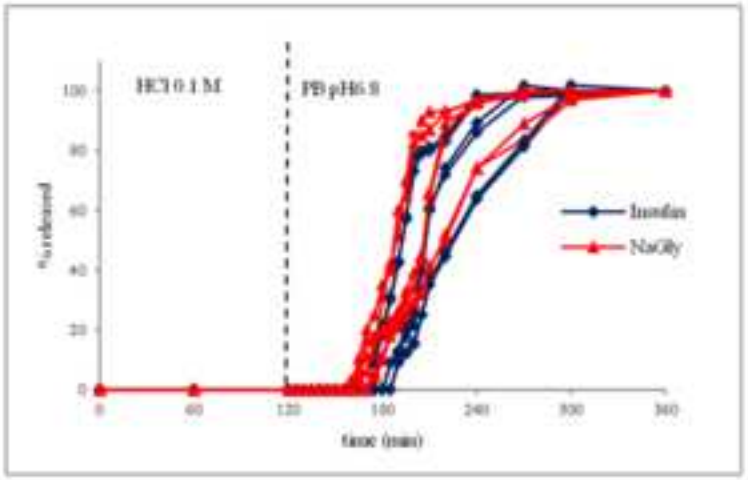

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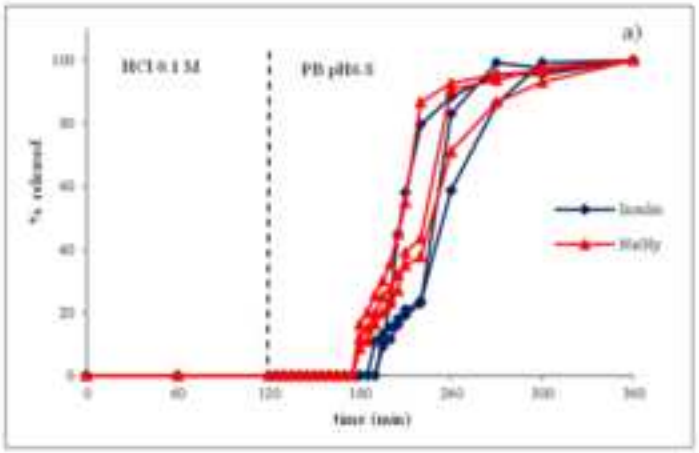

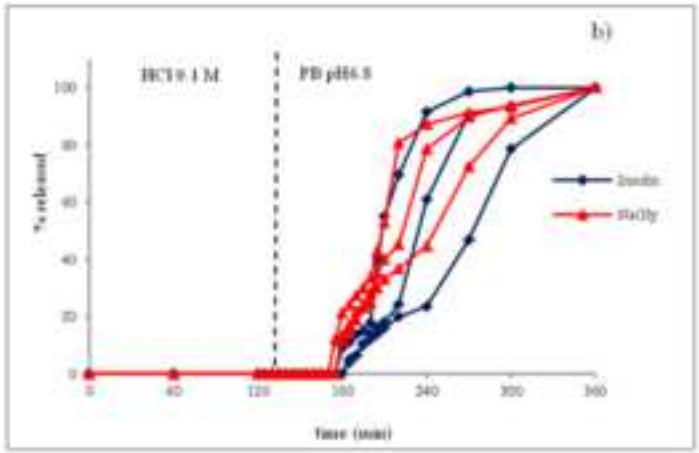

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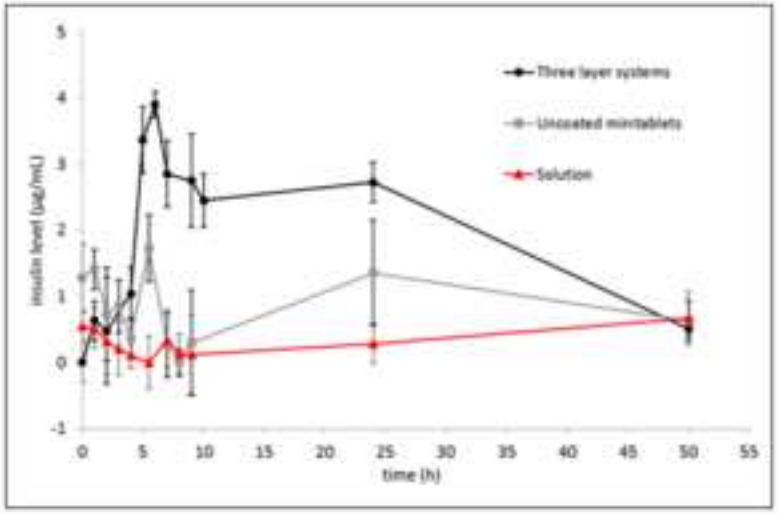

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