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Abstract

Background and Aims

The oral administration of insulin has so far been precluded by gastro-intestinal enzyme degradation and poor intestinal absorption. Preliminary evidence for peptide uptake by the gut has recently been obtained, by our research group, following the administration of nanostructured lipid-carrier suspensions loaded with glargine insulin in healthy animal models.

Methods and results

In this experimental study, glargine insulin-loaded nanostructured lipid carriers have been converted into solid oral dosage forms (tablets, capsules), that are more suitable for administration in humans and have prolonged shelf-life. The liquid and solid oral dosage forms were tested for glargine insulin uptake and glucose responsivity in healthy and streptozotocin-induced diabetic rats (6 animals in each group).

A suitable composition gave redispersible solid oral dosage forms from glargine insulin-loaded carriers, using both spray-drying and freeze-drying. It was observed that the liquid and solid formulations had relevant hypoglycaemic effects in healthy rats, while only capsules were efficacious in diabetic rats; probably because of gut alterations in these animal models. Detected glargine insulinaemia was consistent with a glycaemic profile.

Conclusion

The formulations under study showed their potential as oral glucose-lowering agents, particularly when used as capsules. However, further study is needed to produce a useful orally-active insulin preparation.

Key Words

Insulin therapy; oral therapies; nanostructured lipid carrier

Introduction

Alternative insulin administration routes have been keenly investigated because of the distress that daily injections cause to people with diabetes. The oral route would be the most advantageous for long-term administration, but has been hampered by gastro-intestinal enzyme degradation and poor intestinal absorption [1]. Lipid nano-carriers can be used as gut-uptake enhancers thanks to a variety of mechanisms [2]. Our group has recently published preliminary results on the absorption of orally administered glargine insulin that is loaded into nanostructured lipid carriers (NLC). The NLC were produced via fatty acid coacervation [3,4], which is a solvent-free technology that makes use of biocompatible materials and mild operating temperatures. Glargine insulin was encapsulated inside stearic/oleic acid NLC using hydrophobic ion-pairing (HIP), which is based on the interaction between an anionic surfactant and a positively charged protein [5,6]. *Ex-vivo* and *in-vivo* experiments in healthy animal models have demonstrated the uptake of glargine insulin by the gut, and this is probably due to the promoting effect of oleic acid on the paracellular route, and to protein release at the intestinal mucosa [4].

However, major issues still need to be addressed, including glucose responsivity in healthy and diabetic animal models, the effects of administered doses, and variables such as insulin sensitivity [7-9], and gut mucosa alterations [10,11]. Furthermore, NLC suspensions are not suitable for use in humans, primarily because of the large volumes that are administered, whereas solid-dosage forms (capsules, tablets) may be suitable for use in humans and prolong product shelf-life. Water, which makes up nearly 97% of NLC suspensions, can be eliminated by freeze-drying and spray-drying and thus the NLC can be converted into solid form. However, these processes can impair formulation stability, leading to protein degradation and nanoparticle aggregation [12,13]. Moreover, the resulting powder should be redispersible, that is able to reconstitute the original NLC suspension in the gastro-intestinal environment. A suitable cryoprotectant mixture must be engineered for all of these tasks.

In this study, NLC that were loaded with glargine insulin have been converted into solid dosage forms for oral administration, and underwent preliminary biopharmaceutical characterisation. The liquid and solid oral dosage forms were then tested for blood glucose responsivity in healthy and diabetic rats.

Methods

Chemicals

Lactic acid, sodium hydroxide, sodium carbonate and citric acid were obtained from A.C.E.F. srl (Fiorenzuola d'Arda, Italy). Porcine insulin, bovine serum albumin (BSA), 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate, 80% hydrolysed polyvinyl alcohol 9000–10000 MW (PVA9000), sodium stearate, sodium dodecyl sulfate (SDS), trehalose, ethanol and acetonitrile were obtained from Sigma Aldrich (Saint Louis, Missouri, MO, USA). Hydrochloric acid, sodium chloride, sodium bicarbonate, Tween 20, sodium dihydrogen phosphate, disodium hydrogen phosphate, tris(hydroxymethyl)aminomethane (TBS), oleic acid, eosin and phloxin were obtained from Merck (Darmstadt, Germany). Kollicoat® IR was a kind gift from BASF (Ludwigshafen am Rhein, Germany). Gohsenol® was a kind gift from Harke Pharma (Mülheim an der Ruhr, Germany). Trifluoroacetic acid (TFA) and Streptozotocyn (STZ) were obtained from Alfa Aesar (Haverhill, MA, USA). The 4% formaldehyde solution, slides and coverslips were from Kaltek srl (Padova, Italy). The xylene substitute, paraffin and slide-mounting media were obtained from Leica Microsystems Srl (Buccinasco, Italy). Hematoxylin was obtained from Carlo Erba Reagents S.r.l. (Cornaredo, Italy). Mixetan alcohol (alcoholic mixture based on ethanol) was obtained from Alcoolital s.r.l (Fossano, Italy). The anti-insulin glargine antibody (mouse monoclonal antibody, MAb) was obtained from Antibodies-online (UK), while anti-human insulin mAb, which was labelled with horse radish peroxidase, was purchased from Novus Biologicals (CO, USA). Nunc Maxisorp microtiter plates were purchased from Fischer Scientific (Milan, Italy). Glargine insulin was precipitated from commercial products (Lantus®) via dilution with phosphate buffer 0.1 M

pH=7.40, followed by centrifugation (17.000 g, 5417 Eppendorf Centrifuge, AG, Hamburg, Germany), washing with distilled water and freeze-drying overnight. Distilled water was purified using a Milli-Q system (Millipore, Bedford, MO). All other chemicals were of analytical grade and used without further purification.

Animals

10-week-old male Wistar rats (Charles River, MA, USA), weighing nearly 250 g, were housed in standard facilities, and were handled and maintained according to the ethical regulations of our Institutional Animal Care and Use Committee. The rats were kept under controlled environmental conditions (24 ± 1 °C, 50–60% humidity, 12 h light and dark cycles, lights on at 7:00 a.m.) and given *ad-libitum* access to food and water (Table s1).

Diabetes was induced by STZ intraperitoneal injections (65 mg/kg dose). This model has been validated for glucose-responsivity studies when hyperglycaemia is established, i.e. when fasting blood glucose levels are > 200 mg/dL (11 mmol/L) and/or statistically higher than in healthy control rats [14].

In order to assess the integrity and functionality of the gut wall, diabetic rats were sacrificed at scheduled times after STZ injection, and the duodenum and jejunum were subjected to histological examination, as described in the Supplementary Materials, Section 2. Healthy animals were employed as controls for histology studies.

A separate cohort of animals was used (Table s1) for the administration of insulin-loaded formulations. Formulations were administered 20 days after STZ diabetes induction in order to simulate late diabetes [14].

All procedures conformed to the institutional guidelines on animal welfare (DL 26/2014 implementation of directive 2010/63 UE) of Turin University's Ethics Committee, as well as to International Guidelines. All effort was made to minimise the number of animals and their discomfort. All experiments on animal models were performed according to an experimental

protocol that has been approved by the University Bioethical Committee and the Italian Ministry of Health (Aut. N. 32/2016-PR).

Nanoparticle formulation and characterisation

See Supplementary Materials, Section 3.

Formulation and biopharmaceutical characterisation of solid oral dosage forms

See Supplementary Materials, Sections 4, 5.

Administration protocol to healthy and diabetic rats

Glucose responsivity was studied in both healthy and diabetic rats. Six animals were used for each experimental condition.

Healthy animals were fasted for 12 h prior to the experiment, but allowed *ad-libitum* access to water. Diabetic animals were fasted for 5 h prior to the experiment, but allowed *ad-libitum* access to water. Fasting times were different for healthy and diabetic animals because of their differing glucose metabolism. Indeed, basal glucose is insensitive to long fasting periods in healthy rats, whereas spontaneous decrease occurs after nearly 12 hours of fasting in diabetic ones. As the experiments were scheduled on a 6-hour timeframe, 5 hours of fasting was enforced before experiments.

Glucose and glargine insulin levels were measured on blood samples that were collected via bleeding of the tail vein, 10 min before (baseline) and at scheduled times (30, 60, 90, 120, 150, 180 and 360 minutes for healthy animals; 60, 120, 180, 240 and 360 minutes for diabetic animals), up to 6 hours after administration. The blood glucose level was measured using an Accu-chek Aviva glucometer (Roche) with reactive strips, and the volume needed for blood glucose measurements was approximately 2 µL. Insulin glargine was quantified with an in-house validated ELISA assay, using approximately 50 µL of blood. The Tailveiner® restraint system was used to hold the animal in order to perform tail bleeding.

NLC suspensions were administered by gavage. The effects of two different doses of glargine insulin (30 *vs* 60 U/Kg) were compared in healthy rats. Redispersible powders were obtained from

NLC suspensions by freeze-drying/spray-drying. These powders were either loaded into gelatin capsules or, alternatively, underwent direct compression to form tablets. The solid form dosage limit (30 U/Kg) was based on the maximum size allowed for administered tablets and capsules. Size 9 Torpac® gelatin capsules were utilised to administer the powders as their use in a Torpac® dosing syringe is a suitable method for oral dosage to laboratory rats that weigh 250 g. Before starting the study, the animals underwent a short training period of 3-4 days; the insertion of the syringe tube did not appear to cause undue discomfort to the rats, nor did it lead to tissue damage. The procedure, which can be performed rapidly by trained personnel, is ideally suited for dispensing solid materials to fully conscious animals [15]. The tablets that were obtained via the direct compression of the powders were flavoured with ground feed so that they would be eaten spontaneously by the rats. Control curves were obtained in separate experiments. In healthy rats, the controls were obtained by administration of blank NLC. In diabetic rats, they were obtained by simply monitoring glycaemia for 6 hours, after 5 hours of controlled fasting.

Measurement of glargine insulin in rat serum with sandwich-type in-house developed ELISA

See Supplementary Materials, Sections 6, 7 (Table s7).

Statistical and pharmacokinetic analyses

Animal experiments were performed in healthy and diabetic animals. Measured glycaemia was expressed as both mg/dL and as % of the basal (before administration) for normalisation purposes [16]. Comparisons between animal groups were performed using a two-tailed Student t test (Prism GraphPad v.5.00). The number of rats was consistent with the statistical analysis performed and the size of the parameters to be measured, using G*Power software 3.0.10.

Results

NLC were formulated according to the composition reported in Table 1. The physico-chemical characterisation of the suspensions is also reported in Table 1. Nanoparticles with a glargine insulin concentration of 0.25 mg/mL were used as such for animal studies, whereas NLC loaded with

glargine insulin at 1.0 mg/mL were stable for only a few hours, and underwent spontaneous aggregation. However, prompt 1:1 dilution in the cryoprotectant solution gave stable suspensions, presumably because of the decreased peptide concentration in the aqueous medium. The freeze-drying or spray-drying of the suspension led to redispersible powders (Table s5), which were used to produce tablets via direct compression. While the drying process did not significantly affect the redispersion properties of the powders and the recovery of glargine insulin, spray-drying was associated with a decrease in drug recovery, probably because of the operating temperature (T_{in} = 60 °C). The effervescent mixtures added to the tablet formulation were responsible for reductions in resuspension and disaggregation times, and this helped to reconstitute the original suspension more rapidly in the gastro-intestinal tract (Table s6).

A biphasic serum glargine insulinemic pattern was detected in healthy rats (Figure 1, 2), and a peak was reached 120 min after administration for all the formulations under study. A comparison of the glucose-lowering potential of subcutaneously injected glargine-insulin formulations is shown in Figure s2. All animals displayed an initial rise in blood glucose, presumably consequent to procedure-related stress and nanoparticle intake. The glucose-lowering effect of glargine insulin uptake was more appreciable when glycaemia was expressed as % of the basal, due to individual and intra-day variations in basal glycaemia after fasting [17]. Indeed, significant differences in basal glycaemia can be detected in the different animal cohorts (Figure 1).

A severe form of chronic gastroenteropathy was observed to develop in the diabetic animals (Tables s2, s3, s4, Figure s1), leading to diarrhoea within a few weeks. In particular, villus height (Vh) and the villus-height-to-crypt-depth (Vh/Cd) ratio decreased with diabetes induction in a time-dependent manner. Moreover, lymphoplasmacytic infiltrates, with or without Gut-Associated Lymphoid Tissue (GALT) activation, and diffuse disepithelisation/necrosis were observed. Rats that underwent diabetes induction showed greater gut inflammation than the controls. In these animals, only the capsules exerted a significant glucose-lowering effect, which is consistent with the higher plasma insulin levels obtained (Figure 3, 4). There was a large difference in intestinal

uptake of the capsules and other formulations. Interestingly, negligible gut uptake and glucose-lowering effects were detected when the powders that were used to prepare the capsules (efficacious) and the tablets (non-efficacious) were redispersed in water and the obtained suspension was administered by gavage to the diabetic animals. This suggests that the capsule dosage form is strictly responsible for the insulin uptake and biological effects.

Discussion

This study shows that, in an animal model, insulin can be made to cross the gastro-enteric barrier, reach the bloodstream and lower blood glucose when administered via the oral route, if appropriately loaded into nanoparticles. Indeed, lipid nanoparticles have been proposed for some decades as drug-delivery systems that can enhance the uptake of orally delivered drugs [2]. Glargine insulin is insoluble at neutral pH, and, thus, precipitates after subcutaneous injection, forming micro-crystals that allow the peptide to be slowly released, which is useful as it can be used to provide basal insulin concentrations. However, due to its modified amino acid sequence and different isoelectric point, glargine insulin shows superior HIP capability to human insulin, resulting in more efficient loading in lipid nanoparticles and intestinal uptake, which is probably due to paracellular absorption that is mediated by interactions with mucin [4]. Therefore, it could ideally function as short-acting insulin, in order to replace multiple pre-meal injections. However, the formulations engineered failed to mimic the normal meal-related spikes in insulin secretion, while blood glucose progressively decreased up to 6 hours after administration.

In accordance with previous reports by our group [4], HIP-coupled fatty acid coacervation allowed the drug concentration in the suspension to be modulated. Time-dependant particle aggregation occurred at a drug concentration of 1.0 mg/mL, and this was probably due to excessive drug payload in the suspension. Although meta-stable, it was possible to employ this suspension as a smart intermediate for both drying processes. Indeed, although a high ratio between cryoprotectants and lipids is functional to obtaining redispersible powders, it can cause excessive dilution of the

loaded peptide in the final solid dosage form. NLC with higher peptide/lipid ratios can be used to overcome this problem.

Some key considerations about solid dosage forms should be highlighted. The use of sugars and polymers as cryoprotectants with different properties/functions is well documented in the literature [18], but only the combined use of both allowed us to obtain redispersible powders with glargine insulin-loaded NLC, and this composition worked with both freeze-drying and spray-drying. Using trehalose in the formulation is not likely to be detrimental to diabetes control as there is consistent literature evidence to suggest that this sugar does not elicit rapid increases in blood glucose or the excessive secretion of insulin [19]. Indeed, trehalose has been reported to improve hyperglycaemia control in people with diabetes as it stabilises glucose homeostasis and modulates insulin sensitivity [20-22]. Kollicoat® IR and Gohsenol® polymers are employed commercially in sustained release tablet formulations. Although these excipients are potentially advantageous to the assembly of redispersible powders into tablets by direct compression, they may be responsible for increased tablet-disintegration times. A certain reduction in tablet resuspension and disaggregation times was obtained by adding a small amount of an effervescent mixture (sodium bicarbonate + citric acid).

The glucose-lowering effect observed after the formulations under study were administered to healthy rats may be associated to plasma glargine insulin uptake (Figure 2). This glargine insulinemic profile was different from those reported in a previous study that used a labelled peptide [4]. Instead of a single plasma peak, a biphasic absorption profile was displayed in our present study (Figure 1). It is well known, from the literature, that glargine insulin undergoes rapid metabolisation to M1 (21A-Gly-human insulin), and, to a much lesser extent, to M2 (21A-Gly-des-30B-Thr-human insulin) in biological fluids, such as plasma and the subcutaneous environment [23,24]. Indeed, after subcutaneous injection, most of the circulating insulin is M1, which shows higher bioavailability than the parent molecule and M2 [25]. Nonetheless, enzymatic conversion to M1 should not occur in the gastro-intestinal tract. Unfortunately, commercially available ELISA antibodies for glargine insulin do not specify potential cross-reactivity with M1. However, M1

preserves the metabolic activity of the parent peptide [26]. Therefore, it can be hypothesised that the first peak is due to glargine insulin uptake into the bloodstream, since it is consistent with the peak detected in previous research [4], while the second peak may be partially ascribed to M1 conversion after uptake in the bloodstream. In previous research, performed with fluorescently labelled glargine insulin, absolute bioavailability (F) was approximated to be 4.5% [4]. In this experimental work, plasmatic peaks are consistent with those of previous research, and correlate well to glycaemic decrease [27].

Alterations in the gut mucosa and gastrointestinal motility in the STZ-induced diabetic rats [10,11], may account for the differences in glargine insulin absorption (Figure 4). We hypothesise that STZ intraperitoneal administration might lead to the documented alterations in the gut mucosa; a decrease in Vh and Vh/Cd ratio and an increase in gut inflammation/necrosis (Supplementary Materials Section 2). Mucosal alterations may affect the pharmacokinetics of the formulations under study, as the mucin layer may play a key role in glargine insulin release and uptake by intact gut mucosa, according to our previous observations [4]. The gastroenteropathy that developed in our diabetic rats, which is in accordance with previous data [10,11], coupled with increased insulin resistance in the STZ-induced model [7-9,28], may have affected gut uptake [29], resulting in lower bioavailability and reduced glucose-lowering effects (Figure 4). Capsules, on the other hand, also reduced glycaemia in the diabetic rats, probably because, unlike liquid formulations and tablets (which were disaggregated by spontaneous chewing), their larger size impaired passage through the pylorus, slowing gastric emptying. This might have facilitated the uptake of the glargine insulin-loaded preparation through the stomach-duodenum tract.

In any event, the gut mucosa of humans that potentially receives oral insulin through the formulations tested in this study is more likely to be intact, as diabetic gastroenteropathy in humans generally develops in long-term diabetes with severe chronic hyperglycaemia, autonomic neuropathy and disorders of the neuroendocrine system [30]. Diarrhoea, as observed in the diabetic

animals of this study, was more likely a consequence of alterations induced by acute hyperglycaemia and/or intraperitoneal STZ.

In conclusion, this study has assessed the suitability of the fatty acid coacervation technique to the loading of glargine insulin into NLC to obtain redispersible powders and solid oral dosage forms from NLC. Both the liquid and solid formulations worked in healthy rats, while only capsules were effective in diabetic ones, with the principle obstacle being intestinal mucosa alterations following diabetes induction with intraperitoneal STZ. The obtained data highlight the potential of specific formulations, and capsules in particular, as oral glucose-lowering agents in people with diabetes. However, this proof of concept is still a long way from becoming a useful orally active insulin preparation, and further studies will be needed. Alternative approaches in animals may be used to demonstrate the glucose-lowering potential of all the formulations under study, and further investigation will be dedicated to reducing insulin doses and optimising the therapeutic effects.

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Conflict of Interest

The authors state that they have no conflict of interest concerning this manuscript.

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Table 1 NLC composition and physico-chemical characterisation

NLC			
	Blank	Glargine insulin loaded	
Stearic acid	1%	1%	1%
Glargine insulin	-	0.25 mg/mL	1.0 mg/mL
Composition			
Sodium stearate	50 mg	50 mg	50 mg
Oleic acid	35 µL	35 µL	35 µL
PVA 9000	25 mg	25 mg	25 mg
PVA 9000 milled	50 mg	50 mg	50 mg
Deionized water	5 mL	5 mL	5 mL
1 M Lactic acid	0.25 mL	0.25 mL	0.25 mL
Glargine insulin HIP			
Glargine insulin	-	1.25 mg	5 mg
SDS 1 mg/mL	-	0.5 mL	2 mL
HCl	-	0.35 mL	1.4 mL
Mean size (nm)			
	313.0 ± 25	302.4 ± 35	499.9 ± 45
Polydispersion index			
	0.059	0.129	0.087
% drug recovery			
	-	85 ± 5%	87 ± 7%

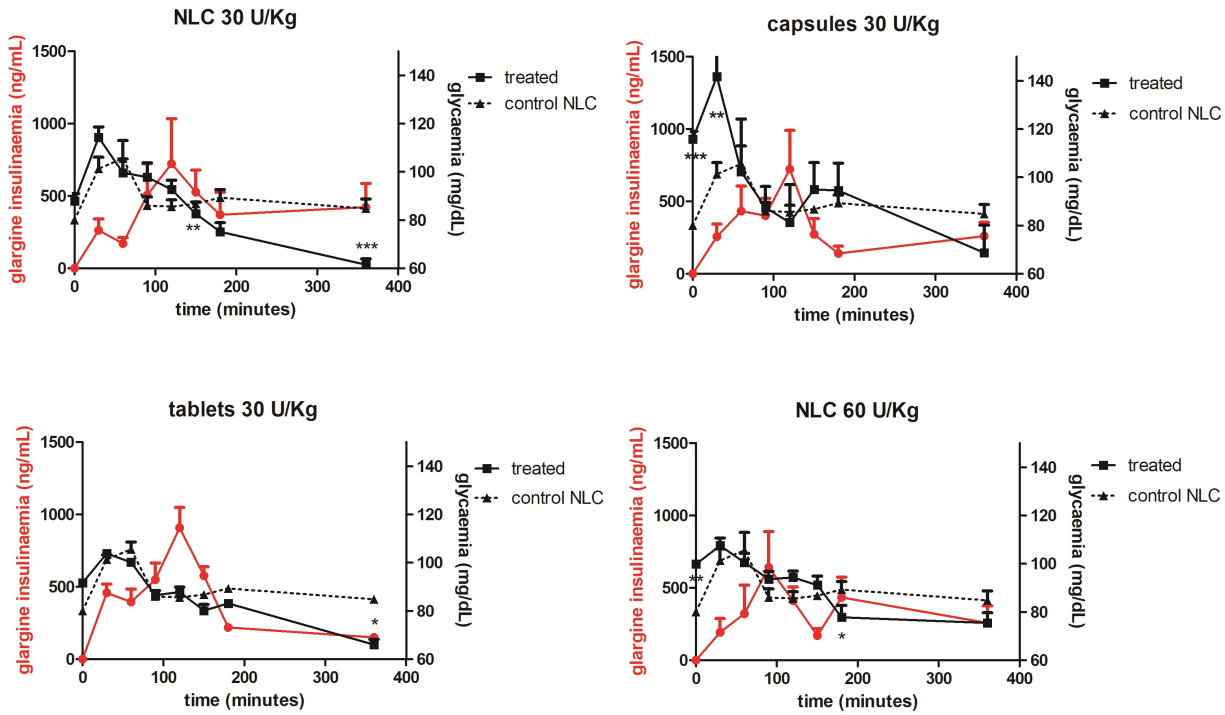

FIGURE 1 Glycaemic (black continuous) and glargine insulinemic (red) profiles in healthy rats after administration of glargine insulin loaded formulations. Glycaemic profiles of rats treated with control NLC are indicated by the dotted black line. Data are expressed as mean ± SEM.

Statistical comparison of glycaemic profiles was performed between the total curves of treated animals vs control NLC, and between the single time points and the basal level of the same cohort of animals.

Statistical analysis on single time points: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

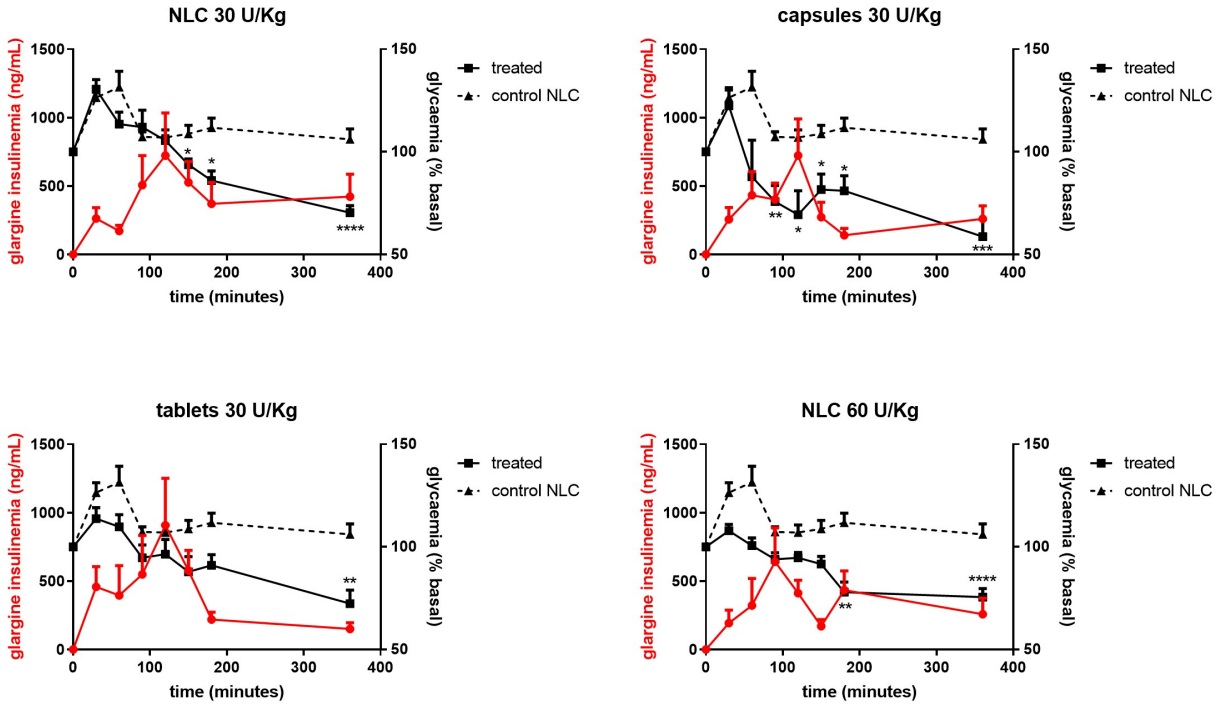

FIGURE 2 Glycaemic % basal (black continuous) and glargine insulinemic (red) profiles in healthy rats after administration of glargine insulin-loaded formulations. Glycaemic profiles of rats treated with control NLC are indicated by the dotted black line. Data are expressed as mean ± SEM.

Statistical comparison of glycaemic profiles was performed between the total curves of treated animals vs control NLC, and between the single time points and the basal level of the same cohort of animals.

Statistical analysis on total curves: NLC 30 U/Kg vs control NLC (last 3 points) $P = 0.035$; capsules 30 U/Kg vs control NLC, $P = 0.005$; tablets 30 U/Kg vs control NLC, $P = 0.025$; NLC 60 U/Kg vs control NLC, $P = 0.005$.

Statistical analysis on single time points: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

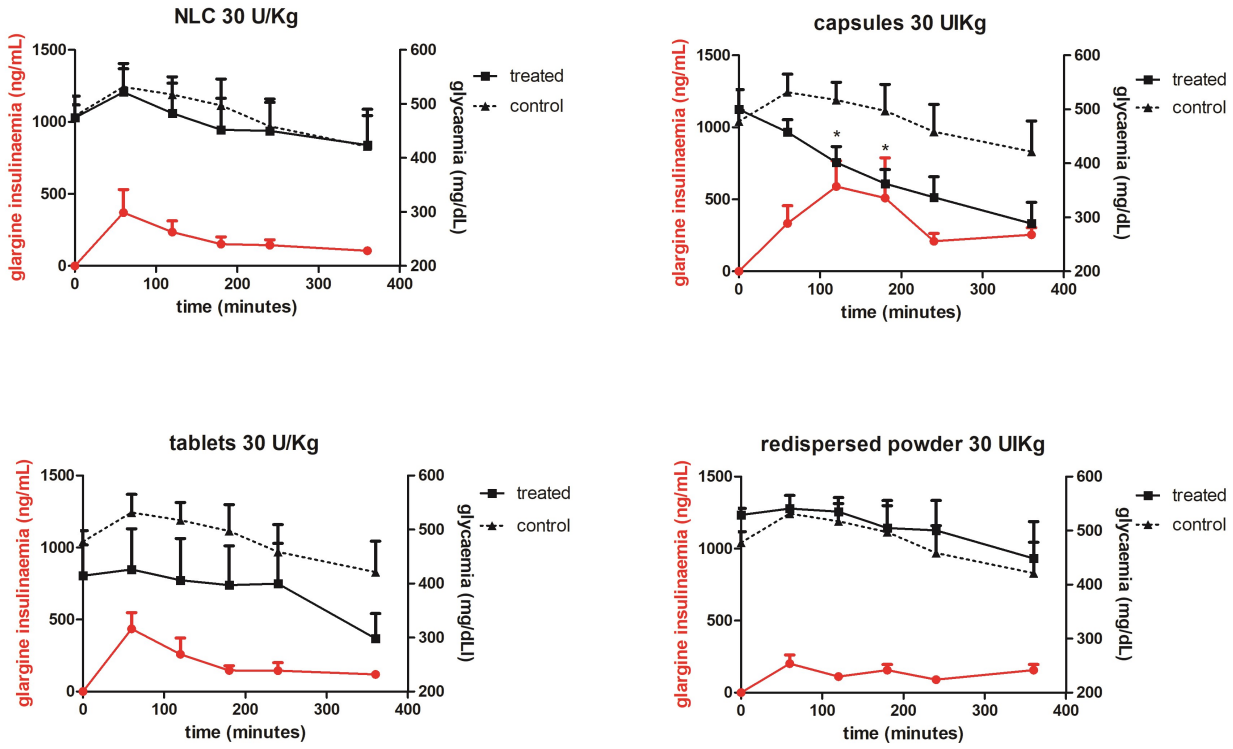

FIGURE 3 Glycaemic mg/dL (black continuous) and glargine insulinemic (red) profile in diabetic rats after administration of glargine insulin-loaded formulations. Spontaneous glycaemic profiles of untreated control rats are indicated by the dotted black line. Data are expressed as mean ± SEM. Statistical comparisons of glycaemic profiles were performed between the total curves of treated animals vs controls, and between the single time points and the basal level of the same cohort.

Statistical analysis on total curves: capsules 30 U/Kg vs controls $P = 0.0275$.

Statistical analysis on single time points: * $P < 0.05$

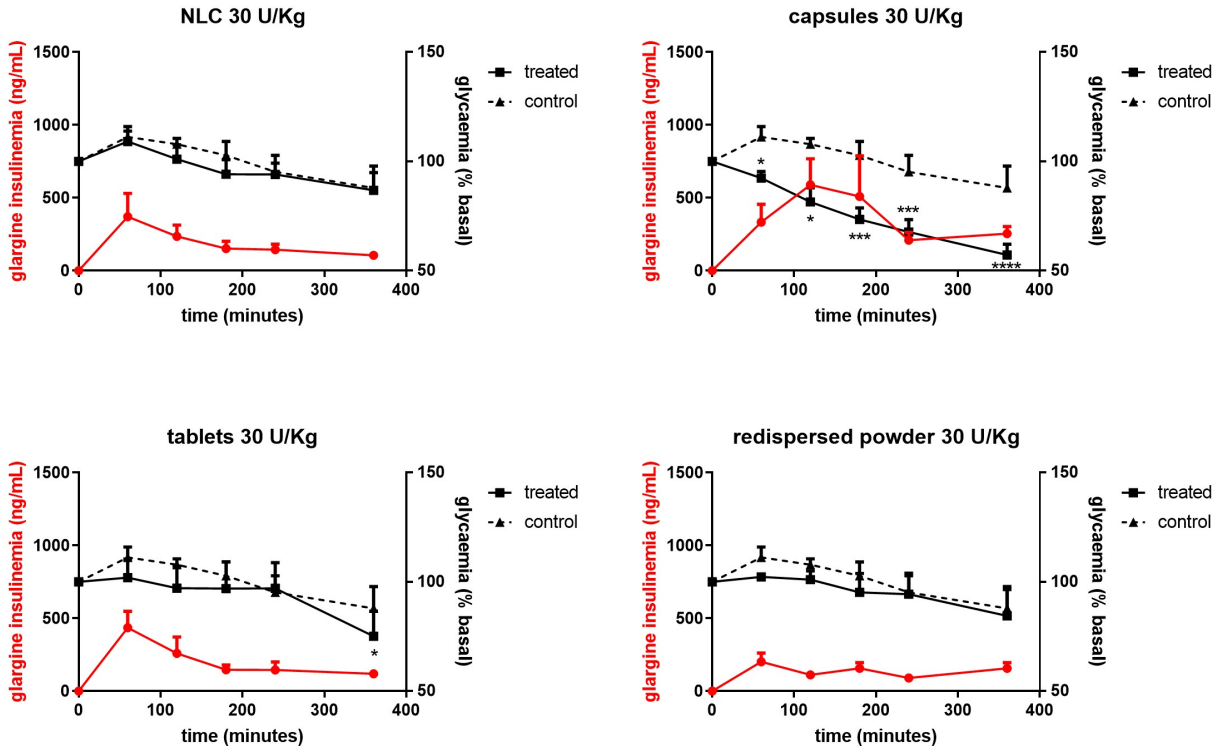

FIGURE 4 Glycaemic % basal (black continuous) and glargine insulinemic (red) profile in diabetic rats after administration of glargine insulin-loaded formulations. Spontaneous glycaemic profiles of untreated control rats are indicated by the dotted black line. Data are expressed as mean ± SEM. Statistical comparisons of glycaemic profiles were performed between the total curves of treated animals vs controls, and between the single time points and the basal level of the same cohort.

Statistical analysis on total curves: capsules 30 U/Kg vs controls $P = 0.014$.

Statistical analysis on single time points: * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$