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Salcaprozate-based ionic liquids for GLP-1 gastric delivery: A mechanistic understanding of *in vivo* performance

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ABSTRACT

Oral delivery of peptides requires formulations with high concentrations of permeation enhancer (PE) to promote absorption, and often necessitates fasting time between dosing and food ingestion. Improved formulations promoting a more rapid absorption would increase convenience of use but requires a faster onset of action. We have developed a salcaprozate-based ionic liquid (IL) formulation, namely choline salcaprozate (CHONAC), for oral delivery of a glucagon-like peptide-1 (GLP-1) analogue *via* gastric absorption. *In vitro* studies confirmed the higher amount of PE accommodated in the same volume of dosage form as well as faster release of the active pharmaceutical ingredient (API) and PE compared to the tablet reference. Storage stability of the CHONAC formulation was demonstrated for up to 3 weeks at 4 °C. The peptide absorption efficacy of the IL formulation was first evaluated *in vivo* in rats and anesthetized dogs, showing a faster absorption compared to the reference formulations. In awake dogs, while the CHONAC formulation still enabled earlier API absorption, its overall exposure was inferior to the tablet reference. This was attributed mostly to the gastric physiology, causing formulation dilution in the presence of additional fluid as well as fast transit of liquids into the duodenum, where peptides liable to proteolytic degradation such as the one used in this study showed a negligible absorption, potentially also due to a lower permeation-enhancing capability of CHONAC in the duodenal region. Exploring these issues, an *in vivo* study in anesthetized dogs involving repeated dosing of a liquid salcaprozate-based formulation in the stomach revealed the potential to sustain peptide absorption throughout the dosing period with a constant absorption rate. In conclusion, combining the advantages of high PE amounts and fast onset of action provided by the IL formulation, and ensuring a prolonged interaction of peptide and PE at a relevant concentration with the stomach epithelium, are necessary to enhance oral peptide bioavailability *via* gastric delivery.

1. Introduction

Oral formulations of biomacromolecules, such as proteins and peptides, can provide a more convenient and less invasive alternative to intravenous or subcutaneous administration. However, there are several significant challenges associated with oral delivery of biomacromolecules, including their enzymatic degradation in the gastrointestinal tract and their low permeability across the absorptive epithelium due to their size and polarity, thereby resulting in poor bioavailability [1–3]. In addition, the absorption rate and extent of oral biomacromolecules can be influenced by multiple factors, including food intake, gastrointestinal motility, and interactions with other drugs or excipients, leading to variability in pharmacokinetics [4,5].

To overcome these obstacles, new strategies for oral delivery of

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biomacromolecules have been developed, such as the use of permeation enhancers (PEs), protease inhibitors and nanoparticle-based delivery systems [6,7]. Among the PEs, sodium salcaprozate (SNAC) has gained attention due to the success in the clinical trials of oral semaglutide [8,9], a glucagon-like peptide-1 (GLP-1) analogue. SNAC enables gastric absorption of semaglutide by fluidizing the epithelial membrane and creating a microenvironment of higher pH which protects the peptide and inactivates the surrounding enzymes [10]. Nevertheless, PE-based tablet formulations are still suboptimal as oral dosage forms, and the achieved bioavailability is not yet close to that of small molecules. Moreover, they often require high doses of PE per dosage form to be effective. For instance, the currently marketed oral semaglutide tablets contain 300 mg of SNAC, whereas oral insulin tablets tested in clinical trials comprise 550 mg of sodium caprate, another PE [11–13]. The dosing conditions are also more restricted compared to standard solid oral formulations, as food intake typically leads to dilution and degradation of biomacromolecules [14]. Therefore, it is important to ensure that most active pharmaceutical ingredient (API) and PE are available for absorption as fast as possible to minimize the time between dosing and food ingestion [15].

In the case of tablet formulations, a fast tablet disintegration is required to increase the rate of dissolution of API and PE [16,17]; however, it is difficult to achieve without the addition of several excipients considering the high doses and hydrophobic nature of the PEs. Alternatively, liquid formulations can provide a faster onset of action by bypassing the disintegration and dissolution steps. Nevertheless, PE-based aqueous formulations require very high volumes to accommodate the large amount of PE per dosage unit, considering the limited PE water solubility in low-to-mid pH environment [18]. Moreover, ensuring the stability of API and PE, especially at high concentrations in aqueous media, is challenging. Therefore, novel liquid vehicles are needed for oral delivery of peptides.

Ionic liquids (ILs) have been recently emerging as a drug delivery system due to their unique physicochemical properties [19,20]. In fact, attempts have been made to use ILs as delivery systems for peptides, such as the choline-geranic acid (CAGE) system used for oral delivery of insulin in rats [21]. ILs are salts where the composing ions are poorly coordinated, resulting in a liquid state below 100 °C, or even at room

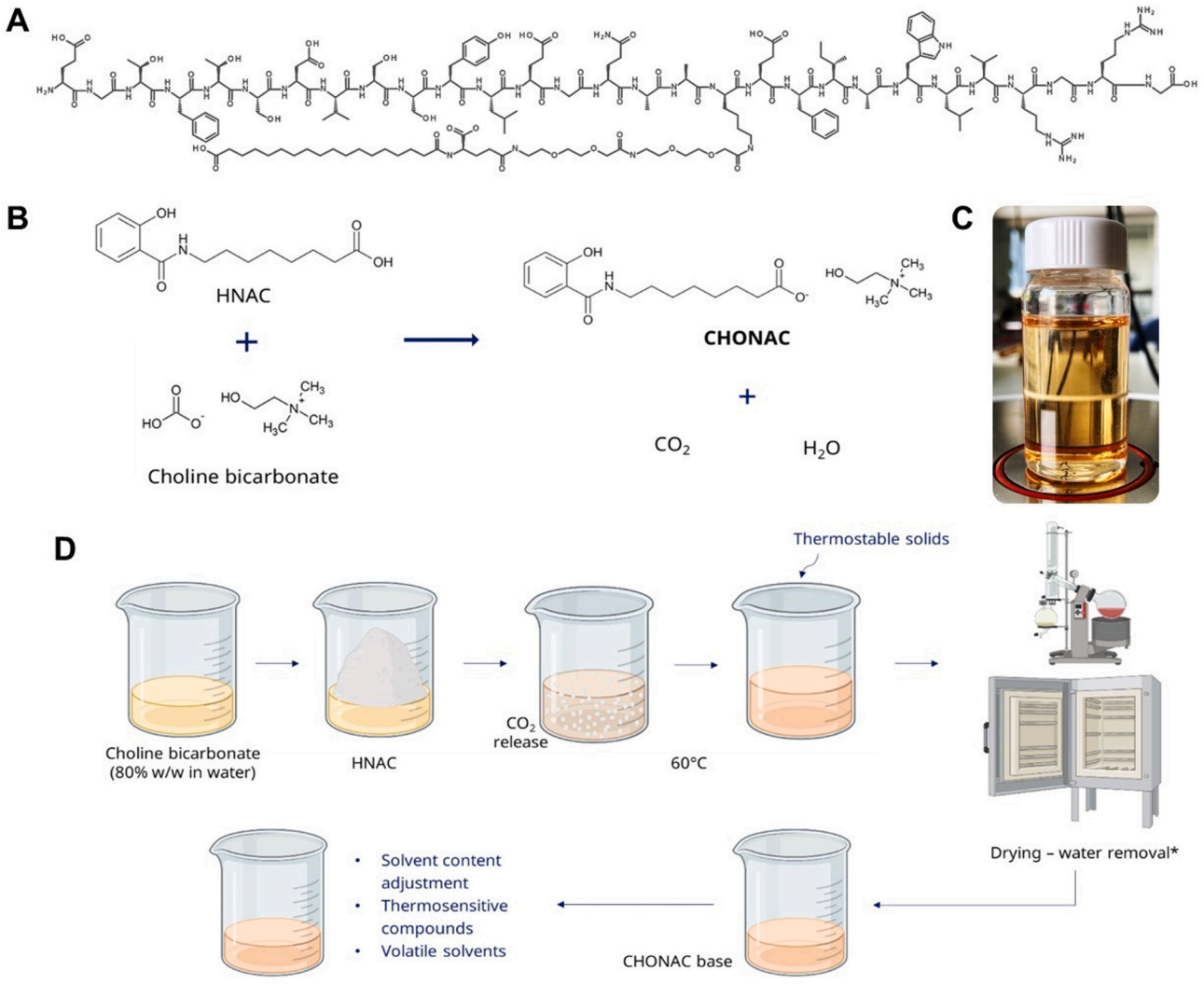

Fig. 1. (A) Chemical structure of the low potency GLP-1 analogue used in this study (B) CHONAC formation reaction, choline bicarbonate is mixed with the acid form of SNAC (HNAC) in 1:1 M ratio, forming carbon dioxide and water as byproducts (C) CHONAC ionic liquid, and (D) Scheme of CHONAC manufacturing process. HNAC is added as powder to an 80 % w/w solution of choline bicarbonate; the neutralization reaction takes place immediately, being tracked by the formation of CO_2 bubbles; the reaction is accelerated by increasing the temperature to 60 °C; in this step any thermostable solid can be incorporated to the formulation; after its completion, the formed CHONAC is dried by rotary evaporation and vacuum heating to remove water from the choline bicarbonate initial solution and formed during the neutralization reaction; the solvent content of the dried CHONAC base can be adjusted then to the desired value; any thermosensitive solids as the API used in this work can be incorporated in this final step.

temperature [21,22]. ILs can dissolve a variety of compounds, including polar and non-polar substances. PEs such as SNAC contain an organic anion, which could lead to the formation of a poorly coordinated salt, *i.e.*, IL, by replacing the sodium cation with an appropriate one. In doing so, the permeation enhancing capabilities would be retained, as the organic moiety is responsible for interacting with the epithelial membrane, while the cation would affect the physical form of the salt, leading to a liquid state which would enable high concentrations of PE per dosage unit.

In this work, we developed a salcaprozate-based IL formulation containing a fatty acid protracted, low potency, long elimination half-life GLP-1 analogue API (Fig. 1A) by using the organic anion N-(8-(2-hydroxybenzoyl)amino)caprylate (NAC) of SNAC and choline as counter ion. We assessed the *in vitro* release of API from the IL and corresponding tablet formulations. Moreover, we investigated an array of formulation parameters, such as ionic strength, viscosity and API loading, potentially impacting the *in vivo* behavior. Ultimately, a series of IL formulations were evaluated *in vivo* in dogs, to gain a mechanistic understanding of such systems and elucidate their potential to serve as oral delivery vehicle for enhancing peptide absorption and bioavailability.

2. Materials and methods

2.1. Materials

The API and SNAC were supplied by Novo Nordisk. NaOH 2 N and HCl 2 N were purchased from Merck (Darmstadt, Germany). Ultrapure water (MQ, 18 MΩ) was prepared in the laboratory with a Millipore-Q system (Darmstadt, Germany) for all aqueous solutions. NaOH 1 N, HCl 1 N and Choline bicarbonate 80 % *w*/w were purchased from Merck (Darmstadt, Germany). Deionized water (18 MΩ) was prepared in the laboratory with a Millipore-Q system (Darmstadt, Germany). Hard shell gelatin-based capsules (Capsugel®) were obtained from Lonza (Basel, Switzerland).

Analytical grade KH_2PO_4, NaOH pellets, NaCl, Titrisol ampoules 1 N NaOH and HCl, HPLC grade acetonitrile were all purchased from Merck (Darmstadt, Germany). HPLC grade trifluoroacetic acid (TFA) was purchased from Fisher Scientific (Loughborough, UK). Canine powder for fasted-state simulated gastric fluid (FaSSGF) was purchased from Biorelevant (London, UK). HPLC grade maleic acid was purchased from Sigma-Aldrich (St. Louis, MO, US).

2.2. Formulation manufacturing

2.2.1. Salcaprozic acid form (HNAC) preparation

SNAC powder was dissolved in MQ Water at a concentration of 115 mg/mL, the pH of the solution was adjusted to 8.5 using NaOH 2 N to fully incorporate the material, once a pale orange transparent solution was obtained, the pH was dropped to 1.5 by adding HCl 2 N. The acid form of NAC, salcaprozic acid (HNAC), precipitated and was then filtrated under vacuum and washed using MQ water. The filtered material was placed on a steel pan and dried for 72 h on an oven (Memmert 100–800) at 65 °C. The powder was characterized by Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA) and X-Ray Powder Diffraction analysis (XRPD) and its identity reported as HNAC by comparing the results with a reference batch (Fig. S6).

2.2.2. CHONAC reaction

Choline bicarbonate was placed on a glass beaker and an equimolar amount of HNAC was added on it, the formation of bubbles indicated the release of CO_2 during the neutralization reaction. Around 20 % of the total mass of choline bicarbonate was added as MQ water to help incorporate the HNAC powder and the mix was then placed over a heating plate and warmed up to 50 °C under magnetic stirring. The beaker was kept under agitation and heat until no more bubbles were formed, and a clear amber liquid was obtained. At this stage, thermostable solids can be dissolved in the CHONAC solution as the viscosity of the system is low and enough free water is present.

2.2.3. CHONAC drying process

The CHONAC solution was now transferred to a round beaker and placed in a rotatory evaporator (Büchi Rotavapor R-114 equipped with a v-100 Büchi vacuum pump and a Büchi Interface I-100 vacuum controller) for 1.5 h at 60 °C and 45 mbar. After the evaporation, the obtained viscous liquid was transferred into steel pans and placed into a vacuum oven (Heraeus Vacutherm VT6025) at 60 °C and full vacuum for 48 h. The remaining water was determined by TGA and confirmed by Nuclear Magnetic Resonance (NMR).

2.2.4. CHONAC completion and capsule filling

The determined water content of the CHONAC base was used to calculate the water volume required to reach the solvent content target for each formulation. This extra water or solvent was used to dissolve the API and/or any other thermosensitive component to be incorporated in the formulation. Once the API solution was ready, it was manually mixed using a spatula with the CHONAC base until complete incorporation, and the formulation was kept at 4 °C.

For specific studies, hard shell gelatin-based capsules (Capsugel®) from Lonza (Basel, Switzerland) were used in the oral administration of CHONAC, the capsules were filled with 1 g of formulation and sealed according to the manufacturer protocol.

2.2.5. API in SNAC solutions

SNAC and API were weighed in a glass container for each solution, and the amount of MQ water required to achieve the desired concentration was measured. For the solution containing PVP, the precipitation inhibitor was dissolved in the MQ water. All the containers were kept in the fridge at 4 °C until the day of dosing, then the materials were taken out and kept at room temperature to equilibrate. 10 min before dosing, the water / water-PVP were poured into the SNAC-API container and magnetically stirred until complete dissolution.

2.2.6. Tablet manufacturing

SNAC was dry granulated prior to tableting using a roller compactor (MINI-PACTOR, Gerteis, Rapperswil-Jona, Switzerland). The granules were blended with the API and then magnesium stearate was added and both blending steps applied a manual geometric mixing followed by mixing in a turbula mixer.

Tablets were produced on a STYL'One tableting press (MedelPharm, Beynost, France) mounted with a single set of punches, and punch size was chosen according to the total tablet weight to well accommodate the powder blends. The press speed was set to 10 %. The fill volume was adjusted to obtain tablets according to target weight. Compression forces ranged from 3 to 25 kN to ensure the same apparent density.

2.3. Chemical and physical characterization

2.3.1. Purity, release profile and chromatographical analysis

Phosphate buffer (Medium A) was prepared by dissolving KH_2PO_4 50 mM in H2O and (aq) 1 M NaOH was added to achieve pH 7.4. Biorelevant canine FaSSGF (pH 5.0) (Medium B) and biorelevant canine FaSSGF (pH 3.0) media (Medium C) were prepared according to the manufacturer protocols from Biorelevant Ltd. and adjusting the pH to 5.0 or 3.0 with HCl (aq) 1 M, before the canine powder addition. When required, media preparations were pH controlled using Meterlab PHM220 pH meter by Radiometer Analytical (Lyon, France), 2-point calibrations were conducted before media measurement by using reference solutions pH 1.679, 4.005 and 7.000 from HACH (Düsseldorf, Germany). All dissolution media were prepared the same day as experiments were conducted.

2.3.2. Formulation stability

CHONAC formulations were stored at room temperature (25 °C) and in the fridge (4 °C) for 3 weeks. At every timepoint, a sample was prepared by weighing out approximately 15 mg of CHONAC formulation, and subsequently diluting it with MQ water to reach GLP-1 analogue's concentration equal to 0.26 mg/mL. For each timepoint and both concentrations, two replicates were made. Samples were shaken until the solution looked clear and analyzed by Ultra-High Liquid Chromatography (UHPLC) without any additional steps.

Timepoint 0 and timepoint 1 week of samples stored at 25 °C together with timepoint 0 of samples stored at 4 °C were analyzed by using a Water Acquity UPLC H-class system (Milford, MA, US), equipped with a quaternary pump, autosampler, temperature-controlled column compartment and UV detector. The rest of the samples were analyzed by using an Acquity UPLC H-class system, equipped with a binary pump, autosampler, temperature-controlled column compartment and UV detector. Column used was a Waters Acquity UPLC BEH C18, 1.7 μm, 50 × 2.1 mm. Eluents were A: 0.1 % *v*/v trifluoroacetic acid (TFA) in water and B: 0.09 % v/v TFA in acetonitrile. Flow rate was 0.7 mL / min, injection volume 2 μL, run time 5.1 min per injection. Column temperature was 30 °C and detection at wavelengths 335 nm for 1.8 min and at 214 nm for the rest of the run time. Autosampler temperature was set to 7 °C throughout the experiment. Gradient program used: 30–55 B% at 0–3.2 min, 55–90 B% at 3.2–3.6 min, 90–95 B% at 3.6–3.7 min, 96 B% isocratic at 3.7–4.0 min, 95–30 B% at 4.0–4.1 min, 30 B% re-equilibration at 4.1–5.1 min. GLP-1 analogue elutes at retention time 3.2 min. Purity of GLP-1 analogue was determined by integrating all peaks corresponding to GLP-1 analogue and determining the proportion of the main peak. The results are reported as percentage of the main peak. Waters Empower software 3 v7.50 was used for chromatogram processing.

2.3.3. Dissolution

Dissolution tests of capsules and solid dosage forms were conducted in sink conditions on a Teledyne Hanson Vision G2 Elite 8 dissolution tester (Chatsworth, CA, US) using a scaled-down USP Apparatus 1 setup. Customized miniature polymer baskets (14 Mesh) were used for the tests (for vessel, shaft and basket dimensions see Fig. S5). Basket rotation speed was set to 40 rpm, bath temperature was set at 37 °C, and 85.0 mL vessel media volume was used. Dissolution medium A-C were prepared as described above. Automated sampling was conducted at timepoints 4, 9, 14, 22, and 30 min. Samples were filtered twice, first by a 10 μm UHMW PE probe filters purchased from Erweka (Langen, Germany). And sequentially in-line by a Millex-HV 33 mm, 0.45 μm PVDF Membrane filter purchased from Merck Millipore (Cork, Ireland), before being transferred to a High-Performance Liquid Chromatography (HPLC) vial. Total sampling volume at each timepoint was 8.0 mL, 7.0 mL used as priming, and 1.0 mL was collected for analysis. The samples were analyzed on HPLC. The vessel volume decrease at each sampling timepoint was corrected for in the fraction released calculation.

Dissolution samples were analyzed for SNAC/NAC and GLP-1 analogue recovery by using a Waters Alliance HPLC 2695 system, equipped with a quaternary pump, autosampler, temperature-controlled column compartment and UV detector. Column used was a Waters Sentry Guard Symmetry Shield C8, 100 Å, 5 μm, 20 × 3.9 mm. Eluents were A: 0.1 % *v*/v trifluoroacetic acid (TFA) in water and B: 0.1 % v/v TFA in acetonitrile 80 % v/v in water. Flow rate was 2.0 mL / min, injection volume 10 μL, run time 7 min per injection. The column temperature was 50 °C and detection at wavelengths at 215 nm and 335 nm for GLP-1 analogue and SNAC/NAC, respectively. Autosampler temperature was set to 5 °C throughout the experiment. Linear gradient program used: 35 B% isocratic at 0–0.7 min, 35–61 B% at 0.7–5.0 min, 61–100 B% at 5.0–5.1 min, 100 B% isocratic at 5.1–5.5 min, 100–35 B% at 5.5–5.6 min, 35 B% re-equilibration at 5.6–7.0 min. SNAC/NAC and GLP-1 analogue eluted at retention time 0.5 and 3.9 min, respectively. Sample recovery was quantified by using a two-point external standard calibration curve for each analyte. Waters Empower software 3 v7.50 was used for chromatogram processing.

2.3.4. Viscosity

Viscosity was estimated using Viscoman™ (Gilson SAS, France). The principle behind the method is the use of a pipette with a well-defined pipette-tip with a force and pressure measuring device. The crosshead speed and the measured force are used to calculate the shear rate and the shear stress respectively. Viscosity can be expressed in relation to shear rate as:

$$\text{Viscosity } (\eta)\ \frac{\tau_W}{\dot{\gamma}} \quad = \quad \frac{(\Delta PD/4L)}{\left(32Q/\pi D^3\right)}$$

where γ is the apparent shear rate, τ_w is the shear stress, P is the pressure resulting from driving the plunger, Q is the volumetric flow rate of the fluid passing through the capillary needle, D and L are the internal diameter and length of the capillary.

The viscosity (η) is calculated from the obtained shear rate (γ) and shear stress (τ_w) values by linear regression. The usable range is up to 20,000 mPa·s. The samples were equilibrated 1 h at 37 °C before measuring. Three measurements were performed for each sample.

2.3.5. Quantification of solvent content in ionic liquids

The solvent content of the ILs was quantified by thermogravimetric analysis (TGA) after preparation as well as upon extended storage, and performed on a Thermogravimetric Analyser 5500 from TA-Instruments-Waters LCC (New Castle, DE, USA) under a nitrogen gas purge of 25 mL/min. A sample of approx. 4–10 mg IL was loaded and distributed in a flame-cleaned platinum pan and heated at 10 °C/min from ambient temperature to 80 °C, followed by a 60 min isothermal step to allow complete desolvation of the sample. Finally, the sample was heated at 10 °C/min to 200 °C. The solvent content was determined as the weight loss at the observed plateau with <0.005 % weight loss/°C and in the temperature range of 90–140 °C, using the Trios software (version 5.6.0.87) from TA-Instruments-Waters LCC (New Castle, DE, USA).

2.4. In vivo studies

Animal studies were approved by the Committee on Animal Care at the Novo Nordisk Environment and Bioethics Committee; conducted with permission from the Danish Animal Experiments Inspectorate (rat studies: 2020-15-0201-00683; dog studies: 2020-15-0201-00731) in accordance with the guidelines of Danish legislation governing animal experimentation (1987) and the National Institutes of Health (publication number 85–23) and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985).

2.4.1. Oral administration to rats

The study was performed on 13 weeks old male Sprague Dawley rats purchased from Charles River (Germany). At day of dosing, rats weighed around 360 g. Prior to dosing, the rats were weighted, mock handled for 8 days, including restrain in the neck skin like the restrain used at the day of blood withdrawal, and fasted for 19 h. 12 rats were divided in two groups of 6 matched on body weight, with one cage containing one rat for each group. After the pretreatment period, all animals were given an oral gavage (dosing volume: 1 mL/kg) of the liquid formulations using a 20 cm tube (BBraun Original-Perfusor® PE, inner/outer diameter = 1/2 mm; Ref. 8,723,060). All syringes and tubes were primed with the relevant formulation for at least 10 min prior to dosing.

2.4.2. Endoscopic administration to dogs

The studies were performed using 1 to 7 years of age male Beagle dogs (mean weight 11.2–12.3 kg; SD 1.4–1.7 kg in the different studies). The housing rooms were maintained at a temperature of 19–23 °C and a

humidity level of 40–70 %. The light cycle was set to 12 h of light and 12 h of dark. Prior to dosing, the dogs were fasted 18–24 h, and were not offered water the last hour prior to dosing (or anaesthesia in case of endoscopy dosing). A venflon was placed in the cephalic vein on the day of dosing to prepare for anaesthesia and blood sampling.

When dosing, the dogs were anesthetized using propofol (after medetomidine sedation in all studies but the one involving dosing 3 times to the same spot). The dogs were then intubated, and the anaesthesia was maintained by isoflurane. At the end of the endoscopic procedure, atipamezole was given to aid the recovery in the dogs sedated with medetomedine. In each dosing, 1 g of the relevant formulations was delivered to the GI tract using a 200 cm tube (BBraun Original-Perfusor® PE, inner/outer diameter = 1/2 mm; Ref. 8,723,060), guided *via* the working canal of an endoscope. All syringes and tubes were primed with the relevant formulation for at least 10 min prior to dosing. The formulation was delivered either to the mid corpus of the empty stomach or to the small intestine (approx. 25 cm beyond the pyloric sphincter). During the dosing, air was used to mildly inflate the dosing region (folds still present), to allow visual orientation. In the study involving dosing 3 times to the same spot in the stomach, the endoscope was kept in place for 60 min, with continuous focus on the dosing area. Regular chow meal and water was allowed 240 min after dosing.

2.4.3. Oral administration to dogs

Male Beagle dogs, 1 to 7 years of age and weighing approximately 10–16 kg (except one around 18 kg) at the start of the studies, were used. The dogs were group housed in groups of 5–6 (12 h light: 12 h dark), fed individually and restrictedly once a day. The housing rooms were maintained at a temperature of 19–23 °C and a humidity level of 40–70 %. Exercise and group social were permitted daily, whenever possible. The dogs were used for repeated pharmacokinetic studies with a suitable wash-out period between successive dosing's. An appropriate acclimatization period was given prior to initiation of the first pharmacokinetic study. All handling, dosing and blood sampling of the animals were performed by trained and skilled staff. Before the studies, the dogs were fasted overnight until 4 h after dosing. Besides, the dogs were restricted to water from 1 h before dosing until 4 h after dosing, but otherwise had *ad libitum* access to water during the whole period. The solid dosage forms containing the therapeutic peptide were administered by placing it in the back of the mouth of the dog to prevent chewing. The mouth was then closed, and 10 mL of tap water was given by a syringe to facilitate swallowing of the tablet. The CHONAC formulations were dosed *via* oral gavage into awake dogs, in each dosing, 1 g of the relevant formulations was delivered to the GI tract using a 50 cm tube (BBraun Original-Perfusor® PE, inner/outer diameter = 1/2 mm; Ref. 8,723,060), guided *via* the working canal of an endoscope. All syringes and tubes were primed with the relevant formulation for at least 10 min prior to dosing.

Blood was sampled at predefined time points for up to 10 h post dosing to adequately cover the full plasma concentration-time absorption profile of the APIs. Blood samples were taken as appropriate, for example from a venflon in the cephalic vein in the front leg for the first 2 h and then with syringe from the jugular vein for the rest of the time points (the first few drops are allowed to drain from the venflon to avoid heparin saline from the venflon in the sample). For each blood sampling time point approximately 0.8 mL of whole blood was collected in a 1.3 mL EDTA coated tube, and the tube was gently turned to allow mixing of the sample with the EDTA. Blood samples (for example 0.8 mL) were then centrifuged at 4 °C and 1500 *g* for 4 min. Plasma was pipetted into Micronic tubes on dry ice and kept at −20 °C until analysis.

2.4.4. Blood sample bioanalysis

The plasma samples from rats and Beagle dogs were analyzed for the GLP-1 analogue using a Luminescence Oxygen Channeling Immunoassay (LOCI). A matched antibody pair (7F1 and 2F6) is involved in the assay, one biotinylated (2F6) and bound to streptavidin-coated Alpha donor beads, and the other conjugated to AlphaLISA acceptor beads (7F1). The binding of the two antibodies to analyte (GLP-1 analogue) brings donor and acceptor beads into proximity, resulting in the excitation of donor beads at 680 nm, triggering chemical reactions in the acceptor beads, and subsequently the emission at 615 nm. This emission signal is measured in the EnVision plate reader for API concentration analysis. The amount of light was proportional to the concentration of active peptide ingredient and the lower limit of quantification (LLOQ) in plasma was 500 pM. Calibrators and controls are in quadruplicates and each sample is measured in duplicates. Calibrators were fitted to a 5 PL curve and samples back calculated from the calibration curve. The samples were tested undiluted, 20× and 400× diluted to ensure that all samples were within calibration range and showed dilution linearity.

2.5. Statistical analysis

Statistical analysis of the animal data was performed by one-way ANOVA using GraphPad Prism version 9.0.1 (GraphPad Software Inc., La Jolla, California, USA).

3. Results and discussion

3.1. Synthesis and in vitro characterization of NAC-based Ionic liquids

To synthesize a NAC-based IL, a number of cations were first screened in a neutralization reaction with the acid form of SNAC, HNAC. Choline and tetrabutylammonium were identified as the two lead candidates forming ILs with NAC at room temperature. Considering the safety profile and availability for large scale manufacturing, choline, and specifically choline bicarbonate, was chosen for subsequent IL development. The neutralization reaction was carried out in stoichiometric ratios of HNAC and choline bicarbonate 1:1, forming carbon dioxide and water as reaction byproducts and obtaining an amber transparent liquid of choline N-(8-(2-hydroxybenzoyl)amino)caprylate, referred to as CHONAC (Fig. 1B and C). A drying process was then executed to remove the excess water arising from choline bicarbonate, thereby ensuring a high concentration of CHONAC and improving the long-term stability by decreasing hydrolysis (Fig. 1D). However, the viscosity of IL increases with solvent removal, leading to poor flowability and potentially slower release. The viscosity of ILs with different solvent content was analyzed at 37 °C to mimic *in vivo* conditions. Expectedly, a negative correlation between solvent content and viscosity was found (Fig. 2A and Table S1). A solvent content of 15 % *w*/w, resulting in a mean viscosity of approx. 1300 mPa s, was selected as the minimum threshold for ensuring a smooth, reliable and reproducible oral dosing as a liquid formulation.

The main advantages of PE-based ILs over tablet formulations are the higher PE concentration achievable in similar volumes of a solid dosage form, as well as the faster release of both PE and API. The maximum achievable NAC amount in 1 g of CHONAC was around 600 mg, which translates into a NAC amount of around 675 mg in 1 mL of CHONAC, given its density of around 1.126 g/mL. Tablets, given their apparent density being inherently lower than the true material density, would require a dramatically higher volume to accommodate the same amount of SNAC. Moreover, tablets would need additional excipients to ensure tablet manufacturability which would add more volume to the dosage form. All this typically translates into slower release of PE and API, dictated by tablet disintegration. To verify the higher availability of PE and API in solution for CHONAC, a release study was carried out in fasted state simulated gastric fluid at pH 3, 5 and 7. The release rate of API and PE from CHONAC with 600 mg/g NAC in a gelatin-based capsule was compared to that from a standard 300 mg SNAC-based tablet, the typical amount used in preclinical and clinical studies. A significantly faster release was observed for the IL compared to the tablet in all tested media, despite the higher PE amount in the IL formulation (Fig. 2B and Fig. S1). Specifically, full API and PE release was obtained at 9 min for CHONAC and at 20 min for the tablet. The

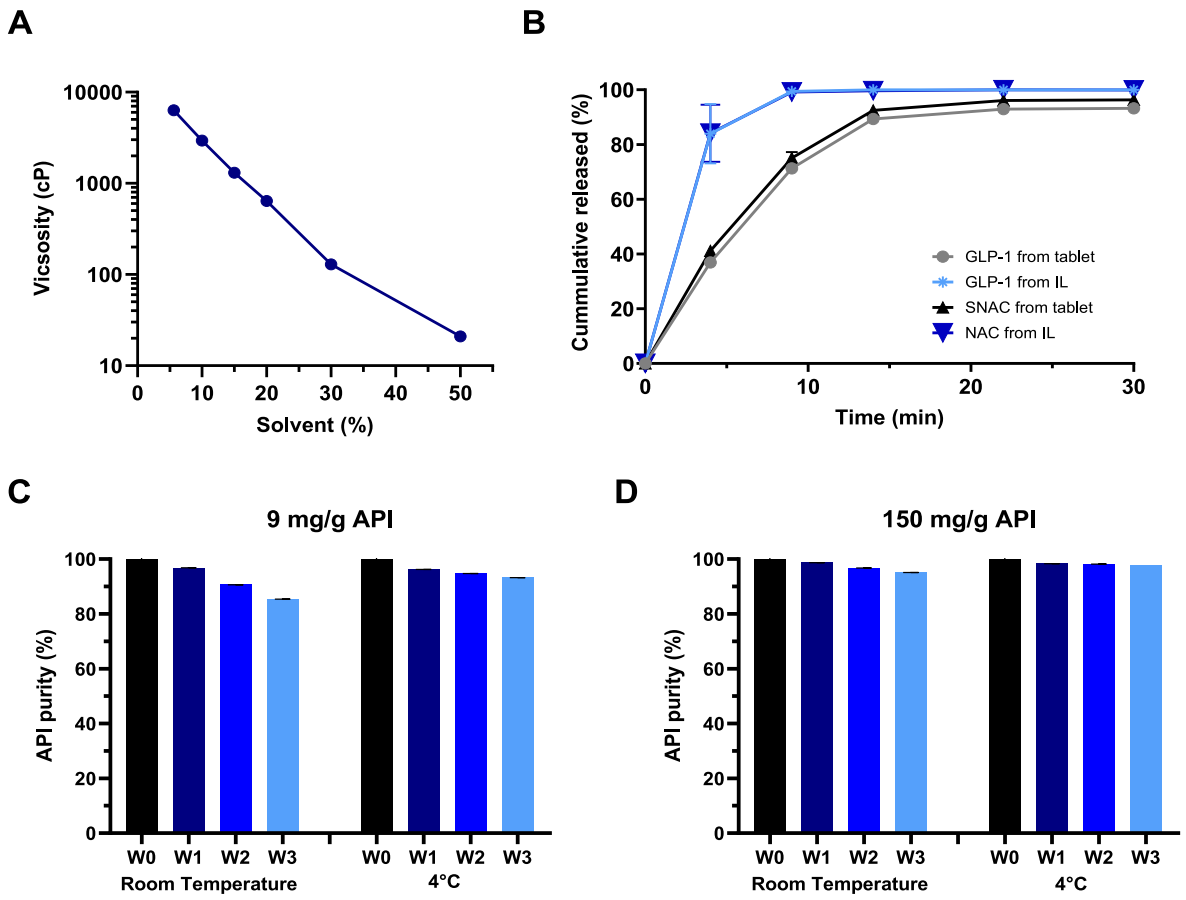

Fig. 2. (A) CHONAC formulation viscosity in function of water content (% *w*/w). Viscosity reported as cP measured at 37 °C. (B) Accumulative release profile (at pH 7) of CHONAC IL containing 3 mg/g API (with 17 % water) and 300 mg SNAC-based tablet containing 3 mg API. (C and D) API main peak purity quantification in CHONAC formulations at concentrations of 9 mg/g (with 600 mg/g NAC and 17 % water) and 150 mg/g (with 545 mg/g of NAC and 25 % water). Results shown from time 0, 1, 2 and 3 weeks stored at room temperature (25 °C) and 4 °C. Data shown as mean ± SD, $n = 3$.

initially slower release for CHONAC at pH 3 and 5 was attributed to the dissolution, and delayed rupture, of the capsule at those pH.

The physicochemical stability of CHONAC formulations containing 10 % to 40 % *w*/w solvent was monitored for 4 weeks at room temperature (25 °C). No apparent physical change was observed, and the NAC concentration remained constant over time. Simultaneously, an API stability study was carried out on the CHONAC formulations containing a GLP-1 analogue. After 3 weeks storage at room temperature (22 °C) or 4 °C, the API purity was analyzed by comparing the main peak under both conditions for each dose. The formulation with higher API concentration was less susceptible to chemical degradation, probably because of the lower water/API ratio. The API at both concentrations was more stable at 4 °C than at room temperature (Fig. 2C and D). Therefore, storage at 4 °C was used for the formulation and *in vivo* development.

3.2. *In vivo safety and efficacy evaluation of CHONAC in rats and dogs*

Considering the potential toxicity of ILs arising from their high ionic strength and the capability of NAC to perturbate cell membranes, the safety profile and efficacy of the CHONAC formulation were initially assessed in rats. A CHONAC formulation containing 3 mg/mL of a low potency, long elimination half-life GLP-1 analogue and 600 mg of NAC was dosed by oral gavage into the rat stomach. The API concentration in plasma was measured up to 3 h after administration. The rats were monitored during the study to see whether any adverse effect would occur. In parallel, an aqueous solution comprising 3 mg of the same API and 300 mg SNAC was dosed as a reference, due to the challenge in incorporating a higher amount of SNAC given its limited solubility in water [18,23]. Instead, IL allows to incorporate higher amounts of NAC in one single dose, overcoming the solubility issue. Over the course of administration, the animals did not show any noticeable abnormality or adverse reaction. The API plasma concentration for the CHONAC formulation was in a slightly higher range to that obtained with the reference liquid formulation (Fig. 3 A), which could be attributed to the lower SNAC content in the reference group. Overall, these results confirmed that CHONAC formulation was safe and effective *via* oral administration in rats, allowing us to test it in larger animal models.

Next, beagle dogs were used to evaluate the efficacy of the same CHONAC formulation compared to a tablet comprising 3 mg of the same GLP-1 analogue and 300 mg of SNAC. The CHONAC formulation was directly dosed into the gastric lumen *via* endoscopy in anesthetized animals. Compared to the SNAC-based tablet, endoscopically dosed in an anesthetized setting, an earlier absorption of the CHONAC formulation was observed (Fig. 3B). However, this did not translate into a higher API exposure, as reflected by the C_{max} (Table S2). In fact, the absorption stopped at 20 min for CHONAC, whereas it proceeded for 1 h in the case of the tablet. The delay of action for the tablet is probably associated with the disintegration and subsequent dissolution steps involved in the classical solid formulations before absorption takes place [24]. Instead, CHONAC as a liquid leads to a quick release of both API and PE, resulting in earlier absorption. Considering the detrimental impact of food intake on oral peptide absorption and the aim to minimize the undesired dosing conditions [14], *i.e.*, to reduce the time between dosing and food ingestion, a quick absorption is desirable. Therefore, further investigations were conducted to understand the key parameters affecting

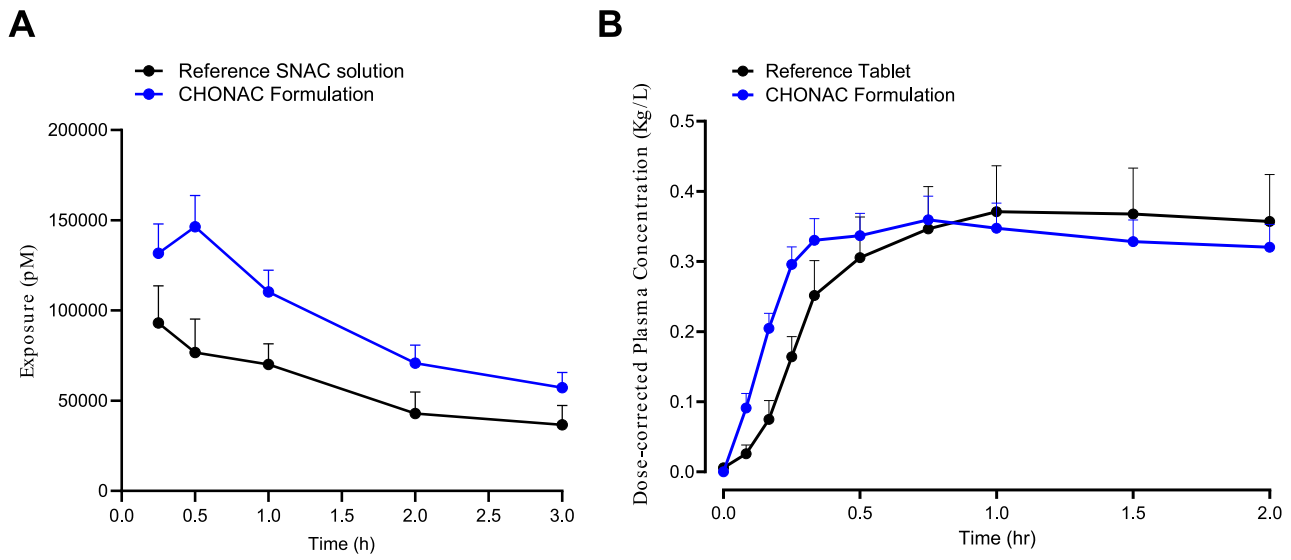

Fig. 3. (A). Rat plasma concentration of GLP-1 analogue from 1 g of CHONAC formulation containing 3 mg API, 600 mg NAC and 17 % water, dosed by oral gavage in the stomach. Mean ± SEM, $N = 6$. (B). Dog dose-corrected plasma concentration of GLP-1 analogue from 1 g of CHONAC formulation containing 3 mg API, 600 mg NAC and 17 % water, and a reference tablet containing 3 mg API and 300 mg SNAC, dosed *via* endoscopy in the stomach under anaesthesia. Mean ± SEM, $N = 8$.

the absorption behavior of the CHONAC formulation.

3.3. Screening of CHONAC formulation parameters by in vivo absorption in awake dogs

Following the tests in anesthetized dogs, an *in vivo* study in awake dogs was performed by dosing CHONAC *via* oral gavage, using the same tablet formulation as control. In this case, even though the plasma API exposure with CHONAC was higher than with the tablet during the first 15 min of absorption, it was notably lower from the 30 min time point over the 2-h frame following oral administration (Fig. 4). In fact, the absorption curve for CHONAC flattened significantly faster than for the tablet. The discrepancy in CHONAC absorption between anesthetized and awake setting may stem from the different physiological conditions. Factors such as gastric secretion and motility, which are greatly reduced under anaesthesia [25,26], may impact the behavior of the formulation.

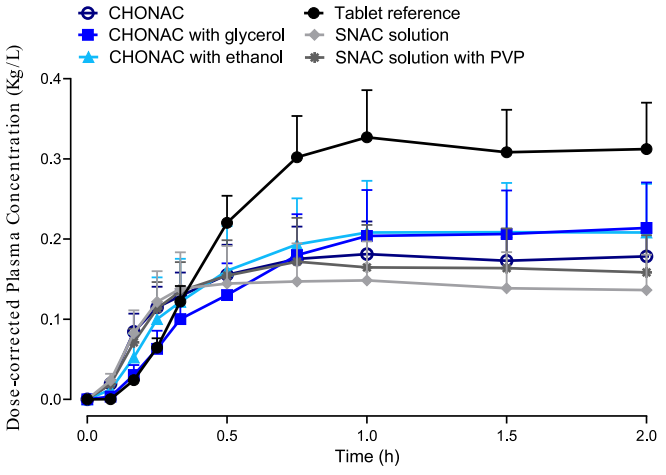

Fig. 4. Plasma concentration of low potency GLP-1 analogue following oral gavage of 1 g CHONAC formulations made with different solvents, a tablet reference comprising 300 mg SNAC, or liquid SNAC formulations with or without 5 % *w*/w PVP K90 to awake beagle dogs. CHONAC contains a total of 17 % w/w solvent, constituting either water alone, water: glycerol 1:4 or water: EtOH 7:10. API dose set to 3 mg per formulation in all cases, data expressed as mean ± SEM, $N = 16$.

First, a higher secretion of gastric acid might result in precipitation of the readily dissolved NAC from CHONAC into HNAC, compromising its permeation enhancing capability [10]. Furthermore, gastric motility may affect the epithelial area covered by the formulation; in this context, the formulation viscosity may also play a major role, as for example a low viscosity could lead to a wider spread of the formulation over the gastric epithelium, causing lower local concentrations of API and PE which may reduce the peptide absorption [9,27]. At the same time, high formulation viscosities may influence the motion of API and PE molecules in the formulation matrix, diminishing the diffusion of API molecules and impacting their migration towards the gastric epithelium [28,29], which could ultimately inhibit the formulation performance.

In an effort to better understand and mitigate these potential effects, two additional formulations made with high and low viscosity solvents, *i.e.* glycerol and ethanol were tested (Fig. S2). In both cases, the API exposure remained unchanged when compared to the standard CHONAC formulation (Fig. 4). This suggested that tuning the mobility and flowability of the formulation was not successful in improving the formulation performance in terms of API absorption. In addition, to potentially mitigate PE crystallization due to gastric secretion, the use of precipitation inhibitors was explored with the aim of maintaining NAC in solution after dosing in a low pH environment. In this case, an aqueous solution of 300 mg/mL of SNAC was prepared in combination with 20 mg/mL of polyvinylpyrrolidone (PVP K90) [30,31], which has proven to efficiently prevent SNAC precipitation into HNAC at low pH in simulated gastric fluid media in a dose dependent manner (Fig. S3). Nevertheless, the absorption profile of the aqueous solutions with and without PVP K90 showed the same evolution (Fig. 4 and Table S3).

Despite multiple compositional changes in these liquid formulations, the variation of plasma API exposure was minimal between different groups (Fig. 4 and TableS3). The earlier absorption onset was observed for each formulation, except for CHONAC with glycerol where the high viscosity might impact the early API release. However, the absorption of the liquid formulations consistently reached the same C_{max} level and stopped earlier than the tablet reference. We speculate that the gradual disintegration of the reference tablet might provide a sustained release of API and PE, leading to a prolonged absorption profile. Instead, liquid formulations, either ILs or API/NAC solutions, are prone to a quick dilution in the gastric fluid followed by spreading over a larger area in the stomach, and potentially a faster transit into the duodenum. This may explain the observed earlier reduction in absorption rate.

3.4. Impact of gastric dilution and residence time on in vivo absorption in dogs

Bækdal et al. demonstrated the detrimental effect of excess water dosed with SNAC-based tablets, indicating the importance of a considerable local concentration of API and PE in the vicinity of the gastric epithelium [24]. Therefore, to understand the impact of dilution for CHONAC in the gastric lumen, we assessed the performance of the CHONAC formulation with and without addition of 10 mL water in awake dogs. When adding 10 mL of water post-dosing, the C_{max} drastically decreased, rendering the API plasma exposure nearly negligible (Fig. 5). Hence, the sensitivity of ILs to aqueous dilution seems far beyond that of a solid dosage form, where only minor reductions were observed [24]. This may partially explain the compromised pharmacokinetics for CHONAC following oral administration in the awake setting. To mitigate the quick dilution of CHONAC while landing in the stomach and provide additional confinement to the formulation, a gelatin-based capsule containing CHONAC was tested *in vivo*. However, only a minor difference was found between the encapsulated CHONAC and the naked CHONAC liquids when dosed orally. Moreover, both formulations resulted in lower API plasma exposure than the tablet formulation (Fig. S4). It thus appears that the physical state of the dosage form, *i.e.*, solid or liquid, remains the dominant differentiator.

Another key aspect potentially impacting the pharmacokinetics of the CHONAC formulation is the quick transit from the stomach to the small intestine. To elucidate its potential intestinal absorption, a CHONAC formulation was dosed endoscopically to the distal duodenum of anesthetized dogs. The API plasma concentration was measured and then compared to intragastric dosing of the CHONAC formulation to anesthetized dogs. The API absorption in the duodenum was significantly lower than in the stomach (Fig. 6A). This could be attributed to the higher proteolytic activity in the small intestine that can more efficiently degrade unstable peptides as well as to the potentially lower permeation-enhancing capability of CHONAC in the intestinal region. Bækdal et al. showed the importance of keeping SNAC-based formulations of semaglutide in the stomach as long as possible to maximize absorption [32]. In another study, Mitragotri and co-workers demonstrated the efficient performance of CAGE, an IL system made of choline and geranic acid, for the delivery of human insulin in the small intestine of rats [21]. However, no direct comparison with our study can be made, given the different API properties, PE mechanisms of action and animal models. To further understand the fate of CHONAC following oral administration, paracetamol was used as a marker of gastric emptying in combination with the formulation. Indeed, paracetamol is a well-studied drug that is primarily absorbed in the small intestine rather than by the gastric tissue [33]. Compared to the SNAC tablet comprising both the peptide and paracetamol, an earlier onset of paracetamol was found with the CHONAC formulation after oral dosing in awake dogs (Fig. 6B). As a result, T_{max} was also reduced, suggesting a faster gastric emptying of CHONAC than the tablet. Considering that the CHONAC performance following intraduodenal dosing was significantly inferior to gastric dosing (Fig. 6A), the decrease in gastric residence time is likely to explain, at least partially, the lower exposure provided by CHONAC than the tablet after oral delivery in awake dogs.

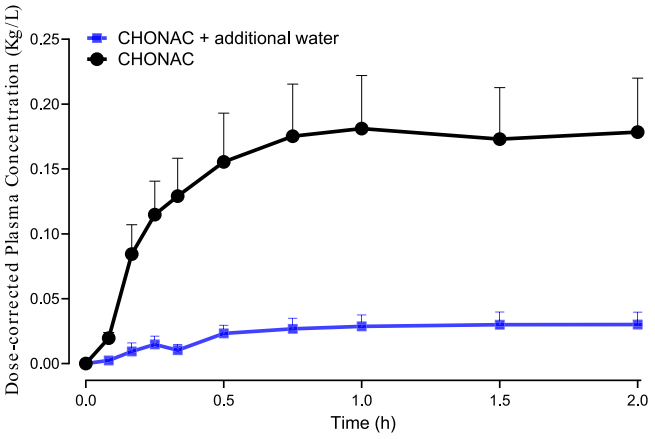

Fig. 5. Plasma concentration following oral gavage administration in awake dogs of 1 g CHONAC formulations with and without post-dosing 10 mL of water. CHONAC contains a total of 17 % *w*/w solvent. Data expressed as mean ± SEM, $N = 8$.

3.5. Opportunities for improved absorption of liquid formulations for gastric peptide delivery

The suboptimal pharmacokinetic profile of CHONAC compared to solid dosage forms revealed two major issues of ILs, and of liquid formulations in general, for gastric peptide delivery: (i) significant dilution by gastric fluids that decreases the concentration of API and PE needed to drive absorption, and (ii) faster gastric emptying leading to compromised absorption in the intestine for peptides liable to proteolytic degradation. Given the need for a prolonged interaction between the gastric tissue and the formulation to improve API absorption, we investigated whether repeated dosing of liquid formulations with a constant SNAC concentration at different time points could sustain absorption. An *in vivo* study in anesthetized dogs was thus conducted by repeatedly dosing endoscopically in the same gastric area, a SNAC solution containing the GLP-1 analogue; the liquid was administered at time zero, after 20 min and 40 min, while monitoring the plasma concentration throughout the study. In this case, a constant and nearly linear absorption rate (R^2 0.991) was obtained throughout the dosing window until 1 h, *i.e.*, 20 min after the last dosing (Fig. 7A). Hence, the supply of fresh liquid formulation at each time interval was able to sustain the API absorption, at both the tested doses of 3 mg/mL and 50 mg/mL. Moreover, no change in absorption rate was found between liquid applications meaning that the absorptive capacity of the tissue was not saturated. Notably, the plasma exposure was significantly higher from the 20 min time point than the previous *in vivo* tests using either a tablet or a liquid formulation (Fig. 7B), inheriting both the quick onset capability of liquid formulations and the long-lasting effect of this multi-time dosing. These results suggest a path for overcoming the inherent drawbacks of liquid formulations such as ILs for gastric peptide delivery as well as call for further formulation strategies based on sustained release.

4. Conclusions

In this work, a stable IL formulation consisting of choline salcaprozate (CHONAC) was developed for gastric delivery of a GLP-1 analogue. While retaining the same permeation enhancing capabilities of SNAC, the corresponding PE used in oral solid dosage forms, CHONAC can offer a higher PE concentration for the same volume of dosage form as well as a faster release of both peptide and PE. This led to an earlier absorption onset of the CHONAC formulation than the tablet reference *in vivo* in both anesthetized and awake dogs. However, the absorption rate for CHONAC decreased faster than the tablet following oral dosing to awake dogs, thereby resulting in lower API plasma exposure. This was attributed to the inherent challenges associated with gastric physiology, namely dilution in the presence of additional fluid as well as fast gastric emptying of liquids. Peptides liable to proteolytic degradation such as the GLP-1 analogue in this study could not benefit by additional absorption in the small intestine, potentially also due to the lower effectiveness of CHONAC in the duodenal region in enhancing permeation. Hence, due to their physical nature and to the gastrointestinal physiology, ILs and in general liquid formulations would struggle to ensure a high local concentration of peptide and PE for a prolonged period in the stomach, which is necessary to drive absorption. To overcome this, a

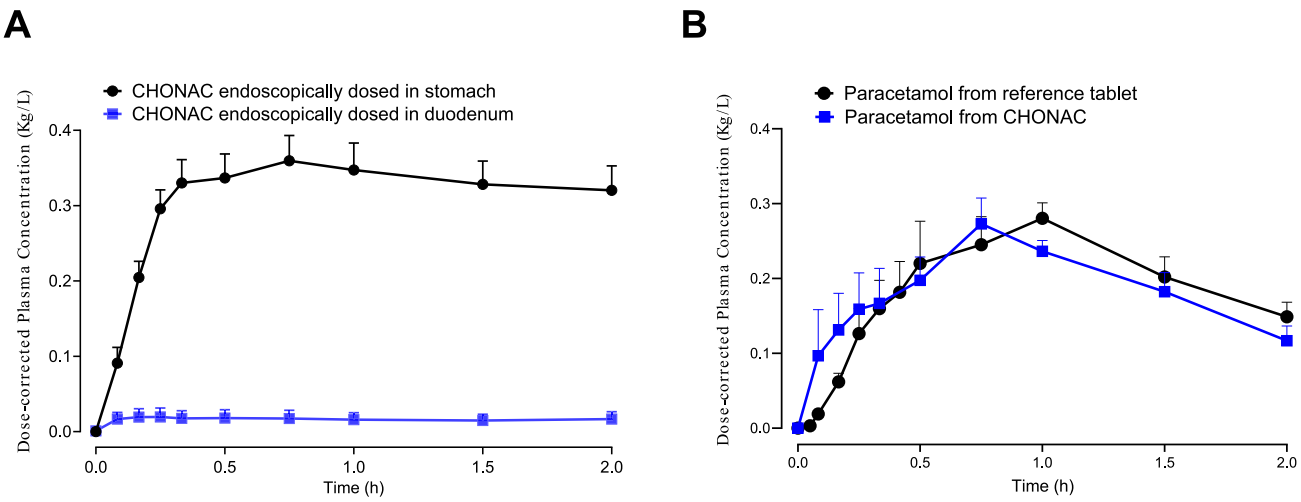

Fig. 6. (A) Plasma concentration of low potency GLP-1 analogue following endoscopic dosing of 1 g CHONAC formulations to the stomach and to the small intestine in anesthetized dogs. (B) Plasma concentration of paracetamol following oral gavage in awake dogs of either 1 g CHONAC formulations or a tablet formulation containing the GLP-1 analogue and paracetamol. CHONAC contains a total of 17 % *w*/w solvent. API dose set to 3 mg per animal. Data expressed as mean ± SEM, $N = 16$.

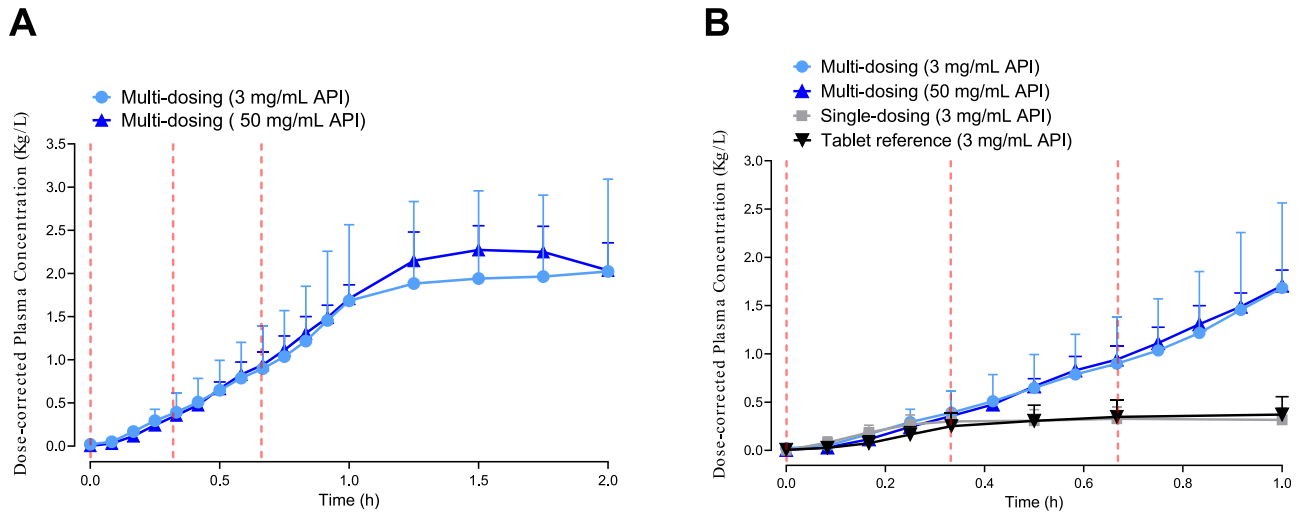

Fig. 7. (A) Dose-corrected plasma concentration of low potency GLP-1 analogue following a continuous endoscopic dosing of 1 mL of liquid formulation to the stomach at 0 min, 20 min, and 40 min, indicated with red lines. Each formulation contains 300 mg/mL SNAC and either 3 mg/mL or 50 mg/mL of API. (B) Zoom into the first hour where the red lines indicate the dosing times, with reference to a single-dosed liquid SNAC formulation and tablet formulation with 3 mg/mL of the same API. Data expressed as mean ± SEM, $N = 16$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proof-of-concept study consisting of repeated dosing of liquid formulations with a constant SNAC concentration every 20 min for three times was carried out, demonstrating the potential to sustain peptide absorption. Therefore, ensuring a long retention time of peptide and PE on the stomach epithelium at a significant concentration is key for gastric delivery of biomacromolecules and a special challenge for IL-based and other liquid formulations.

CRediT authorship contribution statement

René Rebollo: Writing – original draft, Visualization, Project administration, Investigation, Data curation. **Zhigao Niu:** Writing – original draft, Supervision, Investigation, Formal analysis. **Lasse Blaabjerg:** Writing – review & editing, Project administration, Methodology, Investigation. **Damiano La Zara:** Writing – original draft, Visualization, Investigation, Formal analysis. **Trine Juel:** Writing – review & editing, Investigation. **Henrik Duelund Pedersen:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Vincent Andersson:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation. **Michaela Benova:** Writing – review & editing, Methodology, Investigation, Data curation. **Camilla Krogh:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation. **Raphaël Pons:** Writing – review & editing, Methodology, Investigation, Data curation. **Tobias Palle Holm:** Writing – review & editing, Methodology, Investigation, Data curation. **Per-Olof Wahlund:** Writing – review & editing, Visualization, Methodology, Investigation. **Li Fan:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Data curation. **Zhuoran Wang:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation. **Adam Kennedy:** Writing – review & editing, Supervision, Project administration. **Rune Ehrenreich Kuhre:** Data curation, Formal analysis, Project administration, Writing – review & editing. **Philip Christophersen:** Writing – review & editing, Supervision, Resources, Project administration. **Pierre-Louis Bardonnet:**

Writing – review & editing, Supervision, Project administration. **Philip Jonas Sassene:** Writing – review & editing, Supervision, Conceptualization. We thank Dr. Christian Wenzel Tornøe for providing guidance in the development of the NAC base IL, the support from Dr. Stefán Bragi Gunnarsson, Anders Hagedal Uhrenfeldt, Ann-Christin Løff, Trine Rørmose Løjmand, Charlotte Gustafsson, Helle Andersen and Dr. Jeppe Sturis during the in vivo animal studies; and the contribution of Dr. Andrew James Benie, Dr. Jian Xiong Wu, Henning Gustafsson, Elisa Ross and Susanne Bjerggaard Juul-Mortensen in the physical characterization of the formulations.

Data availability

The data that has been used is confidential.

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The graphical abstract and Fig. 1 were created with resources from Biorender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2024.11.036.

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