Improved Oral Bioavailability of Human Growth Hormone by a Combination of Liposomes Containing Bio-Enhancers and Tetraether Lipids and Omeprazole

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ABSTRACT: Liposomes for the oral delivery of human growth hormone (hGH) containing bio-enhancers and tetraether lipids were prepared by dual asymmetric centrifugation. Cetylpyridinium chloride (CpCl), D-α-tocopheryl polyethylene glycol 400 succinate, phenylpiperazine, sodium caprate or octadecanethiol were used as permeation enhancers. *In vitro* data showed that oligolamellar vesicles with average size in the range of 200–250 nm were formed. Performance of the formulations was investigated both *ex vivo* by confocal microscopy scans of sections of rat small intestine and *in vivo* by comparing the area under the plasma curve of hGH after oral or subcutaneous (s.c.) application. The microscopic data reveal an interaction between the liposomal formulation and the intestinal mucus layer. Particularly one formulation, which was designed to be mucus penetrative by addition of a high quantity of TPGS 400 and a ζ-potential close to 0 mV, showed a very strong mucus association in the duodenum and jejunum. Vesicles with CpCl 33% (mol/mol) led to a relative hGH bioavailability of 3.4% compared with s.c. control, whereas free hGH administered orally showed a bioavailability of only 0.01%. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3985–3993, 2014

Keywords: dual asymmetric centrifugation; lipogelosomes; archae; imaging methods; oral absorption; proteins; liposomes; permeation enhancers

INTRODUCTION

Oral peptide and protein delivery remains one of the major tasks in drug delivery, despite the considerable amount of attention paid to this field over the last years.[1,2] On the contrary, because of recent advances in biotechnology, the number of available protein drugs is constantly increasing and therapeutic use becomes more cost-effective.[3] For long and repeated use, oral application is by far the most convenient route for drug delivery. However, proteins, when applied orally, are easily degraded because of low-stomach pH and digestive enzymes, and their size and hydrophilicity lead to a poor intestinal absorption.[4,5] Absorption enhancers, such as small molecule carriers, surfactants and enzyme inhibitors, but also particulate systems, for instance, nanoparticles and liposomes, were used to stabilise proteins in the gastrointestinal tract (GIT) and to improve permeation through the intestinal epithelium.[4,6–11]

Liposomes represent a highly versatile delivery system in terms of composition, size and production method with a good biocompatibility and were already investigated for the oral delivery of peptide drugs.[12–14] Nevertheless, they have some principal drawbacks as oral delivery system for macromolecules such as proteins. Liposomes exhibit a limited stability in the GIT and have to be stabilised, for example by the use of phospholipids with high phase transition,[15] membrane-spanning lipids[16] or polymer coating.[17] Moreover, conventionally prepared liposomes usually show rather low encapsulation efficiencies for hydrophilic drugs.[12,18] This can be overcome by the use of vesicular phospholipid gels (VPGs). These are semi-solid, aqueous phospholipid dispersions that exhibit high drug loading for hydrophilic molecules independent of their charge or size.[19,20] These VPGs can be either prepared by high-pressure homogenisation or by dual asymmetric centrifugation (DAC), a technique recently introduced for VPG preparation by Massing et al.[21,22]

Recently, we showed that liposomes containing bio-enhancers can improve the permeation of a macromolecule *in vitro* [23] and that the tetraether lipid glycerylcaldityl tetraether (GCTE) can stabilise liposomes containing bio-enhancers in simulated gastrointestinal fluids.[24] One goal of the present study was to test the suitability of the DAC technology for the preparation of liposomes containing GCTE and different bio-enhancers and to characterise their physical properties. In addition, the fate of fluorescently labelled liposomes containing bio-enhancers and GCTE in the rat intestine was investigated *ex vivo* by confocal microscopy to gain a better understanding of the correlation between bioavailability improvement and liposome interaction with the mucus barrier and mucosa. The suitability of this type of liposomes for oral protein delivery was examined in rats with human growth hormone (hGH), a 191 amino acids protein with a molecular weight of

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approximately 22 kDa, as model drug. In paediatric use, hGH has to be administered daily over a long time period, which makes an oral delivery form desirable, particularly regarding an application to younger children.[25] Although we could show in a previous study that liposomes stabilised with GCTE do not exhibit leakage of macromolecules at pH 2, experimental results suggest that small, charged molecules such as protons can permeate into the aqueous core of the liposomes,[24] which could lead to the denaturation of encapsulated hGH. Thus, to overcome this stability issue, animals were pre-treated before the administration of liposomal encapsulated hGH either with the proton-pump-inhibitor omeprazole to raise stomach pH or liposomes were further stabilized by incorporating 15% gelatine in the vesicle core or the VPGs were freeze-dried and encapsulated in enteric-coated capsules.

MATERIAL AND METHODS

Materials

EPC was gifted from Lipoid (Ludwigshafen, Germany). GCTE was provided from Bernina Plus (Planegg, Germany). D-α-Tocopheryl polyethylene glycol 400 succinate (TPGS 400) was supplied by Eastman (Kingsport, Tennessee). Cetylpyridinium chloride (CpCl) was purchased from Roth (Karlsruhe, Germany). Cholesterol (Chol), sodium caprate (Capr), FITC-dextran (70 kDa), octadecanethiol (OT) and 1-phenylpiperazine (PP) were purchased from Sigma–Aldrich (Taufkirchen, Germany). hGH (Genotropin®) was obtained from Pfizer Pharma (Berlin, Germany) and omeprazole from 1A Pharma (Oberhaching, Germany). Rhodamine–dipalmitoyl phosphatidylethanolamine (Rh-DPPE) was purchased from Otto Nordwald (Hamburg, Germany). LB gelatine was provided by Gelita AG (Eberbach, Germany). All other chemicals were obtained in the highest purity from the usual commercial sources.

Liposome Preparation

For both the *ex vivo* and the *in vivo* study, liposomes were prepared in a similar way by DAC according to a method previously described.[21] The lipid composition of all formulations for the bioavailability study is listed in Table 1. In case of the microscopic study, the same compositions were used with only a small amount (0.25%) of the fluorescent lipid Rh-DPPE added to the formulation without changing the ratio of the other lipids. Lipid mixtures were prepared beforehand in excess. Therefore, a total amount of 1 g of the different lipids and enhancers were weighed in the exact ratio needed for the different formulations into a round-bottom flask. Approximately 20 mL of a chloroform–methanol (9:1) mixture was added to dissolve all substances. Subsequently, the solvent was removed in a rotary evaporator (Rotavapor-R; Büchi Labortechnik AG, Flawil, Switzerland) and the films were kept for an additional 12 h under high vacuum to remove all solvent traces. For confocal microscopy, 25 mg of lipids mixed in the desired molar ratio with 0.25% (m/m) Rh-DPPE were weighed into a 2-mL cup together with approximately 80 mg of glass beads (1 mm diameter). 37.5 μL of phosphate-buffered saline (PBS) pH 7.4 (NaCl 137 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM, Na_2HPO_4 8.1 mM) were added, and the cups were mixed for 30 min at 3540 rpm in a dual asymmetric centrifuge (DAC 150 FVZ; Hauschild, Hamm, Germany) using a special vial holder as described by Hirsch et al.[26] Finally, the VPGs were further diluted with 0.5 mL of PBS in a 30 s mixing step.

Table 1. Ratio (mol/mol) of the Different Lipids and Bio-Enhancers in the Tested Liposomal Formulations

	EPC	GCTE	Chol	TPGS	CpCl	1-PP	Caprate	OT
CpCl-pos	4	1	2.4		3.6			
CpCl-neutr	4	2	4	4	3		3	
PP/OT	4	1	3.6	1.2		5		1.2
PP/Capr	4	1	2	4		5	5	

As for the *in vivo* experiments, larger batches were prepared, and the mixing protocol was slightly modified. hGH was reconstituted with distilled water to a concentration of 80 mg/mL. One-hundred milligram of lipids was weighed into a 2-mL cup; 250 mg of glass beads (1.5 mm diameter) and 150 μL of hGH 80 mg/mL were added. If mentioned, 15% of gelatine (m/V) was added to the hGH solution prior to liposome formation. Subsequently, the cups were mixed as described above, whereas this time the mixing process was stopped every 10 min and the mixer was allowed to cool down for 20 min to avoid overheating of the lipid protein mixture. VPGs were further diluted with PBS to achieve a protein concentration of 16 mg/mL. In one case, the formulation was freeze-dried after mixing. To remove the glass beads, the VPG was centrifuged through an 80-μm polyamide monofil filter (NeoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg, Germany) fixed on the top of a 2-mL cup at 16.1 rcf for 1 min. Subsequently, the formulation was dried in a Delta 1–20 KD freeze-drier (Christ, Osterode am Harz, Germany) under following conditions: −40°C for 6 h (freezing), −30°C for 40 h (primary drying) and 15°C for 8 h (secondary drying). The dried gel was encapsulated into PCcaps® size 9 capsules (Capsugel, Cambridge, UK) and subsequently capsules were enteric coated by dipping in a Eudragit® L 100 (Evonik, Darmstadt, Germany) solution in acetone/isopropanol/water followed by a short heating period with a common hairdryer to allow film formation.

Size and ζ-Potential Determination

Liposomes were diluted with PBS to an appropriate concentration and Z-average and polydispersity index (PDI) was determined using a Zetasizer® 3000 HS (Malvern, Works, UK) in the automatic mode. Values for each formulation were calculated from three main runs consisting of 10 sub-runs. In terms of ζ-potential, particles were diluted in PBS and values were calculated from 10 measurements.

Determination of hGH Encapsulation

Two-hundred micro-litre of liposome dispersion was given on a Sepharose® CL-4B column to separate non-encapsulated hGH. Liposomes were further diluted 1:10 with Triton-X 1% in PBS, as control-uncolumned vesicles were diluted 1:100 with Triton-X 1% in PBS. hGH concentration was determined by HPLC with a Dionex UltiMate® 3000 system (Dionex, Idstein, Germany) using an Acclaim® 120 C18 5-μm column (4.6×250 mm^2) at 50°C and a UV PDA detector. Flow was kept constant during the run at 1 mL/min with 20% water plus 0.05% trifluoroacetic acid (TFA) and 80% acetonitrile plus 0.05% TFA as mobile phase. hGH concentration was determined at 218 nm against a calibration curve.