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Research paper

Development and in vitro evaluation of a liposome based implant formulation for the decapeptide cetrorelix

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Abstract

Semisolid phospholipid dispersions of vesicular morphology, so-called vesicular phospholipid gels (VPGs), were prepared by high-pressure homogenisation and tested in vitro for their suitability as implantable sustained release system for the decapeptide cetrorelix, a potent LH-RH antagonist. The VPGs contained 300–500 mg/g egg phosphatidylcholine (E80) and 0.5–10 mg/g cetrorelix acetate (CXA). The in vitro release experiments showed a wide variability of the system in release, ranging from complete release within less than 24 h (0.5 mg/g CXA; 400 mg/g E80) to a predicted 80% sustained release over 3 months (8.6 mg/g CXA; 280 mg/g E80). Erosion of the phospholipid matrix, i.e. release of phospholipid vesicles was found to be the main release mechanism, following zero order or first order kinetics depending on the composition of the VPG. CXA-concentration dependent drug–drug or drug–lipid interactions are assumed to be responsible for the change in release kinetics and the decrease of CXA release at high concentrations of the peptide. Multivariate analysis revealed that both lipid concentration and peptide concentration and also the interactions between the two factors are significant factors for the release rate of the peptide. In summary: based on the presented in vitro release data sustained release of therapeutically relevant CXA doses over up to 6 weeks appears feasible. VPGs are thus considered as a promising new approach for the sustained release of peptide hormones.

Keywords: Cetrorelix; Peptide; Vesicular phospholipid gel; Liposome; Depot system; Sustained release; In vitro release

1. Introduction

Many promising new drugs are of peptide or protein origin opening new dimensions in the treatment of diseases. The main challenges in the use of peptide drugs are to overcome their low bioavailability and instability. Peptides are subject to degradation reactions, e.g. by deamidation, Maillard reaction, oxidation and hydrolysis. Degradation, which may also happen in the solid state, is generally accelerated in solution. Extreme conditions like a low pH value, such as in the stomach, usually accelerate degradation leading to a poor bioavailability. Peptides' relatively high molecular mass along with high numbers of charges also limit their per oral bioavailability in terms of absorption. In consequence the oral route is usually not optimal for the administration of peptides and the parenteral route has to be chosen. However, many peptides are still subject to enzymatic degradation by, e.g. peptidases once in the blood compartment, which means that chemical alterations (such as insertion of protective groups or of unnatural amino acids) often have to be performed in order to achieve a drug, which is more resistant against those enzymes. Analogues and antagonists of the luteinising hormone-releasing hormone (LH-RH) have gained growing therapeutical interest over the last years. Various applications have been established in the fields of oncology and gynaecology [1]. The major advantage of LH-RH antagonists, such as cetrorelix, over

Abbreviations: CXA, cetrorelix acetate; PC, phosphatidylcholine; E80, PC-rich fractions of egg lecithin; LHRH, luteinising hormone-releasing hormone; VPG, vesicular phospholipid gel; PCS, photon correlation spectroscopy; PCA, principal component analysis; PLS, partial least square regression; FSH, follicle stimmulating hormone.

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agonists is that they inhibit the secretion of LH and FSH immediately after application [1], while agonists and super agonists lead to an undesired flare-up of hormone secretion in the first line before the desired reduction of hormone level is achieved [2]. Potential applications for LH-RH antagonists can be found in the treatment of, e.g. infertility, benign prostate hypertrophy and sexual hormone-dependent tumours [3–5]. Especially for the latter continuous treatment over long time is usually needed. A recent review on the role of peptide analogues in the therapy of prostate cancer has been published by Schally [6].

The third-generation LH-RH antagonist cetrorelix first synthesized by Bajusz [7], is free of the histamine releasing side effects, which hampered the use of the first generation of LH-RH antagonists. Though the stability has been increased as compared to natural LH-RH, cetrorelix still has to be given parenterally as the bioavailability upon oral administration is below 0.1% and even by various approaches could not be raised above 1.6% [8]. As daily injections are undesirable for long-term treatment, implantable depot formulations appear to be ideal. Several groups are focusing on polymer implant systems for peptide hormones; however, no polymer-based formulation for LH-RH antagonists has yet reached the market. Vesicular phospholipid gels (VPGs), i.e. semisolid, aqueous phospholipid dispersions of (multi-) vesicular morphology, have recently been suggested as novel biodegradable depot systems [9,10]. The aim of this project was to study their potential use as sustained release systems for peptide hormones using the LHRH-antagonist cetrorelix acetate (CXA), a decapeptide consisting of natural and non-natural L and D-amino acids, as model compound.

2. Materials

Cetrorelix acetate (CXA) was a generous gift from Zentaris (Frankfurt, Germany) and contained around 90% cetrorelix. Phosphatidylcholine (PC)-rich fractions of egg lecithin containing at least 80% PC (E80) were supplied by Lipoid GmbH (Ludwigshafen, Germany). Other major compounds contained in E80 are phosphatidylethanolamine (7.0–9.5%), lyso-PC (not more than 3%), sphingomyelin (2–3%) and triglycerides (not more than 3%). E80 is in clinical use for parenteral administration of both i.v. drug emulsions (e.g. for Propofol, Vitamins, Diazepam) and parenteral nutrition (e.g. Intralipid®, Lipofundin®) and has been chosen for this investigation due to its proven parenteral applicability in humans. Trifluoroacetic acid (TFA) and Triton X-100 were purchased from Sigma (Steinheim, Germany).

Ringer solution as acceptance medium for the release test consisted of 8.6 g NaCl, 0.3 g KCl and 0.49 g $CaCl_2 \cdot 6H_2O$ dissolved in one litre distilled water (A. Sarlikiotis, Zentaris, Frankfurt, Germany, personal communication).

3. Methods

3.1. Experimental design (set-up)

The effect of concentration of phospholipid and CXA on the release rate was first studied by a uni-variate approach, but was later extended in order to allow examination of interactions and non-linear behaviour of the factors. VPGs were prepared with phospholipid concentrations ranging from 300 to 500 mg/g, and concentration of CXA ranging from 0.5 to 10 mg/g. The actual amount of phospholipid and CXA were determined in the final gel (Fig. 1).

3.2. Preparation of semisolid phospholipid dispersions

The preparation procedure for VPGs in principle followed the method first described by Brandl et al. [11]. CXA was incorporated by direct loading, i.e. phospholipid and solutions of CXA in distilled water were blended and allowed to swell for about an hour before being fed into the high-pressure homogeniser (APV Micron Lab 40, APV Homogeniser, Lübeck, Germany). The mixture was homogenised 10 times at 70 MPa. After homogenisation the gels were stored in capped glass vials at 2–8 °C. The batch size was between 30 and 35 g.

3.3. Particle size analysis

The size and the distribution of the vesicles upon appropriate dilution of the gels were measured by photon correlation spectroscopy (PCS) as carried out by Ingebrigtsen [12].

3.4. In vitro release test

The method and the custom-made release cells (Peter Wiest, Institute for Pharmacy, University of Freiburg,

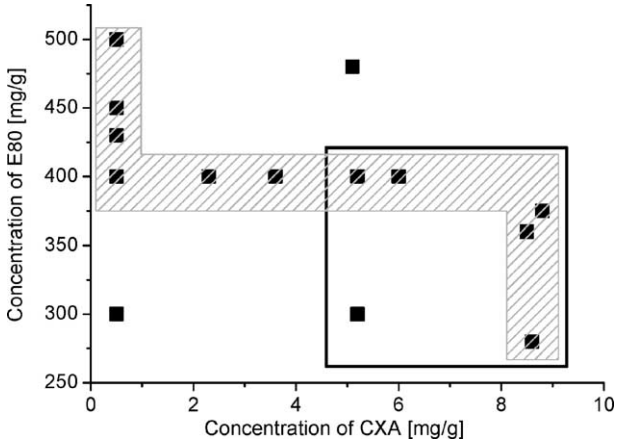

Fig. 1. Combinations evaluated. Grey pattern areas indicate the combinations originally evaluated in a uni-variate manner. The solid square indicates the experimental area for times > 3 days. Result of 300 mg/g E80 and 0.5 mg/g CXA based on educated guess.

Germany) for release testing used in this study were originally described by Tardi [10]. The cells were filled with approximately 1 g of accurately weighed gel and kept at 37 °C during the release test by submersing into a water bath. In accordance with the original method the flow rate was set to 10 ml/h. As not only the diffusion of drug out of the liposomes should be studied, but also the erosion of the vesicular gels, no semi-permeable membrane separated acceptor and donor. The above-mentioned settings (flow rate, temperature, no artificial membrane) resemble in vivo conditions and should provide a reasonable correlation between in vitro and in vivo results; however, VPGs have not been evaluated in vivo yet and a definite statement on in vitro/in vivo correlation is not possible. The fractions were collected in vials which already contained 10% of the final volume of the fraction of 27.5% Triton X-100. This was used in order to disrupt the liposomes and at the same time to reduce the adsorption of cetrorelix to the surface of the vial [13]. At the end of the release experiment, the cells were washed with distilled water and the collected rinsing water was analysed in order to quantify the amount of drug remaining in the chamber. All VPGs were analysed in triplicate. It should be noted that conventional liposome dispersions require the removal of unentrapped drug from the preparation before utilisation. With VPGs not only the encapsulated portion of the drug, but also the free drug, which is entrapped between the vesicles, is released in a controlled manner and a drug loading efficiency of 100% can be assumed. Removal of unentrapped drug is therefore not necessary when working with VPGs.

3.5. Quantification of cetrorelix

The HPLC system consisted of a Waters 2690 Separation Module with a 996 Photo diode array (PDA) detector. The column system consisted of a Symmetry® C18 analytical column (3.9150 mm, 5 μm) combined with a Symmetry® Sentry™ guard column (3.9×20 mm, 5 μm) (all: Waters, Milford, MA, USA). All experiments were performed at 227 nm, the maximum absorption wavelength of cetrorelix. A method for quantification of cetrorelix in presence of phospholipid has been developed, validated and published [14]. In brief: samples of cetrorelix containing 2.5% Triton X-100 were analysed with the chromatographic system described above, using acetonitrile and 0.05% aqueous trifluoroacetic acid (30:70, v/v) as mobile phase with a flow rate of 1 ml/min leading to a retention time of cetrorelix of 3–4 min. A short rinsing cycle was applied after each run.

3.6. Quantification of Phosphatidylcholine

For the determination of the phospholipid content a commercially available enzymatic kit (Phospholipids B, Wako, Neuss, Germany) and a Fluostar Galaxy titre plate reader (BMG Labtechnologies, Offenburg, Germany) were used. The enzymatic determination of the phospholipid content followed the method described by Takayama [15]. The principle of this test is to determine the choline part of the phosphatidylcholine molecule after enzymatic degradation and subsequent coupling of an intermediate to receive a red quinone pigment with an absorption maximum at $\lambda = 505$ nm. In order to analyse large numbers of PC with a micro titre plate reader and to make the test system applicable for PCs with saturated fatty acids chains we implemented some modifications into the test, namely the use of detergent and heat in the sample preparation step [16].

3.7. Encapsulation efficiency

The encapsulation efficiency was determined by separating the liposomes from the non-entrapped drug by ultracentrifugation. Ultracentrifugation was performed for 20 h at 200,000×g and a temperature of 10 °C, which was found suitable to spin down all liposomes but no CXA. The concentrations of CXA and PL were determined in both supernatant and pellet. The encapsulation efficiency was expressed as CXA in the pellet as compared to CXA in supernatant plus pellet.

3.8. Multivariate analysis

Principal component analysis (PCA) followed by partial least square regression (PLS) was performed to identify significant factors including interactions and quadratic terms for release at different time intervals (The Unscrambler, Camo ASA; Norway). The variation of each variable was scaled to unit variance (1/SD). Cross-validation and jack-knifing was used to validate and assess the stability of the models [17]. A more detailed description of PCA- and PLS methods can be found elsewhere [18].

4. Results and Discussion

4.1. Characterisation of the VPGs

VPGs are semisolid, vesicular systems and upon visual examination it could be stated, that all formulations had a semi-solid consistency, except the formulations containing 300 mg/g E80 or less, which behaved more like a viscous fluid. It has been shown that liposome dispersions produced by high-pressure homogenisation often consist of a large number of single unilamellar vesicles and some few larger vesicles with varying morphology [19]. It was decided to characterise the VPGs by their vesicle size distribution, which is routinely done by PCS for liposome dispersions even though it has been shown [12] that PCS is often not capable to reproducibly reveal the true size distribution of sub micron particles if minor amounts of bigger particles are present. A bimodal distribution was found for all VPGs, with more than 95% (by number) of the particles showing

Table 1
Encapsulation efficiency of CXA in diluted VPGs determined by ultracentrifugation

VPG composition	Free CXA (%)	Liposomal CXA (%)	Overall recovery CXA (%)
300 mg/g E80 5.2 mg/g CXA	31.3±0.9	68.5±1.0	98.9±2.7
480 mg/g E80 5.1 mg/g CXA	31.4±1.0	68.8±1.0	102.9±4.3

a diameter around 30–50 nm while less than 5% (by number) show a diameter in the magnitude 100–200 nm. This holds true for all VPGs except for those with 500 mg/g E80 where a larger portion of vesicles (around 12%) with the larger diameter was found. Regarding encapsulation efficiency, preliminary experiments showed no sedimentation of CXA upon ultracentrifugation at concentrations around 125 μg/ml, but slight or significant sedimentation at CXA concentrations of 750 μg/ml and 3 mg/ml, respectively. VPGs were therefore redispersed to CXA concentrations below 125 μg/ml. In the presence of phospholipid sedimentation was observed. The recovery of CXA varied between 97.4 and 107.6%, the recovery of PL between 93.4 and 103.2%. The encapsulation efficiency was analysed for two diluted VPGs with quite high and low PL content at similar CXA content (Table 1). The encapsulation efficiency of 70% is quite high as compared to the 26.5–50.5% found for the hydrophilic model compound calcein [10]. Based on geometrical considerations an encapsulation efficiency of around 45% could be expected [20]. The high portion of liposome-associated peptide in our case is a first indication of drug–lipid interactions, which shall be discussed later on in more detail. This high portion of liposomal drug leads to the assumption that CXA is both encapsulated in the aqueous core of the liposome and incorporated in or adsorbed to the phospholipid membrane. A liposome-associated proportion of CXA around 70% can be expected. The variation of the phospholipid content from 300 to 480 mg/g did not show an influence on the encapsulation/incorporation efficiency.

4.2. In vitro release behaviour of cetrorelix

The most important characteristic for the VPG is its in vitro release behaviour, which was analysed in terms of (1) influence factors, (2) release kinetics and (3) release mechanism by Tardi [10]. Due to the low overall concentration of CXA in the released fractions, a distinction between liposomal and free CXA appeared technically not feasible. It can be assumed, however, that the proportion released in liposomal form is as determined above, i.e. 70%. The efficacy and further destiny of the liposomal CXA has to be evaluated by in vivo studies, which were not subject of this work.

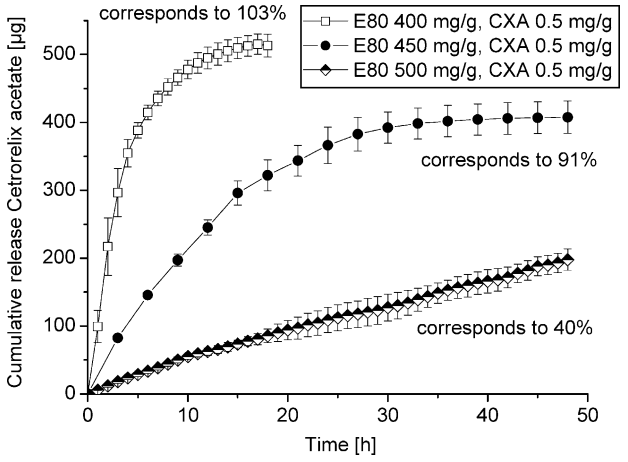

Fig. 2. Cumulative release of CXA from VPGs of different phospholipid contents (400, 450 and 500 mg/g E80) and CXA concentration of 0.5 mg/g versus time. All values given as mean±SD, $n=3$.

4.2.1. Uni-variate evaluation of in vitro release

We first investigated the influence of the phospholipid concentration on the release of CXA. For this set of experiments the concentration of E80 was varied between 400 and 500 mg/g at a low CXA concentration of 0.5 mg/g. The results are given in Fig. 2.

It can be seen that increasing the E80 concentration from 400 over 450 to 500 mg/g decreases the release rate of CXA from the VPG. While the full drug load is released after 19 h with the 400 mg/g VPG, only 40% are released after 48 h with the 500 mg/g VPG and an intermediate position reaching 90% after approximately 30 h is seen for 450 mg/g VPG. This trend compares well to earlier studies reported for the marker Calcein [11], but in our case the release was slower. In Tardi's study [10] 100% release was achieved after approximately 8 h for VPG with 400 mg/g, while here it took approximately 20 h in presence of 0.5 mg/g cetrorelix.

The next step was to investigate whether the concentration of phospholipid has the same influence on the release of CXA at relatively high CXA concentrations. Release studies at higher CXA concentrations were performed for up to 168 h (7 days). Contradictory to the results obtained with 0.5 mg/g CXA, the influence of the phospholipid content on the release rate was not obvious when analysing three formulations with phospholipid contents between 280 and 380 mg/g E80 at a CXA concentration around 8.6 mg/g (data not shown).

Finally the influence of the CXA concentration on the release rate was investigated. For this series of experiments the phospholipid concentration was kept constant at an intermediate level (400 mg/g). Theoretically one would expect to see an increase of the absolute released amount rate with increasing CXA concentration. This holds true for concentrations of CXA up to approximately 5 mg/g. Surprisingly, further increase in the CXA concentration led to a reduced absolute release (Fig. 3), giving a second

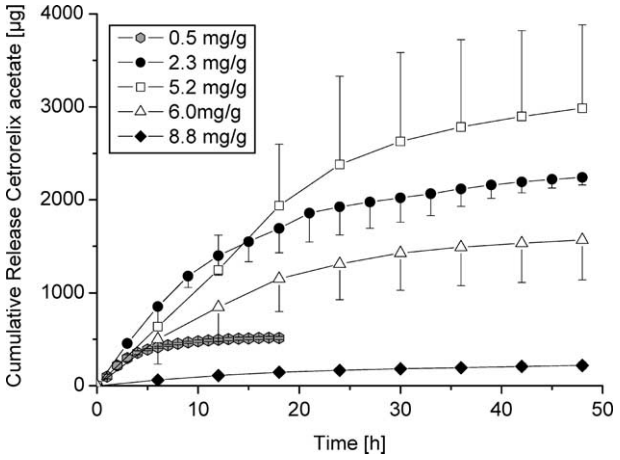

Fig. 3. Cumulative release of CXA from VPGs with 400 mg/g E80 and different CXA concentrations (0.5–8.8 mg/g). All values given as mean ± SD, $n = 3$.

indication for the presence of drug–drug or drug–lipid interactions. With the influence of the concentration of both CXA and phospholipid on the release behaviour proven, it is obvious that univariate analysis cannot completely explain the relationship between each formulation factor and the release. A multivariate evaluation appears necessary taking interactions and non-linear behaviour of the factors into account.

4.2.2. Multivariate evaluation of the in vitro release

PLS analyses were performed on the full matrix shown in Fig. 1 in order to examine possible interactions and non-linear influence of the variables on the release of CXA. First, the initial release of CXA within the first 24 h was investigated. Significant regression factors ($P < 0.05$) determining the release after 24 h were identified to be concentration of peptide (0.626), concentration of lipid (−0.323) as well as the non-linear behaviour of both factors ($c(CXA)^2$ −0.579) and ($c(PL)^2$ −0.469). The PLS model uses two components to explain 68% of the x-variance and 82% of the y-variance. The correlation between measured and model-predicted values was found acceptable (0.88). The calculated response surface is shown in Fig. 4. The highest initial release can be found for formulations with intermediate CXA concentrations (3–6 mg/g) in combination with intermediate concentrations of PL (350–430 mg/g). Lower initial release is found for both high and low concentrations of one variable in combination with high and low concentration of the other variable proving non-linear behaviour of both variables.

Second, the release at later time points (days 5–7) was investigated. Since some of the combinations released the full drug load within a few days, typically those with low concentrations of CXA, and others showed a prolonged release for more than seven days not all combinations were included. For analysis of the factors influencing the release rate at day 5, 6 and 7 the matrix was reduced to CXA

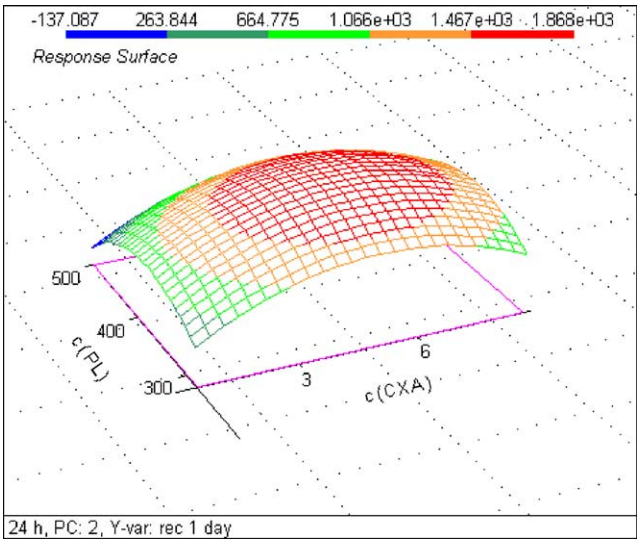

Fig. 4. Surface plot from a PLS of the initial release of CXA measured during the first 24 h (explained x-variance 68%, explained y-variance 82% on two components).

concentrations above 5 mg/g and PL concentration limited upwards to 400 mg/g (solid square in Fig. 1).

Fig. 5 shows the response surface of the PLS model obtained for release rate (µg/day) at day 7 (explained x-variance 62% and explained y-variance 66% on two components). Significant factors determining the release rate are the concentration of CXA (0.757), the concentration of PL (0.577) and the interaction between those (0.447). The same factors were also identified for release rates at days 5 and 6, respectively.

4.2.3. Release kinetics

The release of CXA from VPGs did not strictly follow uniform kinetics like first-order or zero-order. Fast releasing

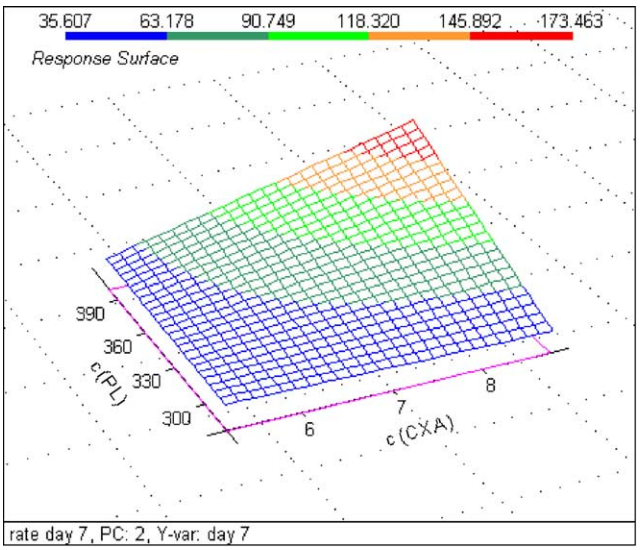

Fig. 5. Surface plot from a PLS of release rate (µg/day) on day 7 (explained x-variance 62%, explained y-variance 66% on two components).

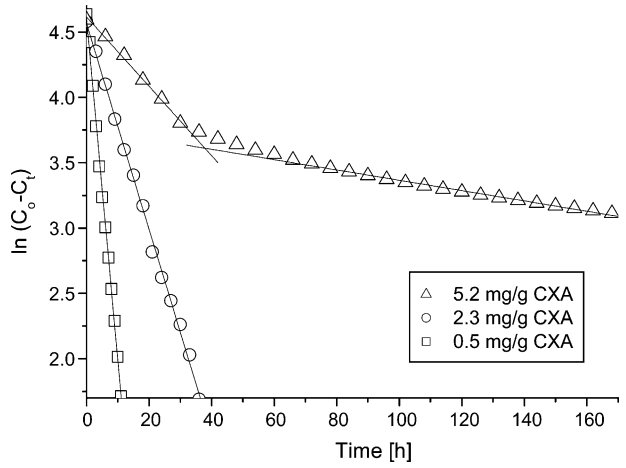

Fig. 6. Kinetic model fit. First order fit for VPGs with 0.5–5.2 mg/g CXA and 400 mg/g E80. Partly biphasic sigma minus plot of logarithmic reduction in concentration versus time. Mean, $n = 3$.

VPGs (such as 400 mg/g E80 and 0.5 mg/g CXA shown in Fig. 2), i.e. VPGs with a low concentration of CXA and E80 are best described by first-order-release kinetics as can be seen from the close to linear behaviour in the logarithmic sigma minus plot (Fig. 6). This behaviour might be explained in two ways: First, by assuming a diffusion-controlled system, which, however, appears to be unlikely due to the properties of the peptide. It is therefore more reasonable to expect an erosion-controlled system, where water can penetrate the whole VPG matrix due to a loose packing of the matrix and the absence of significant drug–drug and drug–lipid interactions. For VPGs with higher CXA concentrations (6 mg/g and above) simple first order kinetics did not lead to a satisfying fit. It was observed for these VPGs that they are probably best described by zero-order kinetics (linear sigma minus plot, Fig. 7) with an initial phase with increased release. Such zero order kinetics

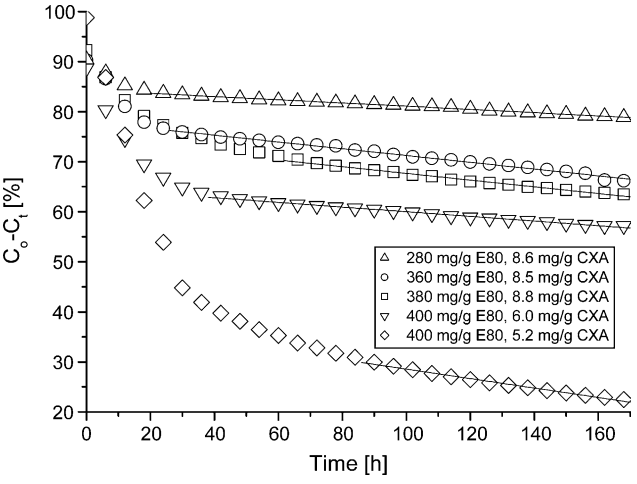

Fig. 7. Kinetic model fit. Zero order fit for VPGs with 5.2 and 6.0 mg/g CXA and 400 mg/g E80 and approximately 8.6 mg/g CXA and 280–380 mg/g E80. Sigma minus plot of linear reduction in concentration versus time. Mean, $n = 3$.

fit well with the anticipation of an erosion-controlled system taking the geometry of the release cell into account. With increasing drug–drug and drug–lipid interactions, the stiffness of the VPG increases and only the surface of the matrix is accessible to erosion. This behaviour can be expected for all peptides showing this kind of interactions. The release cell supports a constant surface during the release test thus leading to zero order process. It has; however, to be stated that the period investigated for the zero order release (one week) is rather short compared to the expected duration of the overall release process. It can therefore not be predicted for sure that the whole process will follow zero order release. Regarding the faster initial release two phenomena may explain this behaviour. First, the portion of CXA on the surface of the matrix may be less strongly bound to the matrix and therefore be released faster. However, this phenomenon appears insufficient to explain the higher initial release rate completely. It is more likely that the surface in addition shows a certain micro-roughness at the beginning of the experiment, thus exposing a larger surface of the matrix to erosion by the release medium. With on-going erosion the exposed parts are eroded first, subsequently leading to a smoother surface and a slow-down of the erosion rate.

The VPG with 5.2 mg/g CXA and 400 mg/g E80 (included in Figs. 6 and 7) appears to take an intermediate position, not clearly following the behaviour of either one of the other two groups of formulations. It can therefore be stated that the release behaviour seems to switch from close to first order to close to zero order at around this composition.

4.2.4. Release mechanism

It was demonstrated earlier with the model compound calcein that the release of drugs from the VPGs in principle may happen by two mechanisms: First, diffusion of the drug out of the phospholipid matrix and second, erosion of the matrix, thus releasing liposomes with encapsulated drug as well as drug that had been trapped in-between the vesicles. These two mechanisms hold true for a hydrophilic, low molecular weight compound such as calcein, where diffusion is seen to a significant extent. In the case of CXA, however, free diffusion through the tightly packed liposome matrix of the VPG cannot be expected. This is first, due to the relatively high lipophilicity of CXA leading to binding to the phospholipid membrane, as already noticed when comparing the encapsulation efficiencies of calcein and CXA, and second, to its rather larger molecular size, which renders permeation through liposome bilayers rather unlikely. For a closer investigation of the release mechanism, the significance of phospholipid erosion, i.e. the amount of released phospholipid was analysed. When comparing the erosion of the phospholipid matrix with the release of CXA from fast releasing VPGs, it can clearly be seen that those two processes are parallel (Fig. 8). This further confirms that erosion is the dominating factor for

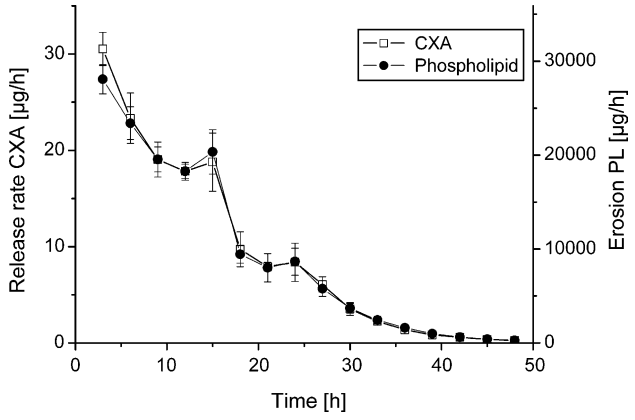

Fig. 8. Release of CXA and PC (erosion of phospholipid matrix), the axes are proportional to the CXA and PC content of the VPG, respectively. VPG with 450 mg/g E80 and 0.5 mg/g CXA. Mean ± SD, $n = 3$.

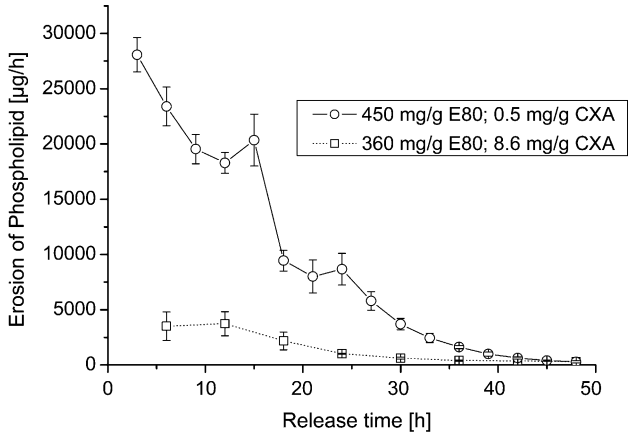

Fig. 10. Erosion of phospholipid from VPGs with high and low content of CXA over release time. Mean ± SD, $n = 3$.

the release of CXA from the matrix. These results are in good agreement with the results reported by Tardi et al. [10]. They found that erosion of the phospholipid matrix and the release of calcein went parallel for VPGs up to 500 mg/g PL and therefore considered these systems as erosion-controlled. It was then investigated if the erosion also plays a major role for the release rate from slowly releasing VPGs, i.e. VPGs with high concentrations of CXA (here: 360 mg/g E80, 8.5 mg/g CXA; Fig. 9). For calcein, the slowly releasing VPGs had been reported to be a diffusion-controlled system with an inert matrix, following square-root-of time kinetics. It was, however, mentioned already that diffusion is unlikely for CXA. It can be seen that these VPGs also release CXA and PC in a parallel manner and thus appear to be controlled by the erosion of the phospholipid matrix. When comparing the erosion of phospholipid in VPGs with high (8.6 mg/g) and low (0.5 mg/g) content of CXA it can be seen that the erosion is lower at high levels of CXA (Fig. 10). This finding may partly explain the decreased release rate with increased

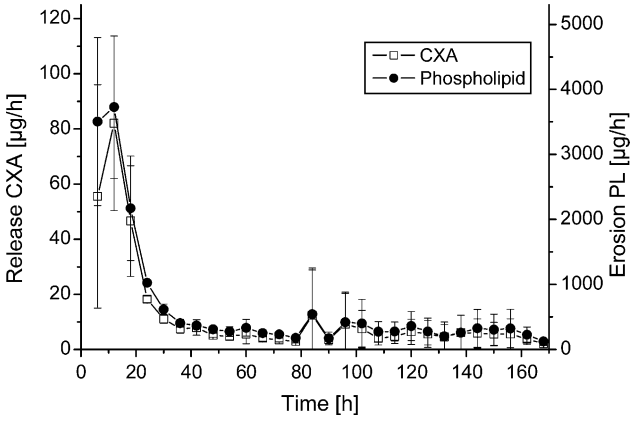

Fig. 9. Release of CXA and PC (erosion of phospholipid matrix), the axes are proportional to the CXA and PC content of the VPG, respectively. VPG with 360 mg/g E80 and 8.6 mg/g CXA. Mean ± SD, $n = 3$.

CXA content (Fig. 3). It is known that CXA tends to self-associate [21] at higher concentrations leading to a gel-like viscosity. This behaviour may also be described as drug–drug and drug–lipid interactions, which in turn increase the overall stiffness of the VPG, thus slowing down the erosion of the lipid matrix and leading to a slower release of CXA.

The findings on the release mechanism can be summarised as follows: for all VPGs an increased initial release is observed which can best be explained by the release of CXA, which was in and between the vesicles on the surface of the matrix. However, no 'burst release' was found for CXA–VPGs. The behaviour observed here is clearly different from that of polymeric systems, where the initial release can be orders of magnitude larger than the subsequent continuous release. From the kinetic studies it was concluded that erosion is the main mechanism for the VPGs, which could be confirmed by the erosion profiles. The type of erosion profile can obviously be changed by varying the composition of the VPG. VPGs with a low concentration of CXA are quickly penetrated by the release medium, erode fast and follow first order kinetics. At higher concentrations of CXA the erosion is slowed down and is approaching a zero order release profile after an initial release. This behaviour can be explained by increased stiffness of the VPG due to drug–drug or drug–lipid interactions. The change in release kinetics indicates that these interactions start to play a major role from around 5.2 mg/g CXA onwards.

What is yet unexplained is the question, why erosion and consequently the release of CXA is decreased when increasing the phospholipid concentration from 280 to 380 mg/g in VPGs with a high concentration of CXA (approximately 8.6 mg/g, see Fig. 9 and Table 1). Two alternative hypotheses might explain this observation: (1) A positive co-operative binding of CXA to phospholipid membranes may occur as reported earlier [8]. CXA may adsorb to the phospholipid membrane, exposing the hydrophobic part of CXA to the solvent with subsequent

Table 2
Released amount of CXA from 1 g VPG of various compositions

Concentration E80 (mg/g)	300	400	400	380	360	280
Concentration CXA (mg/g)	5.2	5.2	6.0	8.8	8.5	8.6
Initial release, day 1 (μg)	1640 ± 550	2340 ± 950	1310 ± 380	1320 ± 140	1160 ± 190	575 ± 25
Release, day 2 (μg)	202 ± 39	596 ± 52	257 ± 61	419 ± 192	182 ± 24	95 ± 6
Mean daily release, day 3–7 (μg)	113 ± 81	163 ± 73	67 ± 11	160 ± 54	124 ± 26	65 ± 9
Predicted duration of release (80% release)	22 days	10 days	50 days	35 days	46 days	98 days

Day 1 and 2: mean of three release experiments ± SD. SD for day 3–7 is given as day-to-day variation of the mean of three release experiments.

adsorption of additional peptide molecules, in order to reduce this unfavourable situation. The exposure of hydrophobic regions would provide the liposome with a more hydrophobic surface and thus reduce the accessibility of the phospholipid matrix for water molecules. A higher concentration of peptide would then lead to a higher surface coverage and increased hydrophobicity. A lower concentration of phospholipid at constant concentrations of peptide means more peptide on the surface and thus increased hydrophobicity. (2) An alternative explanation might be that CXA forms a gel-network in the aqueous space between the liposomes of the VPG. With increasing PL concentration the CXA-network may be increasingly segregated by the vesicles and the release increased. Both hypotheses explain the decreased release rate with increasing phospholipid concentration. It shall furthermore be mentioned that due to the absence of significant diffusion, the lipid composition is not expected to play a major role for the release behaviour, as erosion should be mainly independent from the type of lipid.

4.3. Considerations on therapeutical relevance and outlook

Several implantable depot formulations for LH-RH agonists such as Triptorelin, Leuprorelin, Goserelin and Buserelin are in routine clinical use. All those systems are using poly-lactic glycolic acid co-polymers or poly-lactic acid for the preparation of microcapsules or implants reaching an overall release period of 1–3 months. For LH-RH antagonists, however, no such formulation has reached the marked so far. Following approaches have been described in an experimenal setting: Bauer et al. [22] reported a sustained release formulation for cetrorelix where a counter-ion is formed between CXA and an anionic macromolecule as carrier (e.g. carboxy-methyl cellulose). For this system overall in vitro releases between 23 and 76% over 168 h (7 days) have been reported, depending on the mass ratios of the components. It can be seen that the investigated VPGs provide a wide range of modification of overall releases with 11.5–100% in 168 h. No generally acknowledged dosing scheme has been established for the long-term application of LH-RH antagonists but the following data are helpful for the estimation of therapeutically relevant doses: in an animal model Reissmann et al. [23] reported that 100 μg/kg d^{-1} were sufficient for a full anti-tumour response of a mammary carcinoma. Engel et al. [24] patented a therapeutic regime for the treatment of prostatic cancer using doses of 0.5–5 mg cetrorelix per day. In humans Gonzalez-Barcena et al. [3] were the first to show that 500 μg given subcutaneously every 12 h were efficient in the treatment of benign prostatic hyperplasia. A single injection of 300 μg CXA was investigated as well and led to a suppression of LH for 14 h and FSH for more than 24 h in male subjects [25]. For long term treatment, an increased initial release is beneficial as a high initial release ensures a prompt effect, which can be maintained by a slower continuous release [26]. Based on these results we aimed for a released amount of CXA between 0.5 and 2 mg per day after the initial release in order to maintain therapeutically effective doses. A summary of the releases over one week for 1 g of potentially therapeutically relevant formulations is given in Table 2. As a long-acting formulation was desired it appears obvious that high concentrations of CXA are necessary.

A maximum of 5 g is believed to be feasible for the implantations. Under the assumption that in vivo release is comparable to the above in vitro data, several of the above formulations appear feasible for further studies. Especially VPGs with a higher loading of CXA appear appropriate since their release is expected to last for 1 month or more. So far the formulation 360 mg/g E80 and 8.5 mg/g CXA appears to be the best choice since an implant of 5 g would provide therapeutically relevant plasma levels (e.g. 0.7 mg CXA) for a period of 6 weeks.

5. Conclusion

In vitro release studies revealed that the release of CXA from a VPG is depending on both concentration of the lipid and the concentration of CXA in a non-linear manner. Multivariate analysis revealed that there are important interactions between the two and confirmed that both factors have a non-linear behaviour in the tested area. It should be possible to achieve the desired release behaviour by optimising the concentrations of phospholipid and CXA. The results can be summarised in the following way:

Literature reported strictly decreasing release of the hydrophilic model compound calcein with increasing content of phospholipid, while no influence of the incorporate drug/model-compound was observed. In our study, however, the highest initial release was found for formulations with intermediate CXA concentrations (3–6 mg/g) in combination with intermediate concentrations of PL (350–430 mg/g). Due to non-linear behaviour of both variables other combinations led to a lower initial release. As erosion of the phospholipid matrix was found to be the main release mechanism, this can be explained by an influence of CXA and PL content on the erosion of the matrix. The ability of CXA to form a gel-like structure at higher concentrations might be one main reason for the change in erosion behaviour. Even though no measurements of the viscosity were performed, the increasing viscosity with either increasing phospholipid or CXA concentration was easily observed visibly. In the in vitro experiments release rates between 50 and 300 μg/day per gram VPG were found after an initial release, thereby achieving therapeutically relevant doses. Assuming an implant mass of 5 g, several formulations were identified that could maintain a therapeutically relevant level for 6 weeks. Whether a good correlation between in vitro/in vivo release can be obtained will depend among others on the amount of fluid present at the site of administration and potentially its flux and thus vary from tissue to tissue. A definite statement on in vitro/in vivo correlation will just be available after in vivo experiments have been performed.

It can be concluded that VPGs represent a promising approach for the sustained in vitro release of peptide drugs and a further evaluation of especially in vivo safety and efficacy studies should be performed.

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