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Peptide degradation during preparation and in vitro release testing of poly(L-lactic acid) and poly(DL-lactic-*co*-glycolic acid) microparticles

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Abstract

Biodegradable, tetracosactide-loaded microparticles were prepared by means of (i) spray drying, (ii) w/o/w solvent evaporation method (WOW) and (iii) by the aerosol solvent extraction system (ASES) using poly(L-lactic acid) (L-PLA) and poly(DL-lactic-*co*-glycolic acid) (DL-PLGA) of varying monomer composition or molecular weight. In the absence of the polymer the peptide did not degrade or aggregate irreversibly when in contact with methanol and methylene chloride or under the conditions used in the first step of WOW, as proven by HPLC, electrospray-mass spectrometry (MS) and circular dichroism (CD). During the extraction process, used to isolate the peptide from the microparticles, tetracosactide was partially oxidised. The highest stability of the peptide during microencapsulation was guaranteed with high molecular weight L-PLA, when using WOW or ASES, and with very low molecular weight PLGA, in the case of spray drying and WOW. The burst release of the microparticles, during in vitro release testing, depended on the preparation method as well as on the nature of the polymer and increased in the order ASES < spray drying < WOW and with increasing hydrophilicity of the polymer. Exceptionally, in the case of very low molecular weight PLGA, to which tetracosactide showed a very strong affinity during the in vitro adsorption study, no burst effect was observed. In addition, these microparticles released the peptide continuously, whereas for the others, composed of high molecular weight PLA and PLGA, the burst release was followed by a lag phase. During in vitro release peptide degradation increased with increasing polymer hydrophilicity but could be reduced by increasing drug loading. In polymer-free control solutions tetracosactide degradation was always slower than in the presence of microparticles. Oxidation and hydrolysis were found to be the major degradation pathways.

Keywords: Microparticles; Microspheres; Preparation methods; Poly(L-lactic acid); Poly(DL-lactic-*co*-glycolic acid); Peptide degradation; Peptide adsorption; Tetracosactide; In vitro release

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1. Introduction

The most widely used methods for the microencapsulation of proteins and peptides into poly(lactic acid) or poly(lactic-*co*-glycolic acid) are w/o/w solvent evaporation (WOW), coacervation and spray drying (Aftabrouchad and Doelker, 1992). More recently, ASES has also been proposed for the same purpose (Bleich et al., 1993). Usually, organic solvents such as methylene chloride are necessary to dissolve such polymers. Organic solvents are known to induce reversible and irreversible denaturation of proteins, e.g. unfolding and aggregation (Manning et al., 1989). However, peptides generally lack the many hydrogen bonds and disulphide bridges that stabilise the three-dimensional structure in proteins. Therefore, they display a multitude of conformations in solution and they can re-equilibrate to native conformations so rapidly that denaturing reagents and conditions ultimately have no influence on biological activity. Additionally, peptides often dissolve better in denaturing solvent systems like ethanol, dimethyl sulfoxide or dimethylformamide (Samanen, 1991). Besides organic solvents, the peptide is also exposed to other stress factors during microencapsulation, e.g. mechanical stress and shear forces at the water–methylene chloride or the air–liquid interface. In terms of the possible effects of the preparation methods of microparticles, heat and oxygen exposure are unavoidable in spray drying. ASES offers the advantages that only a moderate temperature (34°C) is used and that the solutions are sprayed into supercritical carbon dioxide, thus avoiding contact with oxygen. Depending on the amino acid composition and the pH, peptides and proteins are also susceptible to other degradation mechanisms, such as deamidation, proteolysis or racemisation (Manning et al., 1989; Samanen, 1991; Cleland et al., 1993). The structure of tetracosactide, which served as a model peptide and is composed of the 24 first amino acids of ACTH, is presented in Fig. 1, together with the major cleavage sites found during in vivo degradation. It contains eight basic amino acids explaining its high isoelectric point of 10.5 (personal communication from Ciba–Geigy). Methionyl is separated from histidyl by only one amino acid, which was reported to catalyse intramolecularly the oxidation of methionyl residues (Li et al., 1993; Schöneich et al., 1993). In vitro, tetracosactide can easily be oxidised at methionyl residues by adding hydrogen peroxide, without formation of other degradation products. Thereby, and also following hydrolysis between AA1 and AA18, tetracosactide loses its biological activity (Dedman et al., 1957; Anon., 1969).

In vitro release testing is mostly carried out in buffer solutions under shaking, which may strongly influence the peptide stability. Atriopeptin III was described to degrade faster in Tris-buffered Ringer's and under shaking than in water and without shaking (Johnson et al., 1991). Additionally, the accumulation of protonated carboxylic acid end-groups generated by the cleavage of polymer ester bonds may enhance the autocatalytic hydrolysis of protein and peptide drugs encapsulated in PLGA microparticles. During in vitro release, the peptide may also adsorb on the surface of the microparticles, leading to an underestimation of the amount of peptide effectively released. For example, insulin was found to have a higher affinity to the lower molecular weight (40000) DL-PLA than to the higher one (140000) (Soriano et al., 1996). The degree of adsorption and the formation of peptide/protein multilayers around PLA/PLGA microparticles are directly related to the hydrophobicity and the concentration of both the polymer and the peptide (Calis et al., 1995; Tsai et al., 1996a,b).

Bearing these factors in mind, it was of interest to study the influence of different preparation methods: spray drying, WOW and ASES and of the nature of the polymer on peptide degradation, adsorption and release during microencapsulation and in vitro testing.

2. Materials and methods

2.1. Material

The model peptide tetracosactide (Synacthen®, M_w: 2933, a gift of Ciba–Geigy, Basle, Switzerland) was encapsulated into the following polymers:

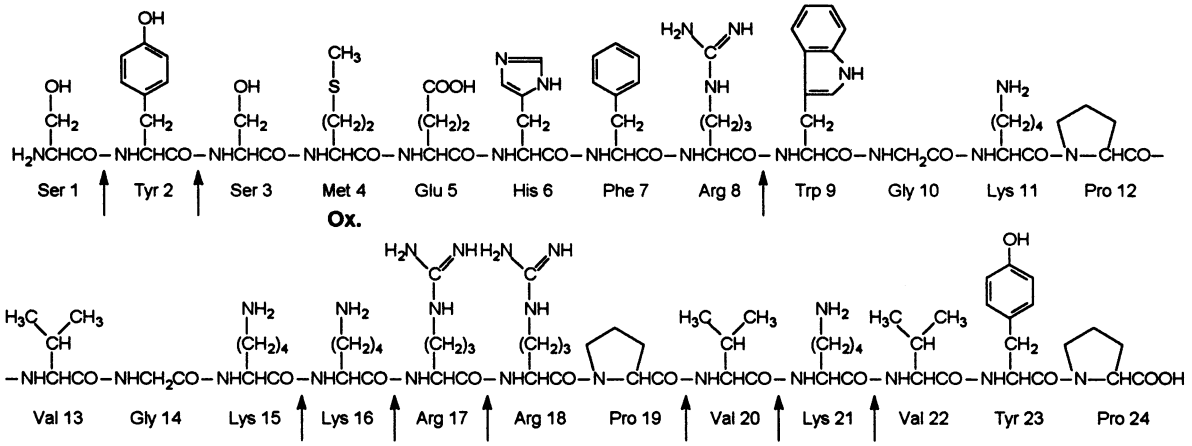

Fig. 1. Cleavage (↑) and oxidation (Ox.) sites of tetracosactide in vivo (Anon., 1969; Hudson et al., 1977; Hudson, 1978).

Table 1
Estimates of oxidised tetracosactide, other tetracosactide degradation products and non-degraded tetracosactide remaining in microparticles after encapsulation of tetracosactide into different polymers by means of SD, WOW and ASES

Microparticle batch	Preparation method	Nominal loading (%)	Oxidised tetracosactide[a] (%)	Other degradation products (%)	Non-degraded tetracosactide[b] (%)
RG504LMW	SD	1	3.0	3.8	93.2
L206	WOW	1	1.2	4.6	94.2
RG755	WOW	1	0 (−0.2)	11.8	88.2
RG504	WOW	1	4.2	13.3	82.5
RG504	WOW	5	0 (−0.4)	16.5	83.5
RG504LMW	WOW	1	0 (−5.3)	5.5	94.5
L206	ASES	1	3.4	2.8	93.8

The results are expressed as total peak area percent.
[a] Values obtained after subtraction of the amount of tetracosactide which oxidised during the microparticle extraction process.
[b] If the value for oxidised tetracosactide was negative after correction (values in parentheses in the fourth column of the table) it was set to zero for calculating the amount of intact tetracosactide after microencapsulation.

L-PLA (Resomer® L206, M_w 81200), DL-PLGA 75:25 (Resomer® RG755, M_w 64300), DL-PLGA 50:50 (Resomer® RG504, M_w 52600) and DL-PLGA 50:50 (M_w 3400, abbreviated in the text as LMW). The high molecular weight polymers were purchased from Boehringer (Ingelheim, Germany), the lowest molecular weight DL-PLGA 50:50, named LMW, was synthesised in our laboratory using DL-lactic acid (containing 10% water, Fluka, Buchs, Switzerland) and glycolic acid (Fluka, Buchs, Switzerland) (Witschi and Doelker, 1998). Poly(vinyl alcohol) (PVAL, Mowiol® 4–88, M_w 26000, Hoechst, Frankfurt, Germany) was used as emulsifier in WOW. All solvents were of HPLC grade.

2.2. Preparation of microparticles

The microparticle preparation methods, i.e. spray drying (SD), w/o/w solvent evaporation (WOW) and aerosol solvent extraction system (ASES), were those used previously (Witschi and Doelker, 1998).

Since no microparticles resulted with all three preparation methods when the polymer with the lowest molecular weight PLGA (LMW) was used alone, it was mixed with a high molecular weight PLGA (RG504) in a proportion of 20:80. The nominal drug loading of microparticles was generally set at 1% w/w and in one batch, prepared by WOW with RG504, set at 5% w/w. When no drug

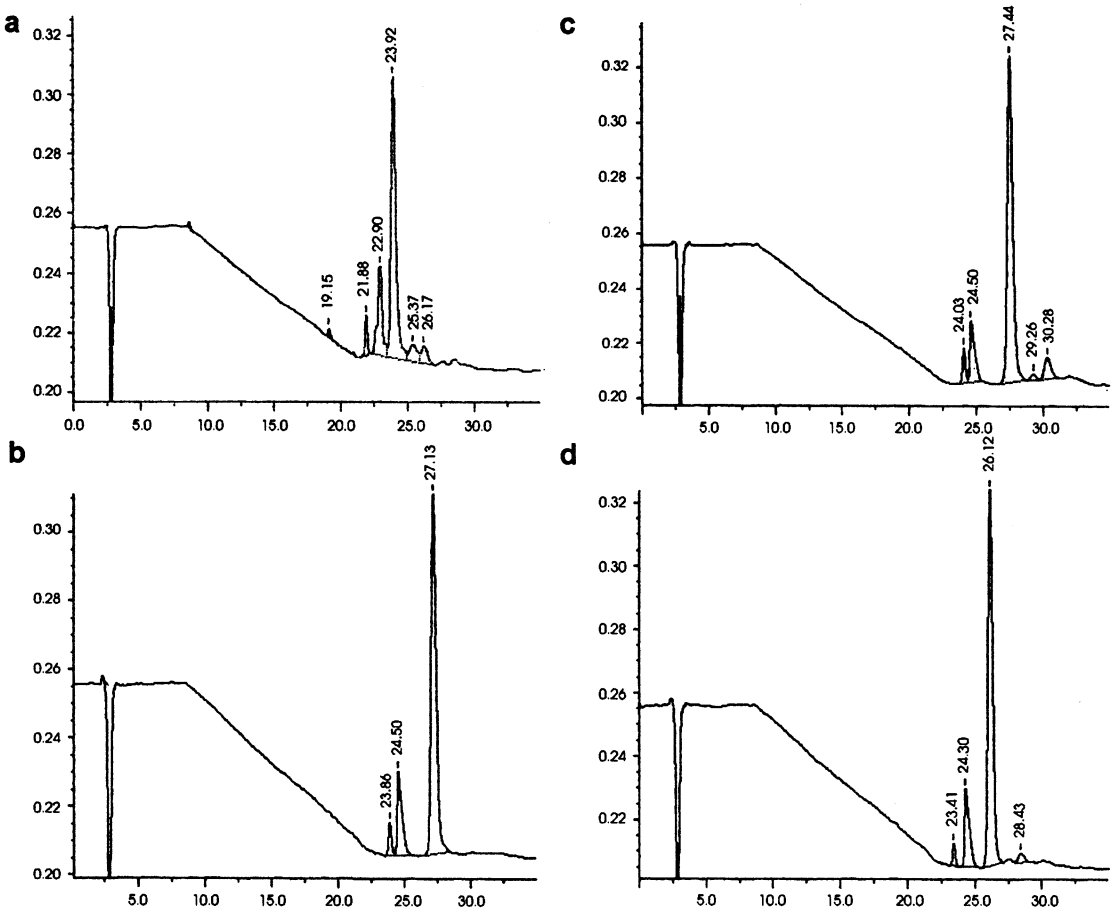

Fig. 2. HPLC chromatograms of a tetracosactide extracted from microparticle batches made from various polymers and prepared using different methods: (a) RG755/spray drying; (b) RG504LMW/spray drying; (c) RG755/WOW; (d) L206/ASES.

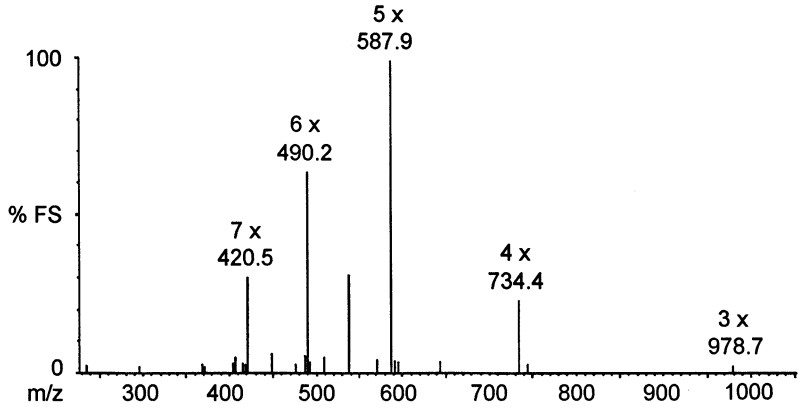

Fig. 3. Mass spectrum of tetracosactide with the charge states indicated.

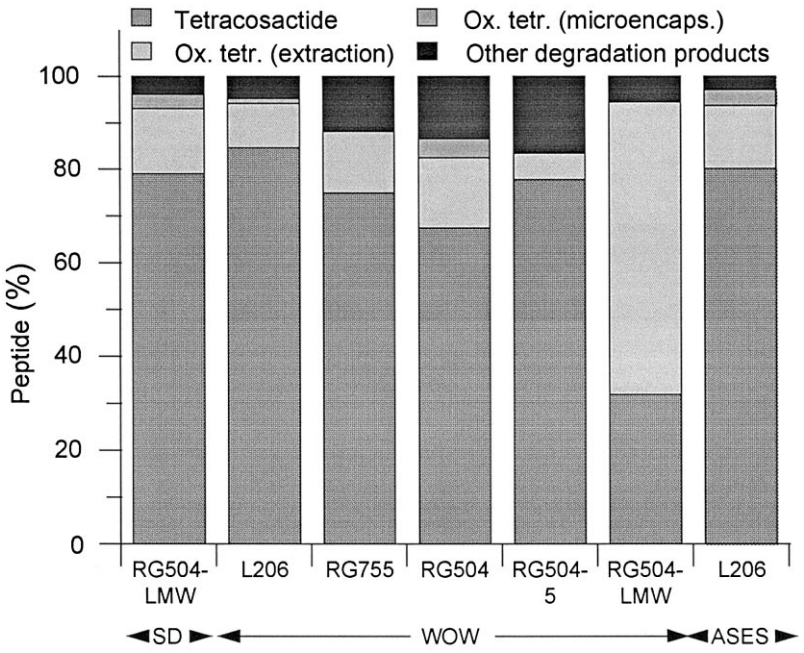

Fig. 4. Intact tetracosactide and its degradation products (singly oxidised tetracosactide and other products) recovered after microparticle extraction. The hatched fields represent the amount of tetracosactide which degraded during microencapsulation. Effective drug loading of the microparticle batches: SD: RG504LMW: 0.85%; WOW: L206: 0.98%, RG755: 0.92%, RG504: 0.61%, RG504–5: 4.26%, RG504LMW: 0.19%; ASES: L206: 0.78%.

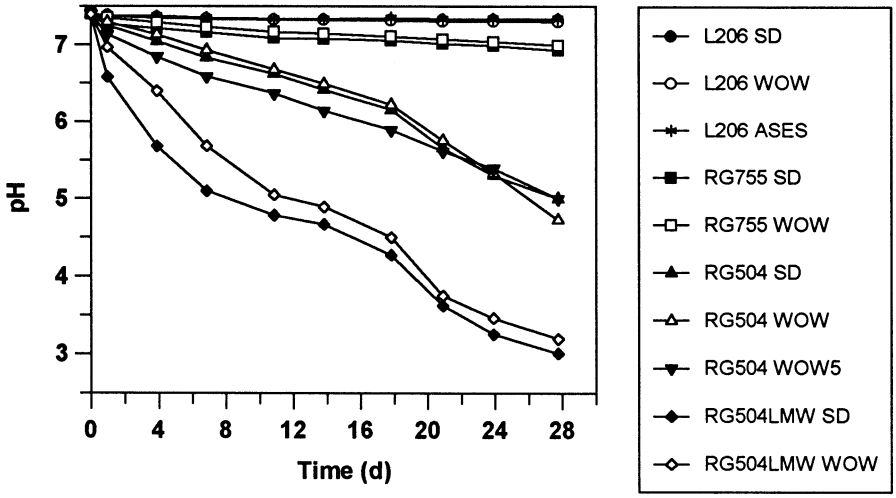

Fig. 5. pH profiles of microparticle suspensions (PBS) during in vitro release testing at 37°C. SD: spray drying; WOW: w/o/w solvent evaporation method; ASES: aerosol solvent extraction system.

was added to the inner aqueous phase in WOW, no stable primary emulsion could be formed. Therefore, the first step of WOW was skipped in the preparation of drug-free WOW microparticles.

2.3. Particle size analysis

The microparticles were mixed with a few drops of polysorbate 80, dispersed in water and sonicated

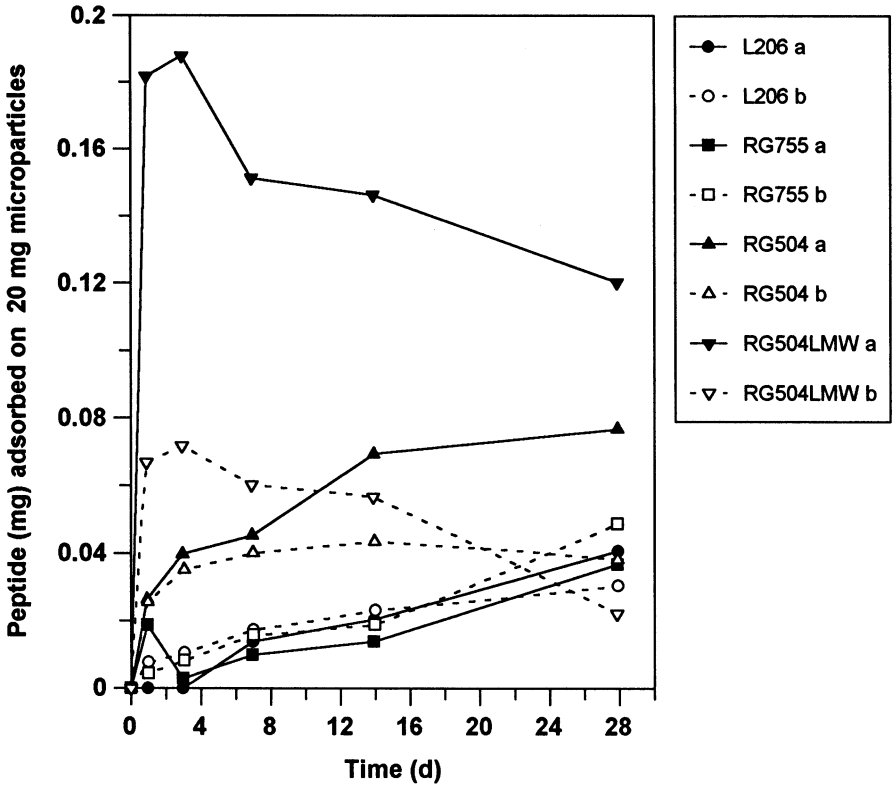

Fig. 6. Amount of tetracosactide adsorbed on 20 mg of microparticles made of L206, RG755, RG504 and RG504/LMW, all prepared by spray drying, after addition of 2 ml of tetracosactide solution with concentrations of 0.10 mg/ml (a) or 0.04 mg/ml (b).

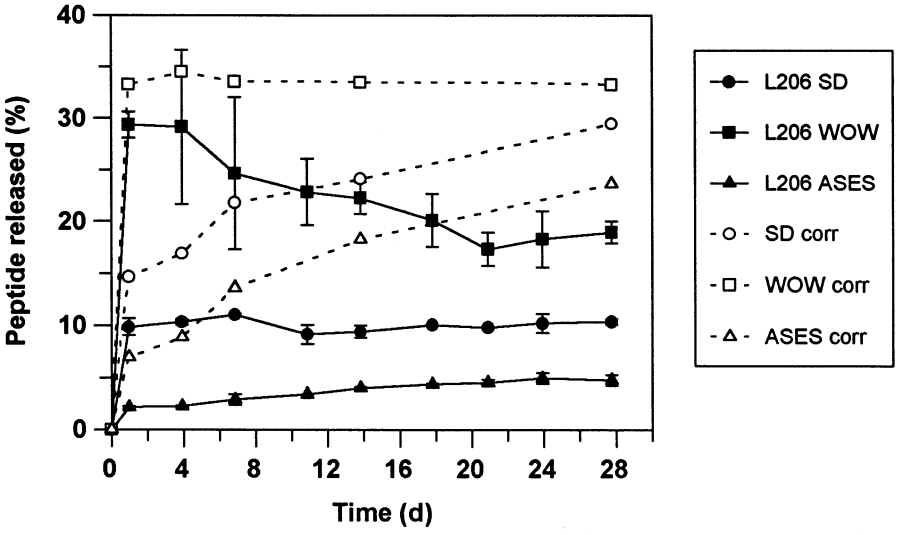

Fig. 7. Cumulative peptide release of microparticles made of L206, prepared by spray drying, WOW and ASES, before and after correction with the results of the adsorption test (corr).

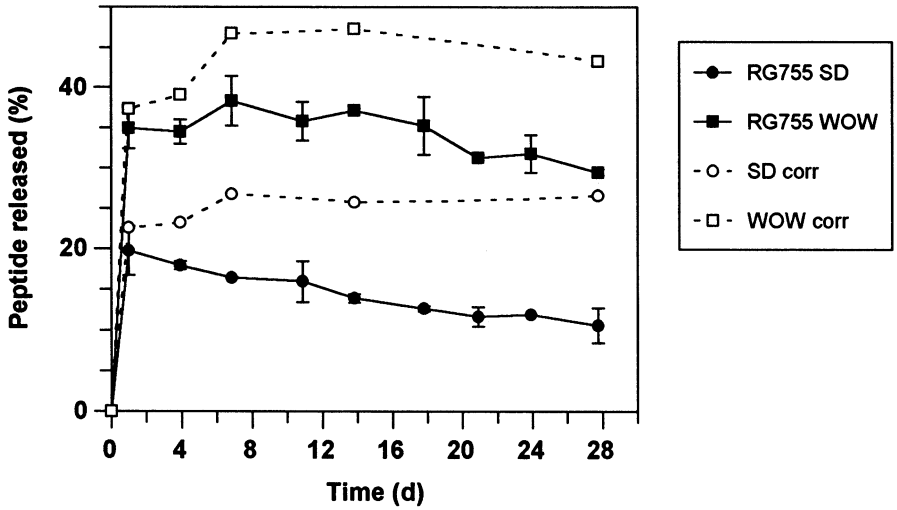

Fig. 8. Cumulative peptide release of microparticles made of RG755, prepared by spray drying and WOW, before and after correction (corr) with the results obtained from the adsorption test.

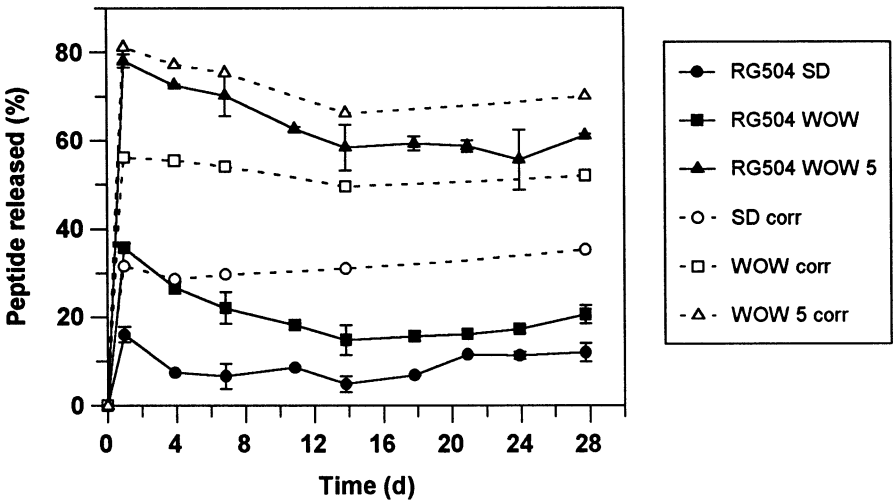

Fig. 9. Cumulative peptide release from microparticles made of RG504 with a nominal drug loading of 1% w/w and, in one case (RG504 WOW5), of 5% w/w, prepared by spray drying (SD) and WOW, before and after correction (corr) with the amounts adsorbed on the microparticles.

for 2 min before determination of particle size distribution by laser diffraction (Mastersizer® X, Malvern, UK).

2.4. Circular dichroism

To check if the organic solvents used during microencapsulation provoke irreversible structure changes of tetracosactide, the peptide was dissolved in different solvent mixtures (Table 1), and furthermore, exposed to the primary step conditions of microencapsulation, but without adding polymers. Tetracosactide was thus dissolved in a methanol/methylene chloride mixture corresponding to the spray drying/ASES solution. It was also dissolved in water and emulsified in methylene chloride using the Ultra-Turrax® homogeniser (first preparation step in WOW). After-

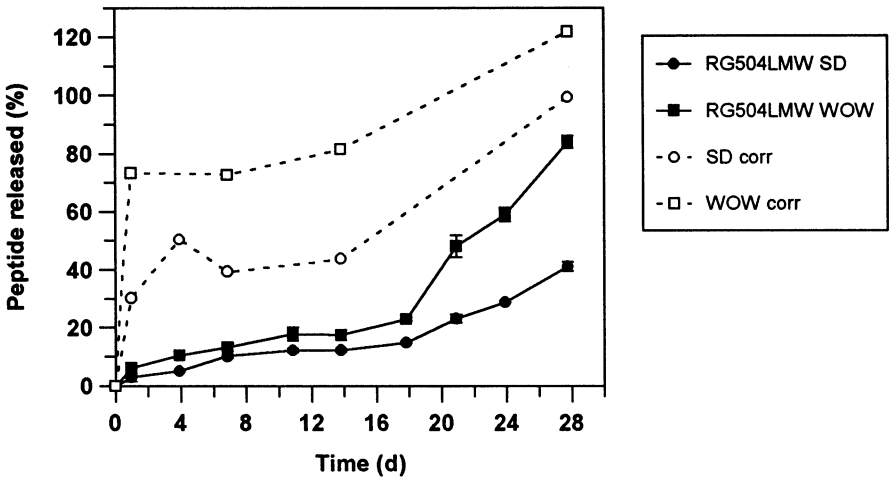

Fig. 10. Cumulative drug release from microparticles made of RG504/LMW, prepared by spray drying and WOW, before and after multiplication with correction factors.

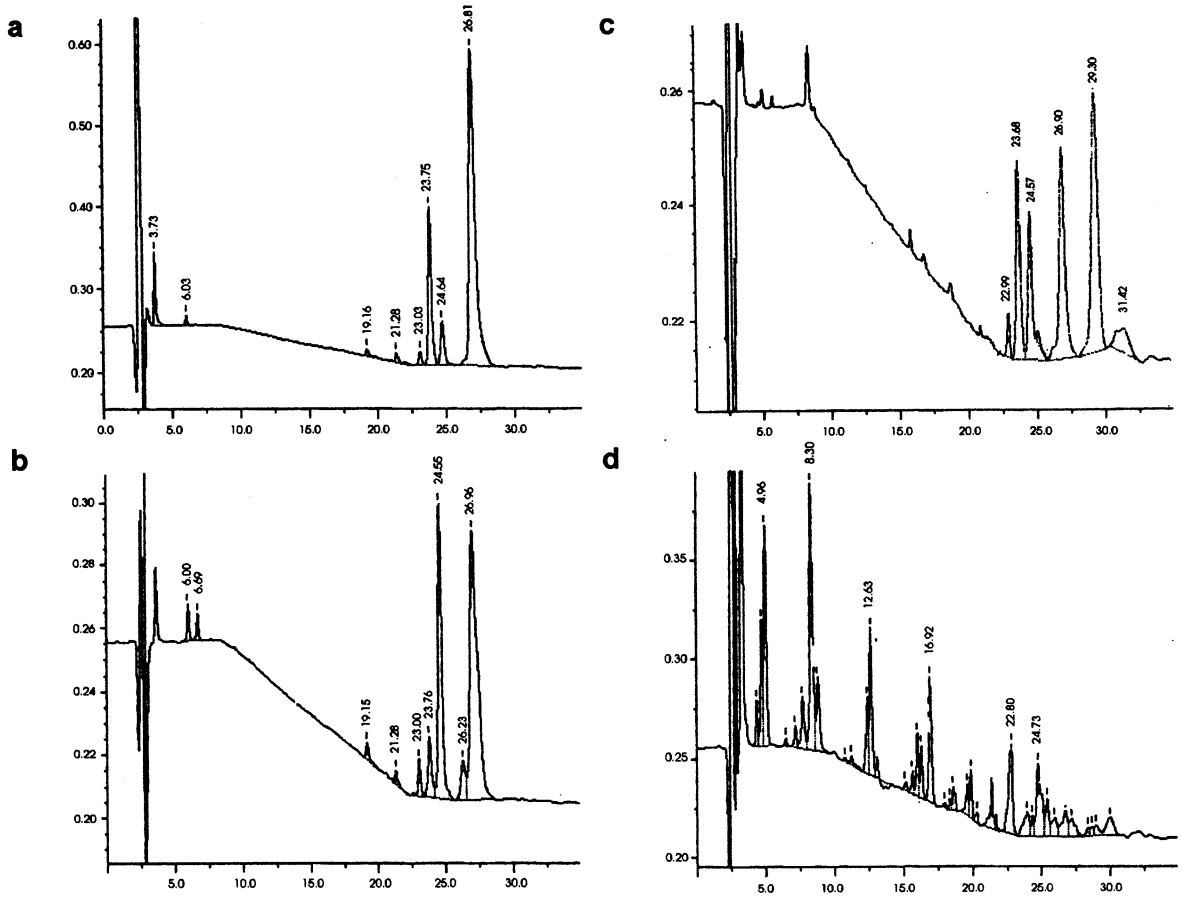

Fig. 11. HPLC chromatograms of various tetracosactide samples after 1-month incubation or in vitro testing: (a) in PBS (0.101 mg/ml) at 37°C under agitation, (b) in PBS (1.0 mg/ml) at 50°C (without agitation), (c) RG504/LMW microparticles prepared by spray drying, with a nominal drug loading of 1% w/w, and (d) RG504 microparticles prepared by WOW with a nominal drug content of 5% w/w.

wards, these preparations were concentrated with a nitrogen flux, lyophilised and redissolved in water to compare their CD spectra with those of freshly prepared aqueous tetracosactide solutions of identical concentrations. All solutions were analysed under the conditions used in the work of Powers et al. (1994) with a Jasco J-715 Spectro polarimeter (J-Tokyo). The spectra ranging from 235 to 350 nm were recorded three times at room temperature with a scan speed of 50 nm/min and a step resolution of 0.5 nm. The response was set at 0.5 s, the band width at 1 nm and the sensitivity at 20 mdeg.

2.5. Determination of peptide content and degradation

Typically, 20 mg (10 mg in the case of the batch with 5% w/w nominal drug loading) of microparticles were dissolved in 4 ml of chloroform, 4 ml of water were added and the mixture vigorously shaken for 30 min to extract the tetracosactide. The samples were then centrifuged for 15 min at 3300 rpm, the aqueous phases filtered and analysed by HPLC. Triplicates were prepared of each batch and two 100 μl aliquots per sample analysed. The HPLC system consisted of a Waters® 600E System Controller, connected to a Waters® 700 Satellite WISP autosampler and a Waters® 486 Tunable Absorbance Detector (Milford, MA). A RP-18 Nucleosil® 5 column (250 × 4 mm, Macherey–Nagel, Oensingen) was used. As mobile phase, methanol and an aqueous solution containing 0.1% v/v trifluoroacetic acid (TFA) were used at a flow rate of 1 ml/min and the temperature was set at 30°C. Methanol was increased linearly from 12 to 30% within the first 10 min, then to 42% in the following 5 min and maintained at this level for 35 min. After analysis, the methanol ratio was increased to 60% (50–55 min) to eliminate more hydrophobic impurities, before being linearly decreased to 12% in 5 min. At the end of the run, to stabilise the system, the solvent composition was maintained for 10 min at the starting conditions, resulting in a total run time of 70 min. Peaks were detected at 216 nm. The system was calibrated by external standardisation. Between 0.001 and 0.100 mg/ml of tetracosactide, the correlation coefficient was 0.999, with the quantitation limit close to 0.001 mg/ml. The day to day reproducibility for tetracosactide test solutions had a variation coefficient of 2% (over a period of weeks). Residual PVAL from WOW microparticles was not found to disturb HPLC analysis.

For monitoring the oxidation power and the drug recovery of the extraction process, 20 mg of drug-free microparticles were dissolved in 4 ml of chloroform, mixed with 4 ml of an aqueous tetracosactide solution and vigorously shaken for 30 min, as in the drug content determination (Witschi and Doelker, 1998). After centrifugation for 15 min at 3300 rpm, the aqueous phase was filtered and analysed by HPLC. The amounts of tetracosactide added in this control experiment corresponded to the amounts extracted from microparticles of different effective drug loading. All samples were prepared in triplicate.

Tetracosactide degradation products were manually collected using HPLC, lyophilised under centrifugation and redissolved in a mixture of acetonitrile (49.5%), water (49.5%) and acetic acid (1%) before electrospray-mass spectrometry

Table 2
Identification of the peaks of the HPLC chromatograms presented in Fig. 11(a) and (b), obtained from tetracosactide PBS solutions which were stored at 37°C (0.1 mg/ml, under shaking) and at 50°C (solution II, at 1.0 mg/ml without shaking) for 1 month

Retention time (min) (HPLC)	Mass (MS)	Product
19.16 (I) and 19.15 (II)	1711	Phe7–Val20
	1523	Pro12–Pro24
21.28 min (I and II)	1014 (main compound)	Arg17–Pro24
	939	Val13–Val20
	1174	Lys15–Tyr23
23.03 (I) and 23.00 (II)	2700	Ser3–Pro24 oxidised (2684+16)
23.75 (I) and 23.76 (II)	2950	Ser1–Pro24 (2934+16) tetracosactide oxidised
24.64 (I) and 24.55 (II)	2684	Ser3–Pro24
26.81 (I) and 26.96 (II)	2934	Ser1–Pro24 tetracosactide

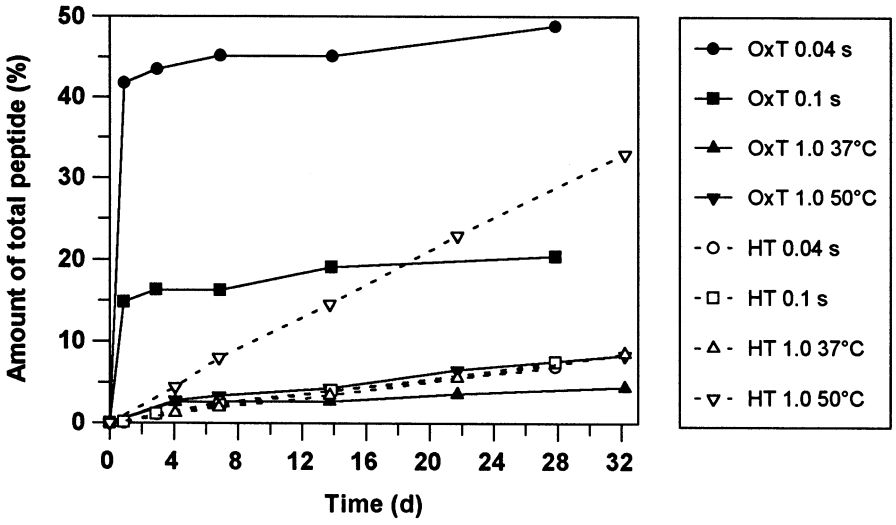

Fig. 12. Rate of tetracosactide hydrolysis (HT) at Tyr2–Ser3 and oxidation (Ox T) during storage in PBS at different temperatures (generally 37°C and one sample at 50°C) and concentrations (0.04, 0.1 and 1.0 mg/ml) with shaking (s) or without.

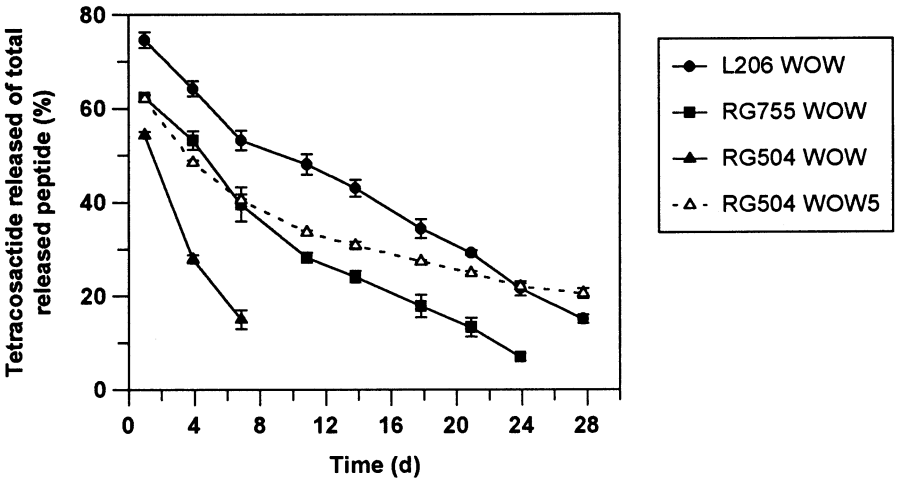

Fig. 13. Intact tetracosactide released from WOW microparticles during in vitro release testing, expressed in per cent of total peptide released.

analysis. A VG Trio 2000 spectrometer (quadrupole, Micromass, Sissach, Switzerland (previously VG Biotech, Altrincham, UK)) operating in a positive-ionisation mode in a m/z range of 200–2000 was used. For each sample, 10 μl were injected with a flow rate of 2 μl/min and 26 scans acquired and treated using an in-house programme. The capillary temperature was set at 60°C and the needle voltage at 3.58 kV. External calibration of the mass scale was performed with a solution of horse myoglobin.

2.6. In vitro peptide adsorption and release from the microparticles

For the in vitro testing, 20 mg of microspheres (duplicates for each batch and sample time) were suspended in 2 ml of PBS pH 7.4, and agitated in

a horizontal shaker (37°C, 120 rpm, type 3022, Gesellschaft für Labortechnik, Burgwedel, Germany). At specific times, samples were withdrawn and centrifuged for 15 min at 3300 rpm. Two 100 μl aliquots of the filtered aqueous phase were analysed by HPLC and mass spectrometry, as described above. The solutions of the samples containing microparticles with a 5% w/w drug loading were diluted five times before injection into the HPLC column.

In the adsorption test, drug-free microparticles (20 mg) were incubated with 2 ml of tetracosactide dissolved in PBS. Two tetracosactide solutions were prepared. The first one (solution a) contained tetracosactide at a concentration of 0.10 mg/ml, corresponding to the maximal tetracosactide release possible from microparticles with a nominal drug loading of 1%. The second one (solution b) had an intermediate tetracosactide concentration of 0.04 mg/ml. Suspensions were prepared in duplicate and microparticle-free control solutions, containing 2 ml of either solution a or b prepared for each sample time. At selected times, samples were taken and the filtered aqueous phases containing the non-adsorbed tetracosactide analysed by HPLC. The amounts of tetracosactide adsorbed (m_{ads}) on 20.0 mg microparticles was calculated as:

$$m_{ads} = (c_i - c_r) \times 2 \quad (1)$$

where c_i = initial tetracosactide concentration (mg/ml), and c_r = tetracosactide concentration recovered by HPLC (mg/ml) during the adsorption test.

During in vitro release testing, the amounts adsorbed on microparticles are not known. Therefore, the values resulting from in vitro release were corrected by the amounts adsorbed during the adsorption test, to obtain an estimate of the tetracosactide effectively released.

3. Results and discussion

The particle size, the encapsulation efficiencies and the amount of tetracosactide, typically recovered during microparticle extraction with the chloroform and water, were presented elsewhere (Witschi and Doelker, 1998).

3.1. Tetracosactide degradation during microencapsulation

When tetracosactide solutions were treated similarly to microparticle preparation, then lyophilised and redissolved in water, their CD spectra were identical with those of freshly prepared aqueous solutions, suggesting that no irreversible structure changes occurred during the microencapsulation simulation run. In addition to CD analysis, the reconstituted solutions were subsequently analysed by HPLC. In samples exposed only to the methanol/methylene chloride mixture, no changes or additional peaks could be detected when compared with the chromatogram of a freshly prepared tetracosactide solution. Agitation for two min at 20000 rpm of an aqueous tetracosactide solution in methylene chloride resulted in negligible oxidation (1.2% versus 0.8 to 1.0% in fresh tetracosactide solutions) of tetracosactide without the formation of other degradation products.

Typical HPLC chromatograms of peptide solutions obtained after microparticle extraction are presented in Fig. 2(a)–(d). Tetracosactide eluted at 26–27 min, which was confirmed by mass spectrometry. Under the selected MS conditions, three to seven charge-bearing sites (see Fig. 3) were found for tetracosactide containing three arginyl and four lysyl residues which are typically charged during ES–MS (Covey et al., 1988). The resulting mass obtained for tetracosactide was 2934–2935. To confirm the identity of the oxidised tetracosactide, eluting at 24–24.5 min in HPLC, hydrogen peroxide was added to a tetracosactide solution and subsequently analysed by HPLC and by MS. Under these conditions tetracosactide is oxidised at the thioether group of methionine (Dedman et al., 1957), resulting in a mass of 2950–2951 in MS.

When microparticles were prepared by spray drying using the high molecular weight polymers L206, RG755 and RG504, chromatograms similar to the one presented in Fig. 2(a) were obtained. The masses of these main peaks were all above the mass of tetracosactide, possibly due to oxidation of tetracosactide at different sites during spray drying in the presence of the high molecular

weight polymers. Consequently, almost no intact tetracosactide could be recovered in the corresponding spray dried microparticle batches. In contrast, by using the polymer mixture RG504 LMW (Fig. 2(b)), degradation of tetracosactide during spray drying could mainly be avoided (see also discussion below). Comparable chromatograms of tetracosactide were obtained from microparticles prepared by ASES (Fig. 2(d)). From these results it can be concluded that very low molecular weight PLGA protects tetracosactide against oxidation during spray dying. This may be explained by interactions between the carboxylic acid groups of the polymer with the amino groups of the basic amino acids of the peptide, sterically hindering oxygen attack. Additionally, by replacing air with supercritical CO_2 in ASES, tetracosactide is not exposed to oxygen and is therefore protected against oxidation when sprayed together with high molecular weight PLA in a methanol–methylene chloride formulation. The small peak at 28.43 min (Fig. 2(d)) was not observed in spray dried microparticles prepared with RG504LMW.

A chromatogram of tetracosactide extracted from RG755 microparticles prepared by WOW is shown in Fig. 2(c). It contains all the peaks typically found in microparticles prepared by WOW. Depending on the polymer used to prepare the microparticles, the peak eluting at 30.28 min (Fig. 2(c)) was very small in the case of L206 but increased for RG755 and RG504 batches in the cited order and with increasing the drug loading from 1 to 5%. During microparticle extraction, as found in control experiments, oxidised tetracosactide was formed, but there were no products eluting later than tetracosactide. Consequently, the peak at around 28.4–30 min was a degradation product typically formed during microencapsulation. As a certain amount of tetracosactide was oxidised during microparticle extraction (finely punctured fields in Fig. 4), the results obtained for tetracosactide degradation during microencapsulation were corrected with the values of the extraction control experiments. In the control experiments, empty microparticles, prepared by the three methods and composed of the different polymers, were used and tetracosactide amounts added, which corresponded to the effective drug loading of the different microparticle batches. It was found that an almost constant absolute amount of tetracosactide was oxidised during extraction, which was hardly affected by the concentration of tetracosactide in the aqueous phase during the extraction process. The amount of oxidised tetracosactide recovered (expressed as total peak area %) was in all samples very close to that of the polymer-free solution ($14.1 \pm 2.4\%$, when the same amount of tetracosactide was added to the various samples) and was lowered by the presence of drug-free WOW and ASES microparticles (results not shown). Exceptionally, drug-free microparticles prepared by spray drying with the L206, RG755 and RG504 polymers (not the mixture RG504LMW) caused complete oxidation of tetracosactide during extraction. The reason for this catalytic effect (only singly oxidised tetracosactide was formed and not the variety of degradation products found in drug-loaded spray-dried microparticles) of spray-dried drug-free microparticles is not known (the original non-treated polymer did not show this effect). The amounts of tetracosactide which degraded during microencapsulation are summarised in Table 1. In Fig. 4, the amounts of tetracosactide which oxidised or degraded otherwise during microencapsulation and microparticle extraction are presented. It can be concluded that the amounts of degradation products, other than singly oxidised tetracosactide, increased in WOW microparticles depending on the polymer in the order L206 < RG504, as well as with increasing the drug loading, whereas the addition of LMW (in RG504 LMW WOW samples) reduced the formation of such products. In RG504LMW SD and L206 ASES microparticles, degradation was only 3.8 and 2.8%, respectively, but the amount of oxidised tetracosactide was greater than in WOW microparticle batches. The highest degradation of tetracosactide occurred during spray drying, when the high molecular weight polymers L206, RG755 and RG504 were used. Best results, in other words lowest tetracosactide degradation, were observed with WOW and ASES microparticles made of L206 and for

the polymer mixture RG504LMW when microparticles were prepared by WOW as well as by spray drying. In the other WOW batches, the amount of intact tetracosactide decreased with increasing amount of glycolic acid and decreasing molecular weight of the polymer.

3.2. Peptide adsorption and release during in vitro testing

As the sample buffer solutions were not replaced over time during the in vitro release experiment, the pH decreased depending on the nature of the polymer as presented in Fig. 5. Whereas only a slight pH decrease was observed in samples containing L206 or RG755 microspheres, the pH fell to 5 in RG504 samples and was close to 3 in RG504/LMW samples, as already observed elsewhere (Witschi and Doelker, 1998).

The amounts of tetracosactide adsorbed on 20 mg of microparticles made of L206, RG755, RG504 and RG504/LMW, prepared by spray drying, are presented in Fig. 6. It can be seen that tetracosactide only adsorbed to a small extent on microparticles composed of L206 or RG755, whereas its affinity to RG504 microparticles was increased and even very high in the case of RG504LMW microparticles. On the latter, tetracosactide was adsorbed to over 90% in the case of solution a (0.10 mg/ml) and b (0.04 mg/ml), after one day. On these RG504LMW microparticles, the total amount adsorbed was increased with increasing peptide concentration of the solution. In contrast, an increase of peptide content in samples containing L206 or RG755 microparticles did not influence markedly the amount of total tetracosactide adsorbed. This led to the conclusion that at concentrations equal or below 0.04 mg/ml tetracosactide, the surfaces of L206 and RG755 microparticles were saturated with tetracosactide. Consequently, maximal adsorption capacity was reached at the lower investigated peptide concentration, pointing out the absence of peptide multilayers around the microparticles. Due to the many basic amino side groups of tetracosactide and the resulting high isoelectric point (10.5), tetracosactide is not expected to show aggregation under the adsorption test conditions.

For RG504 microparticles, the curves of adsorbed tetracosactide were similar for both solutions a and b during the first phase, but in the second phase, the amount of tetracosactide adsorbed increased with increasing peptide concentration. In RG504LMW samples, the amount of free polymer carboxylic end groups is much greater than in the other microparticle batches composed of L206, RG755 or RG504, respectively, due to the presence of very low molecular weight polymer. Consequently, the number of free carboxylic end groups at the surface of the microparticles, capable of interacting strongly with the amino side groups of the peptide, will be much increased and therefore more peptide molecules adsorbed. Additionally, as the amount of adsorbed peptide increased with increasing tetracosactide concentration from 0.04 to 0.10 mg/ml, the polymer groups interacting with the peptide at the microparticle surfaces, were not thought to be saturated at the lower peptide concentration. The fact that the amount of adsorbed tetracosactide increased in L206, RG755 and RG504 samples over time may be mainly related to the surface of microparticles which increases over time (Crotts and Park, 1997) and to the manner of calculating the total peptide amount (the degradation products were considered to have the same UV absorption coefficient in HPLC analysis). In RG504LMW samples, a significant amount of water-soluble polymer degradation products was released during microparticle degradation, leaving only small amounts of solid material in the samples at the end of the experiment. The carboxylic acid groups of the released oligomers were assumed to compete with those at the microparticle surfaces. Consequently, less peptide adhered on microparticle surfaces and more peptide remained 'free' in the solution, resulting in descending adsorption curves. Under the selected in vitro test conditions tetracosactide did not adsorb on the polypropylene tubes, used for the experiment. As tetracosactide degraded to varying extents during in vitro testing, the sum of the peak areas was used to calculate the amount of peptide that had been either adsorbed or released. Water-soluble polymer hydrolysis products were found to elute before 4.5 min in HPLC chro-

matograms and were not included in the calculations.

The cumulative in vitro peptide release curves of L206, RG755, RG504 and RG504LMW microparticles, prepared by the three methods, are presented in the Figs. 7–10 together with the corrected values. These values were obtained by adding the amount of peptide which was adsorbed on the microparticles during the adsorption experiment to the amount of peptide recovered during in vitro release testing. With the exception of RG504WOW, with a drug loading of 5%, the results were corrected using the values of solution b (0.04 mg/ml). Due to the significant adsorption on RG504LMW samples, exceeding 90%, which additionally depended strongly on the peptide concentration in the range of interest, the values were corrected by multiplying the recovered peptide concentrations with correction factors. These factors (results not shown) were obtained by dividing the initially added peptide concentrations by the recovered peptide concentrations, detected at fixed times during in vitro adsorption testing. The results for the WOW samples required the least correction, since the amounts of peptide recovered in the release media were close to the non-adsorbed amounts found in the adsorption test samples. For the other batches, additional adsorption experiments at lower tetracosactide concentrations would be necessary to obtain more precise correction data.

After the burst release from L206 microparticles (Fig. 7) no more peptide was released from the WOW batch over the investigated time period, whereas low quantities were still released from ASES, and probably also from the spray dried microparticles, after the burst release. In RG755 and RG504 microparticles, the burst release was followed by a lag phase which lasted until the end of the experiment. In contrast, microparticles made of RG504LMW (Fig. 10) exhibited a continuous release with a higher release rate during the last phase. With the corrected values (for 1% nominal drug loading) the burst effect increased depending on the polymer in the order L206 < RG504 ($\leq$ RG504LMW) for spray dried (15–31%) and WOW (33–73%) microparticles. The logical consequence of this order, which is in agreement with the findings of Heya et al. (1991), Mehta et al. (1996), is to expect a very high burst effect for the most hydrophilic microparticles containing the very low molecular weight PLGA. But, due to very strong interactions between this type of polymer and the tetracosactide, as demonstrated in the adsorption experiment, the RG504LMW microparticles exhibited no burst effect.

When comparing the three preparation methods, the burst effect increased in the order ASES < spray drying < WOW. For WOW, the burst release was always 1.5–3 times higher than for spray dried microparticles. Due to the very high porosity of RG504 WOW microparticles with 5% w/w drug loading the corresponding high drug release was not surprising. Consequently, spray drying was suggested to result in less porous microparticles with a more homogeneous peptide distribution than WOW. Porosity may even be reduced when using ASES for microencapsulation.

3.3. Peptide degradation during in vitro testing

During the adsorption test, aliquots of solution a and b were incubated under the same conditions as with the sample tubes containing drug-free microparticles. The degradation profile of the control solution a after 1-month incubation is presented in Fig. 11(a). In another experiment, solutions containing 1 mg/ml tetracosactide in PBS were stored at 37 and 50°C without shaking. The chromatogram obtained after 1-month incubation for the solution stored at 50°C (diluted before HPLC injection) is presented in Fig. 11(b). From the chromatograms shown in Fig. 11(a) and (b), it can be seen that the same peptide degradation products were recovered, but in varying quantities. The identities of the different degradation products are listed in Table 2. In Fig. 12, the rates of tetracosactide oxidation and hydrolysis (at Tyr2–Ser3) expressed as per cent of total peptide amount are presented. During storage, the amount of oxidised tetracosactide increased slightly over time and additionally it was found to hydrolyse at Tyr2–Ser3, similar to tetracosactide. Consequently, the total amount of oxidised

(singly oxidised tetracosactide plus oxidised hydrolysed tetracosactide) and that of hydrolysed peptide (hydrolysed tetracosactide plus hydrolysed oxidised tetracosactide) were used for calculating the values of oxidised and hydrolysed tetracosactide. When comparing the amounts of oxidised tetracosactide (absolute quantity expressed as peak area and not as % of total peptide) the same values were obtained for the control samples of 0.04 and 0.10 mg/ml and about 1.75 times bigger peak areas for the samples with 1.0 mg/ml peptide (calculated for the non-diluted samples). This indicates that oxidation at the methionyl residue depended directly on the reactive oxidising species available in the samples, which were obviously limited and almost consumed after one day, and to a much lower degree on the peptide concentration. However, in agreement with Fransson et al. (1996), an increase of the temperature from 37 to 50°C resulted in increased oxidation (4.4–8.3% after one month for solutions of 1.0 mg/ml). Oxidation of methionyl was described to follow second-order kinetics (Fransson et al., 1996), and to be caused by different reactive oxygen containing species, of which hydroxyl radicals ($HO\cdot$ and $HOO\cdot$) are the most reactive (Cleland et al., 1993), and to positively correlate with the oxygen content and phosphate content (Fransson and Hagman, 1996). Fig. 12 shows that the curves for the hydrolysis product expressed in per cent of total peptide were superposed for the various solutions stored at 37°C. The peptide was assumed to cleave following first order kinetics into Ser1–Tyr2 and Ser3–Pro24 with diketopiperazin formation at the two N-terminal amino acids (Powell, 1996). Increase of the temperature from 37 to 50°C markedly increased the rate of this reaction, whereas shaking, previously described as increasing the degradation rate of atriopeptin(III) (Johnson et al., 1991), did not influence the suggested intramolecular reaction.

Unfortunately, the AA sequence Ser1–Tyr2, which was reported to elute very early under comparable HPLC conditions (Hudson, 1978), was not detected but corresponded probably to the peak eluting at around 6 min, which was not identified. The Ser1–Tyr2 diketopiperazine lacks a free protonatable amino group, which is necessary to get a signal under the described MS conditions.

During in vitro release testing, intact tetracosactide was only recovered in samples containing microparticles prepared by WOW. The amounts of intact tetracosactide released, expressed as per cent of total released peptide, are presented in Fig. 13. For batches with a nominal drug loading of 1% w/w, tetracosactide stability decreased with increasing polymer hydrophilicity (glycolic acid proportion) and with decreasing polymer molecular weight in the order: L206 < RG504. The formation of carboxylic acid end groups, correlating with the pH decrease in the samples over time (Fig. 5), during polymer degradation, increases in the same order. When the nominal drug loading of RG504 microparticles was increased from 1 to 5% w/w, tetracosactide stability was enhanced. In any case, tetracosactide was better protected until day 24 in L206 WOW microparticles with 1% w/w nominal drug loading than in RG504 WOW microparticles with a 5% w/w nominal drug loading (Fig. 13). Only at the end of the in vitro testing, was the amount of recovered intact tetracosactide higher for RG504 WOW5 samples. In control solutions of 0.10 and 0.04 mg/ml, 74 and 47%, respectively, of tetracosactide remained intact after 1-month testing, oxidised tetracosactide being the major degradation product. A chromatogram of tetracosactide released from RG504 WOW microparticles with a nominal drug loading of 5% w/w, after 1 month, is presented in Fig. 11(c). The peak eluting at 23.68 min was identified as a single oxidised tetracosactide by mass spectrometry. Intact tetracosactide eluted at 26.90 min. For the peaks at 24.57 min and 29.3 min masses higher than for tetracosactide were calculated (3006 and 2992) and could not be interpreted. Because the peak with the high mass of 3006–7 eluted generally at the same time as did AA 3–24, it is uncertain if peak masking occurred or if it was another degradation product. The peak at 29.30 min, never observed in polymer-free control solutions, increased depending on the polymer in the order L206 < RG504, similar to degradation during microencapsulation, as discussed under section Section 3.1. After one day of in vitro

testing, the peak at 24.57 min was very small, but the area of the peak with a retention time of 29.30 min was already 25% of the total peak area in RG504 WOW-5 samples.

In RG504/LMW samples, either prepared by spray drying or WOW, the peptide amounts recovered in the release medium were too low to allow an evaluation of the extent of the oxidation, due to the high peptide affinity for the microparticles. However, as a consequence of fast polymer degradation and the significant pH decrease in the latter samples, tetracosactide was found to be severely degraded into multiple degradation products at the end of the experiment, as can be seen in Fig. 11(d).

No intact tetracosactide, only the oxidation product, was detected in L206 microparticles prepared by ASES. The amount of peptide released from these microparticles was very low and therefore assumed to oxidise when coming in contact with the phosphate buffer saline solution, independently of the presence of the microparticles.

For spray-dried microparticles, the situation was particular. During microencapsulation of tetracosactide into L206, RG755 and RG504, all tetracosactide was oxidised as discussed under Section 3.1. Consequently, no intact tetracosactide could be recovered from these samples. However, during the adsorption study, when tetracosactide was added to spray-dried empty microparticles, the degradation profiles of tetracosactide, excepting the increased oxidation (see Section 3.1), was comparable to those of tetracosactide released from WOW microparticles.

4. Conclusion

Oxidation was found to be the major tetracosactide degradation pathway caused by the conventional microparticle extraction method, using chloroform and water. Control experiments allowed the estimation of the amount of tetracosactide which oxidised during extraction but not during microencapsulation. Lowest tetracosactide degradation occurred during WOW and ASES when L206 was used as polymer, and with the polymer RG504LMW in the case of spray drying and WOW. When microparticles were prepared with the higher molecular weight polymers L206, RG755 and RG504 using spray drying, tetracosactide degraded into various products. These results indicate that the very low molecular weight PLGA interacted with tetracosactide during spray drying and protected it against degradation. In the batches prepared by WOW, the amount of degradation products other than singly oxidised tetracosactide increased depending on the polymer in the order L206 < RG504. Again, in WOW, addition of very low molecular weight PLGA was beneficial for tetracosactide stability.

The simple contact of tetracosactide with methanol and methylene chloride, as well as the exposure to mechanical stirring, similar to the first preparation step in WOW, did not markedly alter the integrity of the peptide.

The burst effect of the microparticles was governed by the preparation and the nature of the polymer and increased in the order ASES < spray drying < WOW and depending on the polymer in the order L206 < RG504 with increasing the hydrophilicity of the polymer. However, when the very low molecular weight PLGA was added, the burst effect disappeared due to very strong peptide polymer interaction. A lag phase generally followed the initial peptide release. Continuous tetracosactide release was only observed in batches made of RG504LMW. Peptide adsorption on drug-free microparticles increased in the order RG755 ≤ L206 (next to zero at the beginning) < RG504LMW (over 90% after one day). During in vitro release from WOW microparticles, peptide degradation increased with increasing polymer hydrophilicity and decreasing polymer molecular weight in the order: L206 < RG504 (< RG504LMW). By increasing drug loading, peptide stability was also improved.

In all polymer-free solutions, the absolute amount of oxidised tetracosactide was the same and hardly influenced by the concentration of tetracosactide but was increased by raising the temperature. In contrast to oxidation, hydrolysis at Tyr2–Ser3, the other main degradation reaction, was the same in all samples stored at 37°C

when expressed as per cent of total peptide, indicating a first order reaction mechanism. Shaking did not accelerate tetracosatide degradation.

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