ABSTRACT

Structural stability of the entrapped leuprolide acetate (LPA) in poly(DL-lactide-co-glycolide) (PLGA) was studied after in-vitro release of the drug using UV-Vis, fluorescence, circular dichroism, $^1$H NMR spectroscopic and HPTLC techniques. To prepare the injectable implant, 3% (v/v) of LPA was dissolved in a mixture of 33% (v/v) of PLGA (intrinsic viscosity of 0.5 dL/g) in N-methyl-2-pyrrolidone (NMP) as solvent. Upon introduction of 1 g of the above mixture (containing 30 mg of LPA) into 4 mL of aqueous carbonate buffer at pH 7.2, NMP diffuses out from the matrix causing the polymer to precipitate. Release of the drug into 4 mL of the fresh buffer solution at 37°C was measured every 24 h for one month. The UV-Vis and intrinsic fluorescence spectra of the solutions were recorded right after collection. Then, the release media were lyophilized and studied by $^1$H NMR spectroscopy. Although a clear interpretation of the CD spectra was faced to some ambiguities, the first derivative of the UV-Vis spectra and the results of fluorescence studies indicated that the tertiary structure of the peptide had suffered little destruction during its stay in PLGA. $^1$H NMR studies of the released medicine showed no change in the peptide primary structure. HPTLC analysis of the release medium showed one band with similar Rf to the original sample of LPA. The collected results indicate that the entrapped LPA suffers no structural changes during its stay in polymer formulation and remains stable during the first 24 h of release.

INTRODUCTION

In recent years, recombinant proteins, peptides and antibodies have established themselves on the pharmaceutical market for the treatment and prevention of numerous diseases [1]. A prerequisite for their clinical application is the development of stable formulations because proteins are complex and susceptible molecules [2]. In order to assure protein integrity during bioprocessing, formulation, storage and handling, the use of different analytical techniques to detect the degraded or deformed protein is inevitable. Especially characterization of the conformational variants induced by environmental stress,
aggregation, or chemical reactions such as oxidation or deamidation, which can change shape, morphology and size of the molecules and make them immunogenic, requires a selection of complementary methods [3].

Leuprolide acetate (LPA, Figure 1a) is a potent gonadotropin-releasing hormone (GnRH or LHRH) agonist which is used in treatment of some sex hormone related disorders including malignant prostate cancer, endometriosis and precocious puberty [4]. Its short biological half life (nearly 4 h) is considered as the major shortcoming of the medicine which requires frequent injection in a daily dosage schedule [5]. This problem can be overcome using a degradable polymeric drug delivery system that encompasses and releases a sufficient amount of the active substance for a desired therapeutic period (1, 3 and 6 months in this case). However, this type of delivery system has its own concerns regarding the kinetics of protein release as well as its stability.

The copolymer of lactide/glycolide (PLGA, Figure 1b) is considered as a patient-friendly and cost-effective material which is easily dissolved in a biocompatible organic solvent like N-methyl-2-pyrrolidone (NMP, Figure 1c) [6]. After injection of the polymer solution into the body, the solvent diffuses into the aqueous environment resulting in the polymer precipitation which forms an implant at the injection site. Since the polymer gradually degrades, the technique has been used for in-situ forming biodegradable drug delivery purposes [7-8]. In case of LPA, a formulation of the medicine (Eligard®) in PLGA dissolved in NMP has been commercialized [9]. As a result, the polymer intrinsic properties, the release medium properties and the formulation properties have been extensively studied [10-11]. The stability of the

Figure 1. The chemical structures of leuprolide acetate (LPA), poly (lactide/glycolide acid) (PLGA), N-methyl-2-pyrrolidone (NMP).
entrapped peptide has also been studied, but mainly by means of the liquid chromatographic (LC) methods [12-13]. However, these methods fail to reveal the impact of the polymeric matrix on the structure of an entrapped peptide like LPA. To achieve this goal a combination of techniques should be applied. Using an in-vitro release model, it was the objective of this research to study the structural durability of LPA in the aforementioned formulation by means of UV-Vis, fluorescence, circular dichroism (CD) and $^1$H NMR spectroscopy. HPTLC technique was also used to support the results.

**EXPERIMENTAL**

**Materials and Methods Chemicals**

PLGA resomer RG 504 H [poly(DL-lactide-co-glycolide), 50:50, MW=13,000] was purchased from Boehringer (Ingelheim, Germany), leuprolide acetate CAS-74381-53-6 was purchased from Bachem (Bubendorf, Switzerland) and stored according to the label guidelines. NMP was obtained from Merck distributor. All the other chemicals used in this work were taken from the authentic samples.

**Formulation Preparation and Release Study Programme**

To prepare a solution (3% w/w) of the injectable implant, a mixture of PLGA and NMP (330 and 640 mg, respectively) was made and shaken continuously for 72 h at room temperature. The resulting mixture was $\gamma$-irradiated at a dose of 25 kGy. To this solution, then, 30 mg of LPA was added and shaken for 30 min before use [14]. Upon introduction of 1 g of the obtained formulation into 4 mL of aqueous carbonate buffer at pH 7.2, NMP diffused out from the matrix and polymer was precipitated. The resulting system was kept at 37°C. To study the released drug, the whole 4 mL of the buffer solution on the matrix (RD solution) was collected after 4 h and the same volume of the fresh buffer was put on the remained matrix. Afterwards, the RD solution was collected every 24 h in the same way.

In order to have blank samples for comparative purposes, 970 mg of the $\gamma$-irradiated mixture of PLGA and NMP (330 and 640 mg, respectively) was introduced into 4 mL of aqueous carbonate buffer at pH 7.2 and kept at 37°C. A 4 mL of the buffer solution on the drug-free-matrix (DP solution) was collected in the same manner as the RD solutions.

**UV-Vis and Fluorescence Spectroscopy**

To obtain good quality UV-Vis and fluorescence spectra, samples were subjected to proper dilution before experiments. UV-Vis spectra of the RD solutions were recorded right after collection in a range of 240 to 320 nm against the corresponding DP solutions by a double-beam instrument of Shimadzu 1650 PC model at room temperature using conventional quartz cuvettes. First derivative of the desired spectra were obtained by means of the instrument soft ware, UV Probe (Version 2).

The extinction coefficient of LPA in carbonate buffer solution (pH 7.2) at 280 nm and room temperature was 5995 (data is not shown here). The data was used for estimation of the released drug into the RD solutions.

Intrinsic fluorescence spectra of the RD samples were acquired by a Varian Cary (Eclipse Australi) instrument in the conventional 400 $\mu$L quartz cuvettes at 25°C using an excitation wavelength of 280 nm.

**Circular Dichroism**

Circular dichroism (CD) spectra were recorded on an Aviv 215 spectrophotometer (USA). The data were smoothed by applying the JASCO J-715 software, including the fast Fourier-transform noise reduction routine, which allows the enhancement of most noisy spectra without distorting their peak shapes. The concentration of the LPA samples was adjusted to 0.14 mg/mL and Far-UV CD experiments were carried out in a range of 190 to 260 nm.

**$^1$H NMR and HPTLC Experiments**

$^1$H NMR spectra were acquired on a 400 MHz Bruker Avence-400. The selected samples of RD and DP solutions were lyophilized. The collected precipitates were then dissolved in 0.8 mL of D$_2$O and studied by the instrument.

HPTLC Chromatography was performed on 10cm $\times$5cm 60 F254-silica gel HPTLC plates (Merck #5642). The layers were cleaned by development to the top with chloroform-methanol (1+1) prior to chro-
matography, then dried in a fume hood and heated on a TLC plate heater at 80°C for 10 min. Sample and standard zones were applied to the plates as bands by means of an Automatic TLC sampler, (linomate IV, Camag, Muttenz, Switzerland). Plates were developed with chloroform-methanol (70:30 v/v). The visual examination of the bands was facilitated in the presence of iodine vapour.

RESULTS AND DISCUSSIONS

Figures 2a and 3 illustrate the overlaid UV-Vis and fluorescence spectra, respectively, of the released LPA in the RD solutions collected at different times. These spectra were used for further structural analysis in this study. Figure 4a shows the 1H NMR spectra of the solvent, degraded matrix containing no medicine in the applied buffer. Figure 4b also demonstrates the overlaid 1H NMR spectra of some precipitates obtained after lyophilizing the corresponding RD solutions. Finally, Figure 5 shows the results of HPTLC experiment carried out on some collected RD solution.

Literature survey reveals that the possible structural changes of the released LPA from the PLGA matrix have not been extensively studied [13]. The structural features of LPA in solution have been studied by different means of spectroscopy [15-16]. The stability of the LPA structure after release has been also studied but mainly by different LC methods [12-13,17], which are not informative from a structural point of view. However, as mentioned earlier, peptides and proteins are vulnerable molecules which might suffer structural changes after being entrapped in the micro-environment of a matrix. Since LPA is a single chain peptide, changes can happen in its either primary or secondary/tertiary structure. To prove that the structural features of an entrapped peptide has been left unchanged during its stay in a degradable matrix like PLGA, it is essential to collect LPA right after its release from the formulation. Structural studies of the freshly released peptide and comparing the results with those of the original LPA should shed light on the structural durability of the entrapped LPA, as well as, the impact of the PLGA matrix on the LPA structure. There are a few different spectro-
scopic methods for studying the structural stability of a peptide. CD, for instance, is a powerful tool which can be used for examining the secondary structure of the peptides [18]. However, in the case of this study, the application of such method is severely limited by the fact that the degradation of the polymeric matrix releases molecules which contain chiral carbon, thereafter interfering with the CD behaviour of the sample [19].

Similarly, FTIR pursuing of the released LPA in the corresponding RD solution would not be free of interferences. Therefore, to achieve the goal of this study, the UV-Vis spectra of the expected components of an RD solution were examined at the beginning. As Figure 2b shows, the maximum absorption of a solution containing the degraded matrix stands clearly away from the maximum absorption of a buffer solution containing LPA. Therefore, the released drug can be studied directly in solution with little interference of the solvent or other molecules. Figure 2a contains the results of such experiments.

Since the UV-Vis spectra of the released LPA are almost free of interferences, they can be used for further studies. Assuming that the trapped LPA involves in a chemical reaction like hydrolysis, then, the products of such reaction are released in the medium alongside with the original LPA. As a result, not only will the shape of the resulting spectrum change, but also the first derivatives of such a spectrum and the original LPA will not be superimposed and cross zero at different places [20]. As it is seen in Figure 2c, the intersections of the corresponding first derivatives of the overlaid UV-Vis spectra, Figure 2a, with the horizontal axis centre almost on one point which points to the existence of only one chromophoric substance in the studied solutions [21].

The interference free UV-Vis spectrum of a peptide indicates that a meaningful fluorescence spectrum of the sample can be obtained with little difficulty. Figure 3 shows the overlaid spectra of some RD solutions and a solution containing the authentic LPA collected from the intrinsic fluorescence experiments. Comparing these spectra, the occurrence of any disruption is rejected in the three-dimensional structures of the released LPA.

Considering the chemical structure of LPA, it is also possible to investigate the integrity of the released medicine structure by monitoring the $^1$H NMR spectrum of RD solution content as described in the experimental section. After lyophilizing an RD solution, one expects to find the degradation products of PLGA, NMP, and the released LPA beside its possible degradation products. Figure 4a shows the $^1$H NMR spectrum of PLGA in NMP containing no medicine. Peaks at $\sigma = 1.878$ (q), $2.278$ (t), $2.68$ (s), and $3.578$ (t) belong to the c, d, a, and b protons, respectively, of NMP and peaks at $\sigma = 3.958$ (q) and $3.795$ (s) belong to the f and h protons of the degraded PLGA. The protons of g are apparently covered by the strong peaks of NMP protons. Figure 4b also shows the $^1$H NMR of freshly prepared LPA in D$_2$O. As it is seen in Figure 4b, the pattern of the downfield of the spectrum produced by the protons of histidine, tryptophan and tyrosine can be used for the structural stability investigations with little interferences of the solvent or PLGA molecules. Figure 4c illustrates the spectrum of the content of some RD solutions which had been mixed after lyophilization and dissolved in D$_2$O. Comparing the insets of 4b and 4c spectra also supports the idea that the released LPA has managed to maintain its original structure after 24 h of release in the experiment medium.

Finally, the content of RD solutions was checked by HPTLC. All the studied samples produced only one band with the $R_f$ value similar to the original LPA sample, Figure 5. Therefore, the results of this research indicate that the entrapped LPA has experienced no important structural changes during its stay.

![Figure 3](image-url)
Figure 4. (a) $^1$H NMR spectrum of PLGA in NMP containing no medicine, (b) HNMR spectrum of freshly prepared LPA in D$_2$O, (c) $^1$H NMR spectrum of the content of some RD solutions.
in the PLGA matrix and maintains its original structure after release in the carbonate buffer, pH 7, for 24 h at 37ºC.

CONCLUSION

The HPTLC analysis and $^1$H NMR studies of the freshly released LPA from PLGA confirm that the primary structure of the peptide has suffered no changes during its stay in the polymeric matrix. In addition, the UV-Vis and fluorescence studies of the freshly released LPA from PLGA indicate no major changes in the tertiary structure of the released LPA. These results support the idea that the degradable copolymer of lactide/glycolide is inert towards LPA and can be used safely for the implant formulations.

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