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European Journal of Pharmaceutics and Biopharmaceutics 49 (2000) 253–257

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Research paper

Formation of peptide impurities in polyester matrices during implant manufacturing

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Received 24 September 1999; accepted in revised form 24 January 2000

Abstract

Most peptides are susceptible, in vivo, to proteolytic degradation, and it is difficult to formulate and to deliver them without loss of biological activity. In addition, it is often desirable to release them continuously and at a controlled rate over a period of weeks or months. For these reasons, a controlled release system is suitable. Poly(lactic acid) (PLA) is a biocompatible and biodegradable material that can be used for many applications, including the design of injectable controlled release systems for pharmaceutical agents. Development of these delivery systems presents challenges in the assessment of stability, specially for peptide drugs. By means of an extrusion method, long-acting poly(lactic acid) implants containing vapreotide, a somatostatin analogue, were prepared. The nature of the main degradation product obtained after implant manufacturing was elucidated. It was found that the main peptide impurity was a lactoyl lactyl–vapreotide conjugate. Because lactide are found in small quantities in most commercially available PLA, the influence of residual lactide in the polymeric matrix, on the formation of peptide impurities during manufacturing, was specially investigated. This work demonstrates that the degree of purity of the carrier is of great importance with regard to the formation of peptide impurities.

Keywords: Peptide; Polylactic acid; Melt-processing; Impurity; Vapreotide

1. Introduction

With the recent advances in biotechnology and chemical synthesis, the large-scale production of peptides for pharmaceutical purposes has become feasible. Therefore, there has been a great deal of research focused on the delivery of peptide drugs in a controlled manner [1]. A variety of polymers have been utilized as matrices to incorporate these peptides. Among them, poly(lactic acid), poly(glycolic acid) and their copolymers are well known biocompatible biodegradable materials with wide applications, including the design of controlled-release systems for pharmaceutical agents [2]. Especially interesting for sustained delivery of peptides are rod-like formulations obtained by various manufacturing techniques [3]. However, manufacturing of these delivery systems presents many problems in the area of stability assessment, since peptides possess chemical and physical properties, which present unique difficulties in the processing and delivery of these drugs [4].

The formation of peptide impurities during processing is among the potential difficulties associated with the incorporation of peptides into polymeric systems [5]. Indeed, during implant manufacturing, the peptide is often exposed to various unfavorable conditions, in particular exposure to elevated temperatures, pressure and shear [6]. So far, few studies have attempted to characterize the formation of peptide impurities associated with peptide extrusion within poly(esters) [7] or to study how the polymeric matrix affects the stability of the incorporated peptide. Typically, depending on suppliers, commercial polyesters contain various amounts of residual products, which can interact with the peptide. Although a number of protein or peptide stability studies have been carried out in solution, the issue of the stability in polymeric matrices has not received much attention [8]. Thus it is of great interest to understand the under-

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0939-6411/00/$ - see front matter
PII: S0939-6411(00)00066-7

lying mechanisms and the effects of external factors on the formation of peptide impurities. In this study, we used the somatostatin analogue vapreotide, a cyclic octapeptide as a model. This peptide has been formulated and extruded under harsh conditions in a poly(lactic acid) matrix. The major peptide impurity formed was isolated and identified. The influence of the polymeric matrix purity and in particular of residual lactide on peptide impurities formation was evaluated.

2. Materials and methods

2.1. Materials

The poly(lactic acid) L 104 (100 L PLA, MW 6000) was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany).

The somatostatin analogue vapreotide (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-amide) in the form of the pamoate salt was obtained from Novabiochem (Basel, Switzerland). The L-lactide was purchased from Aldrich (Steinheim, Germany).

All other chemicals were of analytical grade and used without further purification.

2.2. Methods

2.2.1. Implants preparation

The implants were obtained by extruding mixtures of L 104 and vapreotide pamoate at a core loading of 18.5% (w/w) (vapreotide, calculated as base) with a laboratory ram extruder as described previously [8]. The powder mixture was introduced into a barrel of 10 mm internal diameter, into which a piston rod (10 mm in diameter) was first inserted and then moved further into the barrel under an appropriate pressure. The drug/polymer mixture remained at 120°C during 1 h. The extruded rod had a diameter of 1.5 mm.

2.2.2. Peptide impurity extraction and isolation: analytical methods

The rod was grounded and 50 mg were dissolved in 20 ml of a mixture of acetonitrile and acetic acid (35 mM) in a ratio of 16:4. Thirty milliliters of acetic acid (35 mM) were then added (pH 3.1). An aliquot was centrifuged at 10 000 rev./min during 10 min, and the supernatant was collected and analyzed.

Vapreotide impurities were determined using a gradient reversed phase HPLC assay (column ET 125/4 Nucleosil 100-5 C18, Macherey–Nagel) with a UV detection wavelength at 220 nm (Waters module Iplus). The Millenium 2010® chromatography manager was used to integrate the data. As mobile phases, an aqueous solution containing 0.075% (w/v) trifluoroacetic acid (TFA) (pH 2.3) was used as solvent A and a mixture of this solution with acetonitrile (400/484) (w/w) was used as solvent B. The flow rate was 1 ml/min and the column oven temperature was 45°C. The linear gradient was increased from 44 to 80% B within the first 8 min, maintained at this level for 4 min and then decreased from 80 to 44% in the following 8 min.

The major impurity has been isolated and analyzed using the analytical HPLC method to confirm the identity and to establish purity. Its purity (>99%) was sufficient to allow further analysis without additional purification.

2.2.3. Determination of lactide content in polyester

Initial lactide content in the polyester was determined using an isocratic reverse-phase HPLC assay (precolumn KS 11/4 Nucleosil 100-5 C 18, Macherey–Nagel, column ET 250/4 Nucleosil 100-5 C 18, Macherey Nagel) with UV detection at 210 nm (Waters module Iplus). The Millenium 2010® chromatography manager was used to integrate the data. As mobile phase, an aqueous solution containing 0.1% trifluoroacetic acid (TFA) and acetonitrile (60/40) (v/v) was used at a flow rate of 1 ml/min. The temperature was set at 25°C, and a precolumn (KS 11/4 Nucleosil 100-5 C 18, Macherey–Nagel) was used.

2.2.4. Impurity analysis by chemical sequencing and mass spectrometry (MS)

An ABI model 477A protein sequencer was used to perform automated Edman sequencing. Mass data was obtained either with a matrix assisted laser desorption time of flight mass spectrometer (MALDI-TOF MS) from Perseptive Biosystems (model Voyager Elite) using the reflectron mode, or for MS-MS experiments, a VG Quattro II mass spectrometer.

2.2.5. Impurity analysis by NMR

The impurity (0.4 mg) was dissolved in 0.5 ml of d_4-methanol/d_3-acetonitril (1:1 v/v). The ^{1}H-NMR and the two-dimensional homonuclear DQF-COSY spectra [9] were recorded on a BRUKER AMX-600 spectrometer operating at 14.1 Tesla. 8K data points were acquired in the t_2 domain with 512 time incrementations in the t_1 dimension. The spectra were compared with the corresponding data recorded for a 1 mg sample of vapreotide pamoate.

2.2.6. Polymer purification

The native polymer (L104) (2 g) was solubilized in 6 ml chloroform. The polymer was then precipitated by addition of 100 ml methanol and filtered on a porosity 4 filter and dried under vacuum during 24 h.

3. Results and discussion

An essential part of pharmaceutical drug development is the assessment of the stability of the active ingredient in the therapeutic system, in order to comply with the ICH requirements at the time of submission [10]. However, the approval of peptides as therapeutic agents needs defined specification

limits for quality assurance and quality control. This is demonstrated by many guidelines and rules published recent [11]. In the case of synthetic peptides, the identification of impurities at a level of 0.5% or more should be aimed at, according to the USP 23.

3.1. Impurity determination

In order to obtain a sufficient amount of impurity, implants were manufactured by an extrusion process under harsh conditions. The main impurity found after extrusion was isolated and analyzed by an HPLC method. After analysis by chemical sequencing, the amino-terminal appeared to be blocked when the impurity was subjected to four cycles of automatic Edman degradation (Fig. 1). As a positive control, the first three amino acid residues of vapreotide, with the following sequence: Phe-Cys-Tyr-Trp-Lys-Val-Cys-Trp-amide with the two cysteine residues disulfide bridged, were successfully sequenced. The masses of the impurity and the vapreotide were determined as 1275.61 $(M + H)^+$ and 1131.47 $(M + H)^+$, respectively (delta mass: 144) using the MALDI-TOF MS. The impurity was digested with proteinase K and the mass measured 1018.55 $(M + H)^+$ was consistent with the removal of the C-terminal residue, tryptophan amide. Upon reduction, the mass increased from 1018.55 to 1020.55, indicating that the cysteines were involved in disulfide bonding and were not modified. We strongly suspected that (1) a dimer of lactic acid was attached to the amino-terminal residue or (2) both the amino-terminal and the epsilon amino group of the lysine were modified with a monomer of lactic acid. In a MS-MS experiment, a series of daughter b ions were found for the impurity (parent ion *m/z*: 1276) at *m/z*: 395.2, 558.5, 744.2, 873.2, 972.1, 1072.7. This clearly suggested that the lysine was unmodified and that the amino terminal phenylalanine was modified with a moiety of mass 144, corresponding to a dimer of lactic acid.

After NMR analysis, the DQF-COSY spectrum of the main impurity showed, in addition to the peptide pattern, two cross-peaks at δ_1/δ_2, 1.50/5.16 and 1.17/4.92 respectively. Their fine structures were indicative of two new AX_3 spin systems corresponding to a lactyl–lactamide moiety.

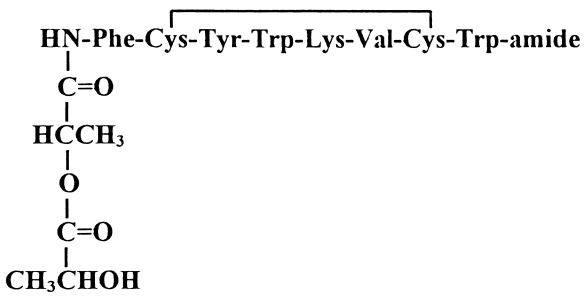

Fig. 1. Lactoyl lactyl–vapreotide conjugate: suggested structure of vaprotide main impurity.

This data corroborates the constitution suggested by MS for the main impurity.

3.2. Influence of starting material purity on peptide impurities formation during manufacturing: effect of residual lactide

Strategies to reduce protein and peptide byproducts during manufacturing are based on a well understanding of peptide degradation mechanisms. In our case, the major impurity formed after extrusion, was a lactoyl lactyl–peptide conjugate. A detailed study of the influence of the remaining L-lactide in the polymeric matrix on the formation of peptide impurities during the manufacturing process, was investigated. In these experiments, implants containing vapreotide were prepared with poly(lactic acid) matrices containing various amounts of L-lactide, varying from 0 to 10.8% (w/w).

Fig. 2 shows the influence of increasing L-lactide content, on the formation of the main impurity in the implants after extrusion.

The matrix containing L-lactide below the detection level was highly purified L 104, whereas the matrix containing 0.8% of L-lactide was the native polymer obtained from Boehringer Ingelheim. The other polymer matrices containing L-lactide were obtained by mixture of the native polymer with various amounts of pure L-lactide. In a preliminary study, we made sure that the L-lactide was not degraded after extrusion.

As shown in Fig. 2, it can be seen that the percentage of L-lactide in the polymeric matrix is directly proportional to the formation of the main impurity. Furthermore, it can be seen, that even small amounts of L-lactide (<1% w/w) have to be taken into account, since they are sufficient to lead to noticeable amount of the impurity (>5% w/w).

The mechanisms underlying the addition of lactide on the peptide may be explained by Spassky [12]. Cyclic lactones such as lactide, may be bound to compounds, after ring opening, by the way of a nucleophilic substitution reaction [13]. This reaction is influenced by the ring size, the heterofunctional group, the initiator and the reaction conditions. In

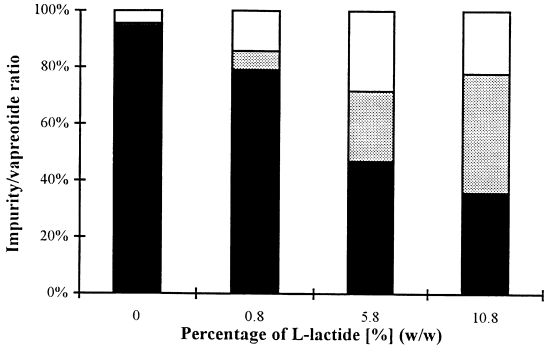

Fig. 2. Influence of the percentage of L-lactide in a poly(lactic acid) matrix, on the formation of vapreotide impurities, during an extrusion process. ■, Vapreotide; ▨, lactoyl lactyl-vapreotide conjugate; □, other impurities.

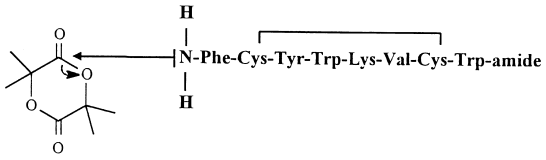

Fig. 3. Suggested mechanism of lactide addition on the peptide.

the present case, the initiator is the Phe-terminal amino group of the peptide, which attacks the C–O bond, leading to the formation of the lactoyl lactyl-vapreotide conjugate (Fig. 3).

It can be observed (Fig. 4), that not only the amount of the conjugate is diminished when the polymer is purified, but also the total amount of impurities. This result indicates that most of the peptide impurities formed during extrusion are depending on polymer purity. Indeed, during the purification process, not only L-lactide was removed, but also other residual products which might have interact with the peptide or have been bound to it during the manufacturing process.

It is interesting to note, that another impurity (impurity no. 2), which appears after 11.4 min on the HPLC spectrum (Fig. 4) increased with increasing amounts of L-lactide.

4. Conclusion

An essential part of pharmaceutical peptide drug development is the assessment of stability of the peptide in the dosage form, not only on storage, but also during manufacturing. Therefore, good knowledge about mechanisms leading to the formation of such impurities during manufacturing is of great importance. In addition, reducing the amount of impurities below a tolerated percentage level as regulated by the ICH guidelines (International Conference on the Harmonization of the technical Requirements for Registration of Pharmaceuticals for Human Use), will prevent their identification and therefore lengthy evaluation and expensive costs linked to their analysis [10]. However, during manufacturing of peptide delivery systems, the appearance of impurities can not be completely avoided.

In this study, implants containing a somatostatin analogue were produced under harsh conditions (high temperature and shear). One of the degradation products was present in amounts larger than 6% of the total peptide peak area. In order to elucidate its structure, this product was isolated and identified by chemical sequencing, mass spectrometry and NMR. It was demonstrated, that this main impurity was a lactoyl lactyl-vapreotide conjugate, resulting from the addition of L-lactide, originally present in the polyester, on the peptide Phe-terminal amino group. In addition, we studied the relation between the percentage of L-lactide in the polymeric matrix and peptide impurities formation during manufacturing. It was shown that the percentage of residual L-lactide was of great importance in the apparition of two main impurity peaks as shown by HPLC. In addition, it was also demonstrated that the total amount of peptide impurities was highly dependent on initial polymer purity.

Peptide degradation during manufacturing processes can hardly be completely avoided, but the knowledge of the mechanisms underlying the formation of impurities are of great interest in the search of how to get an acceptable final drug product.

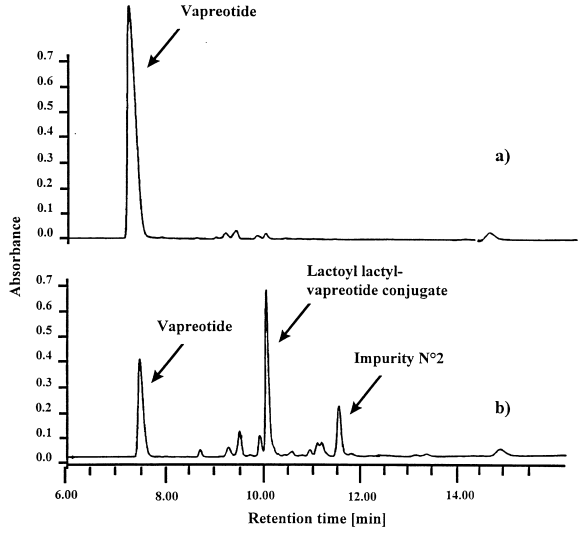

Fig. 4. Influence of the percentage of L-lactide in a poly(lactic acid) matrix during an extrusion process, on the total amount of impurities formed. (a) 0%(w/w) L-lactide; (b) 10.8% (w/w) L-lactide.

Acknowledgements

We wish to thank Dr L.-A. Savoy, M-Scan S.A., Geneva, Switzerland, for performing the MS-MS experiments.

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