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Stability studies of a somatostatin analogue in biodegradable implants

A. Rothen-Weinhold [a,b], K. Besseghir [c], E. Vuaridel [b], E. Sublet [b], N. Oudry [b], R. Gurny [a,*]

[a] *School of Pharmacy, University of Geneva, 30 Quai E. Ansermet, CH-1211 Geneva 4, Switzerland*
[b] *Pharmapeptides, University Campus, F-74166 Archamps, France*
[c] *Debiopharm SA, 1000 Lausanne 9, Switzerland*

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Abstract

In recent years, peptides and proteins have received much attention as drug candidates. For many polypeptides, particularly hormones, it is desirable to release the drug continuously at a controlled rate over a period of weeks or even months, and thus a controlled release system is needed. Polylactic acid (PLA) is a biocompatible and biodegradable material with wide utility for many applications, including the design of controlled release systems for pharmaceutical agents. Pharmaceutical development of these delivery systems presents new problems in the area of stability assessment, especially for peptide drugs. In this study, we aimed to investigate the influence of different steps, during the manufacturing of an implant, on peptide stability in the polymeric matrix. Polylactic acid implants containing vapreotide, a somatostatin analogue, were prepared by extrusion. The effects of time, extrusion and temperature on the peptide stability were studied. The influence of various gamma sterilization doses, as well as the conditions under which the implants were irradiated, were also investigated. Peptide stability in the polymeric matrix was evaluated at various temperatures and at various time intervals up to 9 months.

Keywords: Controlled release; Implant; Polylactic acid; Stability; Vapreotide

1. Introduction

Somatostatin (SS) is a cyclic peptide that has a broad spectrum of regulatory functions in the body. For example, in the hypothalamus it inhibits the release of growth hormone, but in the

* Corresponding author. Tel.: +41-22-702-61-46; fax: +41-22-702-65-67; e-mail: gurny@pharm.unige.ch.

pancreas and intestine it functions as an autocrine regulator. SS has been demonstrated to have inhibitory effects on the growth of certain prostate, breast, secretory pancreatic and carcinoid tumors. For clinical use, primarily in the treatment of acromegaly, several analogs of SS have been developed (Parmar et al., 1993; Gillis et al., 1997). Acromegaly is a chronic and debilitating disorder, resulting from hypersecretion of growth hormone; if untreated, it is associated with significant morbidity and mortality. Increased mortality is due in part to cardiovascular and cerebrovascular causes related to hypertension and diabetes mellitus and also to respiratory ailments (Wass et al., 1986). Results from clinical studies (Vance and Harris, 1991; Liuzzi et al., 1996) have clearly shown that subcutaneous administration of an SS analogue is an effective pharmacological option in the management of acromegaly. However, several daily injections (usually three) or continuous infusions are required to produce an effective therapy, due to the short half-life. Therefore, in order to obtain a long-term, constant therapeutic effect, a controlled delivery system is desirable to avoid repeated drug injections. For the past decade, there has been a great deal of research focused on the delivery of peptide drugs in a controlled manner (Pitt, 1990; Kissel et al., 1991). A variety of degradable and non-degradable polymers have been utilized as matrices to incorporate peptides. Among them, lactic–glycolic acid polymers provide a wide range of degradabilities from months to years, depending on their composition and molecular weight (Rothen-Weinhold et al., 1997). Especially interesting for sustained delivery of peptides are rod-like formulations obtained by various manufacturing techniques, which can eventually be removed. As peptides are often relatively low molecular weight molecules with varying size, solubility and shape, and since most peptides are extremely unstable, it has been very difficult to formulate and deliver them without loss of biological activity. Indeed, during implant preparation, the peptide is often exposed to various unfavorable conditions, particularly exposure to high temperatures or gamma-irradiation. So far, there have been few reports on peptide stability associated with peptide extrusion within lactic–glycolic acid polymers. Although a number of protein or peptide stability studies have been carried out in solution (Pourrat et al., 1995 Barthomeuf et al., 1996), the issue of the stability in polymer matrices has not received much attention. This is partly due to experimental difficulty in analysis of the peptide molecules entrapped within a solid polymeric matrix, since the entrapped peptide has to be extracted in an intact form into an aqueous phase by dissolving the polymer with an organic solvent (Park et al., 1995).

In this study, we focus on the somatostatin analogue vapreotide (RC-160), a cyclic octapeptide. This peptide has been formulated and extruded in a polylactic matrix, under various conditions. We have evaluated the stability after extrusion and after various storage conditions. Its susceptibility to degradation by gamma-irradiation has also been investigated.

2. Materials and methods

2.1. Materials

The polylactic acid L 104 (100 L PLA, molecular weight 6000) was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany).

The somatostatin analogue vapreotide (D–Phe–Cys–Tyr–d–Trp–Lys–Val–Cys–Trp–NH_2 in the form of the pamoate derivative was obtained from Novabiochem (Basel, Switzerland). All other chemicals were of analytical grade and used without further purification.

2.2. Methods

2.2.1. Implant preparation

The implants were obtained by extruding mixtures of L 104 and vapreotide pamoate at a core loading of 18.5% (vapreotide, calculated as base) with a laboratory ram extruder, as described previously (Gurtler et al., 1995). The powder 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 extrusion temperature was always 80°C, except for the temperature studies during which the temperature was increased up to 120°C. The extruded cylinders had a diameter of 1.5 mm, and were cut into short rods 1.5 cm in length.

2.2.2. Sterilization

The solid implants were sterilized with gamma-rays using a ^{60}Co source, at 25 kGy, at −78°C in dry ice and under air. Further, various doses were studied: 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 kGy. For the long-term stability tests, the implants were sterilized in all cases at 25 kGy under various atmospheres: air, N_2, Ar.

2.2.3. Stability study

Implants were first placed in 3-ml glass vessels, which were inserted into 50-ml screw-capped bottles. The bottles were hermetically sealed and stored in thermostatically controlled chambers at 4, 25 and 40°C, and protected from light. At predetermined time periods, implants were removed for analysis. Peptide content (not shown) and purity were determined by high-performance liquid chromatography (HPLC). The assays were performed in duplicate.

2.2.4. Extraction and analytical methods

The implants were completely dissolved in a mixture of 15% sodium perchlorate and 85% acetonitrile; then 100 ml of a buffer solution at pH 2.3 was added. The solution was then centrifuged at 10 000 rpm for 10 min. The supernatant was collected and analysed. Vapreotide impurity levels were determined using a gradient reverse-phase HPLC assay with detection at 220 nm [Waters 600 controller, multisolvent delivery system equipped with a UV detector (Waters 486, tunable absorbance detector), and an automatic injector (Waters 700 satellite WISP)].The Millenium 2010 chromatography manager (version 2.1).was used to integrate the data. The mobile phase consisted of a mixture of phosphoric acid–triethylamine–buffer solution pH 2.3–acetonitrile. The flow rate was 0.8 ml/min. The vapreotide peak area was compared to the peak areas of the total number of peaks and was expressed as a percentage (the areas of the pamoic acid peak and the peak system were not included).

3. Results and discussion

With regard to stability, peptides are susceptible not only to chemical degradation but also to physical degradation. They are sensitive to a variety of environmental factors such as temperature, light, oxidizing agents or oxidation promoting substances, pH, freezing, shaking and shear stress. It is important to consider these factors to ensure stability of the active substance during the production process, as well as during the development and the manufacture of the pharmaceutical product (Cholewinski et al., 1996). Strategies to reduce protein and peptide degradation are based on an understanding of the degradation mechanisms and the effect of changes in manufacturing and storage conditions. Peptide or protein degradation can occur via a large number of reactions involving formation or breaking of covalent bonds. Often these processes will result in partial or total loss of activity (Van Den Oetelaar et al., 1992).

The three most common degradation pathways are aggregation, deamination and oxidation (Pourrat et al., 1995). Vapreotide is a hydrophobic octapeptide with a 2–7 disulphide linkage. Degradation products result from four types of reactions: breaking of the disulphide bond, breaking of peptide bonds, oxidation and deamination of vapreotide and peptide by-products, and particle aggregation. Breaking of disulphide bonds results essentially from thiol oxidation. Experiments (Barthomeuf et al., 1996) showed that in the case of vapreotide degradation the main mechanisms involved are oxidative and aggregative in nature.

3.1. Effects of temperature and extrusion time on peptide purity

Extrusion processes are the most convenient way for implant manufacturing. We studied the evolution of vapreotide purity during extrusion. When using a ram extruder, the mass to be extruded passes through the die continuously. Thus,

the first part of the rod remains in the thermostatically controlled barrel for only a short time, whereas the remainder stays longer at elevated temperature. To study the combined effects of time and temperature on peptide stability, a mixture of peptide and polymer was introduced into the barrel, and a rod was extruded at various time intervals at a fixed temperature. During the intervals, the drug remained mixed with the polymer in the glassy state, at a determined temperature.

It can be seen from Fig. 1 that peptide purity decreases rapidly at high temperatures. This effect is increased with time. Indeed, during the first 10 min, no significant decrease in purity appears independently from the temperatures used. However, with longer extrusion times, a rapid decrease in purity is observed at higher temperatures. It can be deduced that, if the temperature does not exceed 80–90°C, the extrusion time (up to 2 h) does not dramatically influence vapreotide purity. Destruction of the disulphide bond might be one of the mechanisms involved in vapreotide loss of purity (Volkin and Middaugh, 1992). Volkin and Klibanov (1987) found that the destruction of disulphide bonds was indeed one of the causes of loss of purity in proteins involving cystine residues at high temperatures (90–100°C). They studied more than a dozen unrelated proteins with a different number of cystine residues which were found to undergo degradation of their disulphide bonds at 100°C. They demonstrated that thermal stability of disulphide bonds in proteins was not dramatically affected by the primary structure—i.e. by the cystine residues' neighbours. On the other hand, destruction of the disulphide bond was shown to be much slower at acidic pH. Thus, the PLA matrix used in our experiments might be an advantage in this concern. Many comparative studies have suggested that increased hydrophobicity results in enhanced thermostability (Volkin and Middaugh, 1992). As vapreotide is known to be stabilized at an acidic pH (Pourrat et al., 1995) and to be very hydrophobic (five of the eight amino acids are hydrophobes), this could explain the relative stability of this peptide upon exposure to temperatures of 80–90°C for 1 or 2 h.

During the extrusion process, the peptide is confronted by the effects of pressure and shear, which have been shown to lead, to some extent, to peptide destruction, depending on the rate of shear (Groves and Teng, 1992). These problems

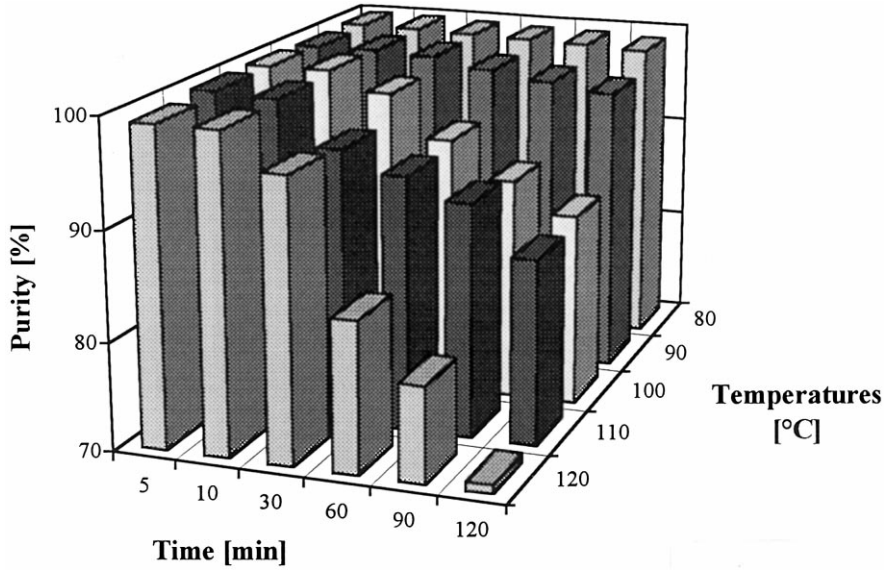

Fig. 1. Influence of temperature and time on vaprotide purity in a polylactic matrix, during an extrusion process.

can also contribute to the loss of purity of the peptide.

3.2. Effects of gamma-irradiation on vapreotide purity

Peptide drugs are currently formulated as parenterals or as biodegradable polymeric matrix systems which are all required to be sterile. Most commonly used terminal sterilization techniques, such as autoclaving, are too harsh for the delicate structure of these drugs and for the polymeric matrix. Thus, radiation sterilization seems to be the method of choice for these drug systems, although it will have harmful effects on peptide purity and stability due to free radical formation. Indeed, peptide or protein molecules irradiated in the solid state absorb radiation energy directly, producing various radicals. Partial modification or a total change in the configuration of the molecules occurs, as a result of the so-called 'direct effect'. However, the modification yield in the solid state is much less than the indirect effect of free radicals in an aqueous solution. This difference may be attributed to the recombination of radicals, due to the low mobility of the molecules in the solid state (Yamamoto, 1992). In order to determine the influence of the irradiation dose on the destruction of vapreotide in a polylactic acid implant, we sterilized implants containing vapreotide at a core loading of 18.5% at various kGy doses ranging up to 50 kGy. No significant decrease in purity was observed, even at high doses. Purity above 98.5% was observed in all cases after irradiation. These results can be partially explained by the composition of vapreotide, which contains five relatively radioresistant amino acids (two Cys, one Tyr, two Trp) (Hatano et al., 1963).

As measurements made immediately after irradiation sometimes show little effect on peptide purity, postirradiation effects have to be taken into account and should be measured after defined time intervals.

In previous work (Rothen-Weinhold et al., 1997), the effects of gamma-irradiation on the polymeric matrix were studied in details.

3.3. Effects of gamma-irradiation and storage under various atmospheres on vapreotide stability

Peptide degradation due to irradiation is mainly via free radical formation. These radicals induce the destruction or modification of amino acid residues, peptide chain cleavage, and cross-linking which results in peptide aggregation (Banga, 1995). The extent of radiation induced changes is modified by environmental conditions (Lim et al., 1988), thus it may be possible to negate the irradiation damage by the use of appropriate conditions during irradiation. In this respect, we aimed to determine the effect of gamma-irradiation on peptide stability over a defined period of time in controlled environments of irradiation, so as to find conditions which would provide sterility but induce minimum peptide damage; 25 kGy (2.5 Mrad) was chosen as the irradiation dose, because it is generally accepted as being satisfactory for sterilizing pharmaceutical products, in accordance with good manufacturing practices (Ferguson, 1988; Richards, 1996; O'Doberty, 1997).

Fig. 2 shows that the implants sterilized and stored under N_2 or Ar atmospheres degrade less rapidly than those sterilized and stored under air. Intramolecular disulphide exchange reactions were shown to be involved in vapreotide instability (Pourrat et al., 1995). Air contains oxygen, which acts as a sensitizer of irradiation effects, and helps in the propagation of a disulphide exchange reaction (Yamamoto, 1992). The first molecular event in the enhancement by O_2 is the peroxidation of radiation-induced radicals: $RH \rightarrow R^{\bullet} + O_2 \rightarrow RO_2$.

This reaction is considered to result in the initiation of a sequence leading to a certain amount of fixed molecular damage. Peroxy radicals formed in the presence of O_2 cannot be repaired by physicochemical mechanisms while $R^{\bullet}$ is capable of being chemically restored by hydrogen donation from appropriate donors in the presence of O_2. It was shown that radiolysis of small peptides containing disulphide bonds caused chain reactions, which resulted in high yields of symmetrical disulphides from mixed disulphides (Purdie and Lynn, 1973). Depending on the relative humidity during the filling process, little H_2O

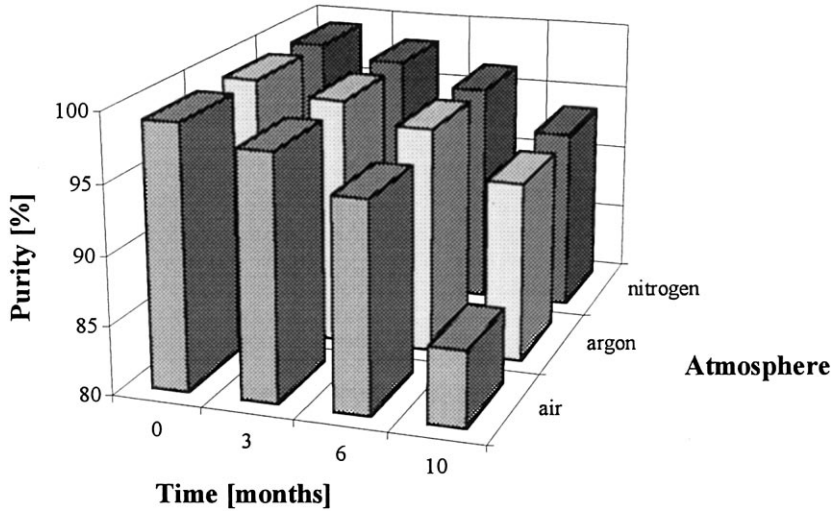

Fig. 2. Influence of irradiating and storage at 40°C under various atmospheres on vapreotide stability after defined time intervals.

may be present. Water molecules produce •OH, which reacts with radiation-sensitive moieties in peptides or proteins, such as sulphur-containing amino acid residues (cysteine, cystine, methionine) and aromatic ring-containing amino acid residues (phenylalanine, tyrosine, tryptophan, histidine). These residues easily become free radicals, and are further oxidized or easily bind to the ring of aromatic amino acid residues, resulting in cross-links or aggregation (Yamamoto, 1992). Tryptophan and tyrosine were found to be the residues first affected by radiolysis. However, in the case of implants, degradation due to •OH radicals should not be an important mechanism of degradation. Indeed, the implants tested contained only 3.7% H_2O after 10 months (determined by a Karl–Fisher method), and it was shown (Banga, 1995) that moisture introduced in a solid-state system to about 6–8% was believed to form a monolayer on the peptide surface, at which level very little change due to irradiation was observed. Furthermore, most excipients are generally hydrophobic and may protect the internal peptide or protein from small amounts of adventitious moisture.

From the data obtained, it can be concluded that, to prevent vapreotide degradation, it is important to operate under N_2 or Ar flow during filling, so as to limit the presence of O_2 and possibly H_2O. No difference was seen between the Ar or the N_2 atmospheres.

3.4. Stability study

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, in order to establish recommended storage conditions. For conventional drugs, rate constants determined at higher temperatures are routinely used to predict the shelf-life at lower storage temperatures by the use of the Arrhenius equation. This allows for the determination of shelf-life without real-time analysis. Until recently, many believed that such accelerated stability studies cannot be done for peptide drugs, since these would degrade at the higher temperatures used. While there is some truth to these observations, it is now believed that such generalizations cannot be made, and limited accelerated stability studies are certainly feasible. With respect to temperatures, the use of fairly moderate conditions not exceeding 45°C is preferred, in order to avoid errors of interpretation that may occur as a result of the fact that the decomposition of active drugs at high temperatures often

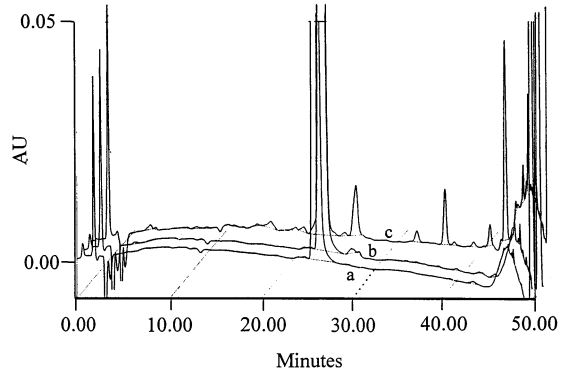

Fig. 3. Effect of 9 months of storage at 4 (a), 25 (b) and 40°C (c) on vapreotide stability in a polymeric matrix under air.

differs from the decomposition at ordinary temperatures (Grimm and Thomae, 1987; Pourrat et al., 1995). The ICH Expert Working Group (ICH Steering Committee, 1993) relates that heat-sensitive drug products should be stored, for accelerated stability studies, under an alternative lower temperature condition, which must be at a temperature at least 15°C above its designated long-term storage temperature.

For accelerated stability studies, we placed the samples in a thermostatically controlled cabinet at 40°C (relatively high temperature conditions for a peptide). Generally, 75% relative humidity (RH) is recommended at 40°C, but it was reported (ICH Steering Committee, 1993) that, for products such as suspensions (peptides in polymeric delivery systems can be viewed as solid suspensions; Hageman, 1992) contained in packs designed to provide a barrier to water loss, specific storage under conditions of high relative humidity was not necessary but that the same range of temperature should be applied. Besides 40°C, we stored samples at 4 and 25°C, in order to evaluate the stability over 1 and 2 years.

Surprisingly, after 6 and even 10 months, the degradation kinetics at 40°C were not sufficiently significant to enable the use of Arrhenius plot (purity $>93\%$), and degradation kinetics determined from this accelerated stability study could not be extrapolated to a 4°C and 25°C long-term stability study.

Furthermore, after 9 months at 4°C or even at 25°C, no significant degradation was observed (Fig. 3). However, a slight difference between these two temperatures could be detected when analysing the number of different degradation products.

After 9 months, at 4 or 25°C, none of the degradation products are present in amounts larger than 0.5%, which is the suggested limit of identification according to the USP 23. However, an increase in the number of degradation peaks can be observed with an increase of the storage temperature from 4 to 25°C. It can also be observed that increasing the temperature from 25°C to 40°C leads only to a very slight increase in the amount of total impurity, whereas the percentage of the peak areas of some impurities increased (Fig. 3 and Table 1). Thus, it can be concluded that, at 4°C, after 9 months' storage, no significant degradation products appear (the peptide purity content was the same at time zero).

4. Conclusion

An essential part of pharmaceutical drug development is the assessment of the stability of the

Table 1
Distribution of the impurity peak areas ($n=3$) after 9 months

Temperature (°C)	Total number of impurity peaks	Number of peaks (x) representing a percentage of the total amount of peak areas, including the vapreotide peak area			
		$x>1\%$	$1>x>0.5\%$	$0.5>x>0.1\%$	$0.1>x>0.01\%$
4	14	0	0	1	13
25	26	0	0	4	22
40	27	2	2	6	17

active ingredient in the formulated dosage form. Each formulation proposed for the dosage form of a new drug substance to be marketed should have its own stability study protocol, since the different active chemical substances can degrade in a number of ways depending upon their structural and chemical characteristics. Thus, the stability study protocols for the drug products will differ depending on the possibility of chemical interaction with excipients and on physical changes associated with the characteristics of the dosage form.

In this study, implants containing vapreotide have been produced, and the influence of the different manufacturing steps on peptide stability has been investigated. It has been shown that, if the extrusion process does not exceed 80–90°C for a period of up to approximately 1 h, no significant degradation of the peptide is detectable. Further sterilization by gamma-irradiation was also shown not to induce peptide damage immediately after sterilization. Accelerated stability studies at 40°C demonstrated that, to prevent vapreotide degradation during long-term storage, it is important to operate under an inert atmosphere (N_2 or Ar flow) during the filling of the glass vessels. Long-term stability studies at 4°C showed that, after 9 months of storage, under N_2 or even air, peptide purity in the polymeric matrix was above 99%. None of the degradation products were present in amount greater than 0.5%.

During the development of a new pharmaceutical dosage form, and especially in the preformulation phase, one only needs to know whether or not the test formulations have potentially good stability. Knowledge about the exact mechanism underlying the instability can be helpful but is not essential. Consequently, this work has proved that it is possible to produce a biodegradable implant containing a peptide without its deterioration during the different manufacturing steps. The stability of the peptide in the polymeric matrix after 9 months is very good and is expected to last for at least 1–2 years under the defined storage conditions.

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