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Development and chemical characterization of biodegradable polymeric implants containing sirolimus for the treatment of malignant solid tumors

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The use of sirolimus and its analogs has been evaluated in studies aimed at combating several types of cancer; however, because of the limited bioavailability of the drug, the search for new forms of administration is required. Biodegradable polymeric implants containing sirolimus were developed and assessed as an alternative method of drug administration. Implants containing 25 % (w/w) sirolimus were prepared employing the polymer matrices chitosan, polycaprolactone and poly(lactic-co-glycolic acid) (PLGA) in two proportions: PLGA 50:50 and PLGA 75:25. Thermal analysis techniques such as thermogravimetry and differential scanning calorimetry, combined with x-ray diffraction were used to characterize and evaluate the compatibility of the constituents of the formulation. No incompatibilities were found between the components, but drug amorphization was observed in all samples. Implants made from the polymers chitosan and PCL may accelerate the degradation of SRL when these polymers are dissolved in methanol at 50 °C. HPLC analysis showed that the implant prepared with PLGA 75:25 did not present degradation products and maintained its appropriate drug content, even when dissolved in methanol and heated to 50 °C. Therefore, it represents the most suitable biodegradable polymer for use in implants developed for the treatment of malignant solid tumors. However, it is still necessary to further study the drug effects after amorphization of the crystal and also to perform stability and solubility analysis.

1. Introduction

In the early 1970s, sirolimus was discovered as part of a screening program for new anti-fungal agents. The compound was first named rapamycin because it was isolated from a soil sample from the island Rapa Nui in Chile (Sehgal et al. 1975; Hartford and Ratain 2007). Sirolimus (rapamycin) is a macrocyclic lactone (Fig. 1) produced as a secondary metabolite by the fungus *Streptomyces hygroscopicus*.It is a crystalline solid with a molecular weight of 914.17 g mol^{-1}, and its color may vary from white to off-white. Rapamycin was originally used as an antifungal agent against *Candida albicans* and was subsequently determined to have potent immunosuppressant and antiproliferative activities (Goodman and Gilman 2006; Pópulo 2011).

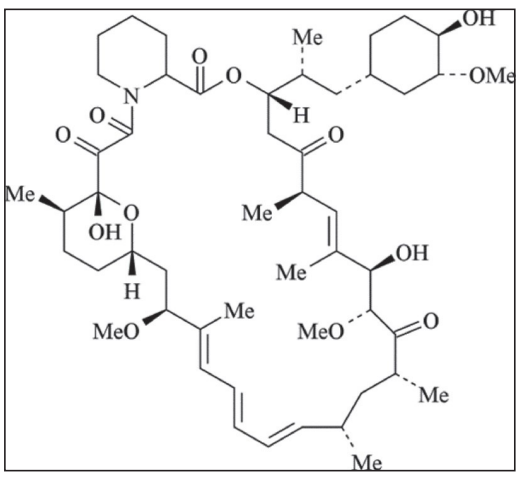

Fig. 1: Structural formula of sirolimus.

Sirolimus (SRL) has a low solubility which reduces its oral absorption (Solymosi et al. 2015). The mechanism of action of SRL is different from other immunosuppressive agents because it binds the cytosolic binding proteins FK-12 (FKBP12) to generate an immunosuppressive complex (complex FKBP-12-rapamycin) which will bind and block a protein kinase called target of rapamycin in mammals (mTOR). The mTOR enzyme is crucial for the survival, growth and proliferation of cells, thus being able to make up the connection of the mTOR signaling pathway in several diseases including cancer (Goodman and Gilman 2006; Onyesom et al. 2013; Pópulo 2011). SRL can also inhibit the proliferation of endothelial cells, the expression of hypoxia inducible factor (HIF-1) and vascular endothelial growth factor (VEGF), and angiogenesis (Cao and Langer 2010). The use of SRL and its analogs has been evaluated for the treatment of cancers, including liver, lung, breast and prostate, both *in vitro* and *in vivo* (Facompre et al. 2012; Liu et al. 2005).

Due to the limited bioavailability of SRL, it is necessary to search for new forms of administration, which has led to the development of controlled release drug delivery systems (CRS), such as biodegradable polymer implants, which would allow a more widespread use of the drug. The development of drug CRS it is an original field as regards the use of polymers that allow sustained release and targeting of a tumor, local tissue, or even a particular location of the blood circulation (Cao and Langer 2010; Saliba 2011).

Currently, the most widely used polymers for applications such as CRS include poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), polyacid glutamic acid (PGA), polycaprolactone (PCL), polysaccharides and poly-amino acids. Natural polymers, including chitosan, collagen, hyaluronic acid, and alginate, can also provide a matrix on which to prepare drugs (Coimbra 2010; Jain 2000; Pillai and Panchagnula 2001).

The development of novel dosage forms demands a detailed assessment of its formulation because the components are likely to undergo reactions and incompatibilities which would influence its effectiveness. Thermal analyses such as differential scanning calorimetry (DSC) and thermogravimetry (TG), associated with high–performance liquid chromatography (HPLC) and x-ray diffraction studies (XRD) are fundamental tools for characterizing novel formulations. These methods allow the detection of incompatibilities among the constituents of pharmaceutical formulations and the characterization of crystal changes such as polymorphism or changes in crystallinity (Oliveira et al. 2010; Yoshida et al. 2010; Yoshida et al. 2011a,b).

Biodegradable polymer implants containing 25 % sirolimus were developed using three different base polymers: chitosan, PCL and PLGA in two proportions, PLGA 50:50 and PLGA 75:25. The study of drug compatibility with the polymers was conducted using TG, DSC, HPLC and XRD.

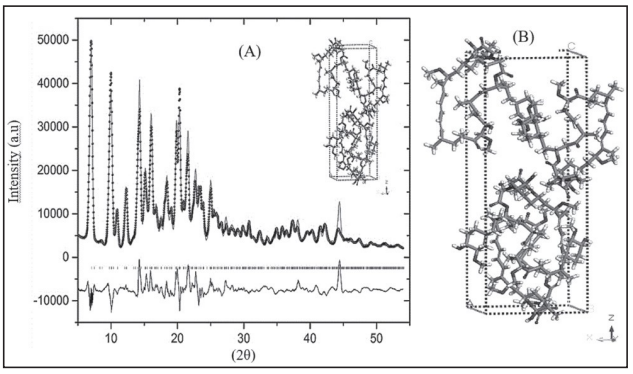

Fig. 3: Diffractogram of SRL adjusted by the Rietveld method (A) and, (B) an SRL unit cell obtained by Rietveld rearrangement.

2. Investigations, results and discussion

2.1. SRL drug characterization by thermal analysis

In the TG curve (Fig. 2), SRL presented thermal stability up to 189 °C. The DSC curve indicates that the SRL thermal decomposition process occurred in two steps, with a total mass loss of 82.0 %. The first degradation was endothermic at 189.1 °C to 208.9 °C and the subsequent degradation was exothermic at 232.9 to 470.8 °C. In the DSC curve of SRL (Fig. 2), an endothermic peak was observed, corresponding to the melting point of the drug, 186.0 °C (T_{onset} = 181.5 ° C, ΔHfus = 61.9 J g^{-1}) and the SRL drug used for analysis showed 98.6 % purity. After fusion, another endothermic event was observed near 191.5 °C, which corresponds to the initial process of SRL degradation and a subsequent exothermic event was observed at a temperature around 199.5 °C, also attributed to the decomposition of the drug, due to loss of mass in both cases. To perform the thermal analysis test for drugs and polymers, the heating rate used as a parameter for the characterization is usually 10 °C min^{-1} (Oliveira et al 2011).

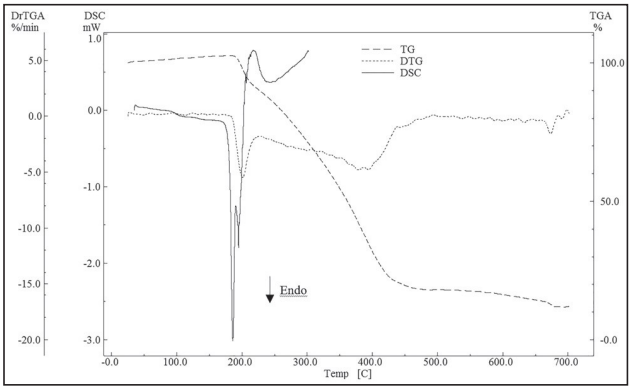

Fig. 2: DSC and TG/DTG curves for SRL obtained at a heating rate of 10 °C min-1 under a dynamic nitrogen atmosphere.

2.2. SRL drug characterization by XRD

The diffractogram of SRL (Fig. 3 A) shows a diffraction pattern typical of the drug with several peaks between 5° and 27° (2θ) and three main peaks of high intensity at 7.2, 10.0 and 14.6° (2θ). The SRL is crystallized according to the space group $P2_12_12_1$, with an orthorhombic system (Fig. 3 B). The network parameters were adjusted according to the Rietveld algorithm match a = 12.065 ± 0.002 Å, b = 12.838 ± 0.001 Å, e c = 34.263 ± 0.002 Å.

2.3. Characterization of polymers by thermal analysis

The characterization of polymers by thermal analysis was performed at a heating rate of 10 °C min^{-1}. The TG/DTG curves for chitosan (Fig. 4 A) showed mass loss in two stages totaling 57.4 %; the first degradation was endothermic and occurred at a temperature between 48.1 and 68.2 °C with initial loss of 9.8 %, and the second degradation was exothermic and occurred between 281.3 and 324.1 °C with loss of 47.6 %. On the DSC curve of chitosan (Fig. 4 A) an exothermic peak, corresponding to the thermal decomposition at 306.6 °C (T_{onset} = 292.2 ° C; ΔH = 118.44 J g), can also be observed. The TG/DTG of PLC (Fig. 4 B) indicated that the decomposition process occurred in two endothermic stages, resulting in a weight loss of 97.8 %, with the first mass loss at temperatures between 287.9 ºC and 300.7 °C, and the second from 389.2 to 421.7 °C. The DSC curve of PCL (Fig. 4 B) showed an endothermic peak at 64.2 °C corresponding to the fusion of the polymer (T_{onset} = 60,1 °C, ΔH_{fus} = 212,6 J g^{-1}).

The TG/DTG curve of PLGA 50:50 (Fig. 4 C) demonstrated that the process of decomposition was endothermic and occurred in a single step between 288.7 °C and 347.2 °C, resulting in a mass loss of 99.1%. The DSC curve of 50:50 PLGA (Fig. 4 C) indicated thermal transitions related to the glass transition of the polymer at a temperature between 45.7 and 52.6 °C, with a midpoint of 49.8 °C.

For PLGA 75:25, thermal events shown in the TG/DTG curve (Fig. 4 D) were similar to those observed to the PLGA 50:50, with the endothermic decomposition process also occurring in one step, from 302.1 °C to 357.4 °C, resulting in a mass loss of 97.1%. On the DSC curve of 75:25 PLGA (Fig. 4 D) the glass transition occurred at a temperature between 54.9 and 60.2 °C, with a midpoint of 58.3 °C.

The DSC curves of PLGA 50:50 and PLGA 75:25 (Fig. 4 C and 4 D) also showed the presence of a small peak of apparent fusion near the region where the glass transition occurred. According to Canevarolo (2007), this phenomenon occurs due to molecular relaxation, which appears as an endothermic transition near the end of the glass transition and can be related to the accumulated stress in the sample, which may be caused by its processing, treatment, or thermal history and is released when the material is heated thus generating a peak of "apparent fusion".

2.4. Compatibility studies of sirolimus with polymers

2.4.1. Compatibility studies by DSC

To study the compatibility of the drug with the polymers, DSC curves were obtained at a heating rate of 10 and 20 °C min^{-1} for drug alone, polymers alone, binary (1:1) drug-polymer mixtures and lyophilized final implants. Preliminary results at a heating rate of 10 °C min^{-1} were not satisfactory to evaluate the compatibility of the formulation, since the thermal events showed low quality. Sharper curves with higher resolution thermal events were obtained using a heating rate of 20 °C min^{-1}; therefore this heating rate was used for all compatibility studies.

Figure 5 shows that, for the binary mixtures, the most significant individual thermal events, such as fusion of SRL and PCL, the glass transition of PGLAs and dehydration with a subsequent decomposition of chitosan, were not changed after binary mixture; what indicates that there is no incompatibility between SLR and

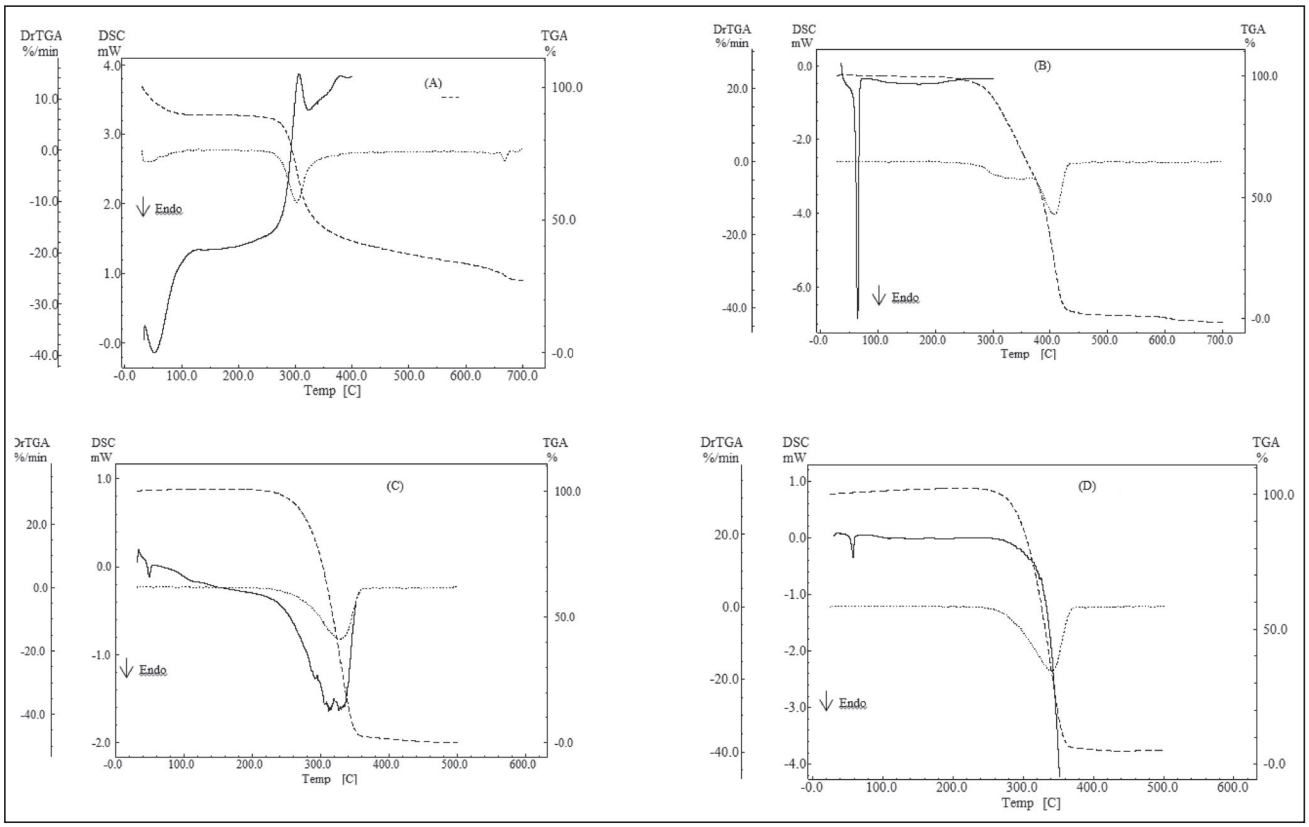

Fig. 4: DSC and TG/DTG curves obtained at 10 ° C min-1 under a dynamic nitrogen atmosphere: (A) chitosan, (B) PCL, (C) PLGA 50:50 and (D) PLGA 75:25.

the investigated polymers. In the results obtained with the implants (Fig. 5) we observed the absence of SRL's melting point, which may be related to possible amorphization of drug during the implant manufacturing process.

The process used to produce the implant contains several steps that are typically also present when producing an amorphous powder, such as solubilization and recrystallization by lyophilization. To confirm the possible amorphization of the drug, XRD studies of the implants were necessary.

2.4.2. Compatibility studies by XRD

The thermal analyses are highly sensitive and responsive for identifying incompatibility amongst pharmaceutical formulations, and they are typically the techniques of choice for compatibility studies (Yoshida et al. 2011). However, XRD can also be used in compatibility studies, which are appropriate to identify the crystallinity of the materials, crystalline polymorphisms and amorphization events caused by mixing the drug with excipients. Figure 6 demonstrates that there is no incompatibility of SRL with the polymers since the crystallinity of the drug can be displayed in the analysis of binary mixtures. However, when analyzing the results of the implants, there is a lack of crystallinity characteristics of the implant SRL, confirming our hypothesis of the drug's amorphization during the formation of the implant.

2.5. Analysis and determination of content of lyophilized implants

The chromatographic conditions were optimized to assess and determine the SRL content in lyophilized implants. After optimization of the method of analysis by HPLC, the chromatographic performance parameters found for the SRL (k'= 4.65, N = 4.520 plates/column; As = 1.02) were satisfactory according to the established limits for each parameter (Ribani et al. 2004).

To determine the drug content of lyophilized implants, samples were prepared at an approximate final concentration of 0.05 mg mL^{-1} drug for each polymer, there is a small change that was considered in the calculations and the standard concentration was prepared with a final concentration of 0.01 mg mL^{-1}. As displayed in Fig. 7, the drug was present in all formulations of implants; however, in the lyophilized implants developed with the polymers chitosan and PCL, the formation of a degradation product (DP) was observed with a retention time (t_R = 1.2 min).

For implants prepared with chitosan and PCL the SRL content is presented in a smaller quantity. In the implant with chitosan the drug content was only 14.2 % of the original and in the implant prepared with PCL a content of 12.5 % drug was found. This may be related to a drug degradation observed by increased formation of DP in 1.2 min. The implants prepared with PLGA showed no increase in the formation area of DP in both the proportions: PLGA 50:50 and PLGA 75:25, however the level of SRL found for PLGA 50:50 was 17.2 % while for implants prepared with PLGA 75:25 the level was 24.6 %, which attests to lack of degradation of the drug in the final lyophilized implant, showing a greater stability of the drug in solution in the presence of PLGA 75:25.

In the compatibility testing of the formulation using DSC and XRD we demonstrated that there is no incompatibility between the polymers and the SRL solid state. According to the results of HPLC content of drug, it could be inferred that the degradation of SRL observed in implants made of chitosan and PCL may be related to the solubilization of methanol in materials with such polymers, associated with the column temperature for the analysis that was 50 °C, given that the speed of a liquid state reaction is faster than the speed of a solid state reaction.

2.6. Conclusion

Compatibility testing using the DSC technique confirmed that there was no incompatibility between SRL and the polymers in solid samples. The implant manufacturing process leads to amorphization of SRL, which causes a change in solubility and product stability. If the implant manufacturing process cannot be altered, attention should be paid to stability and release studies of the

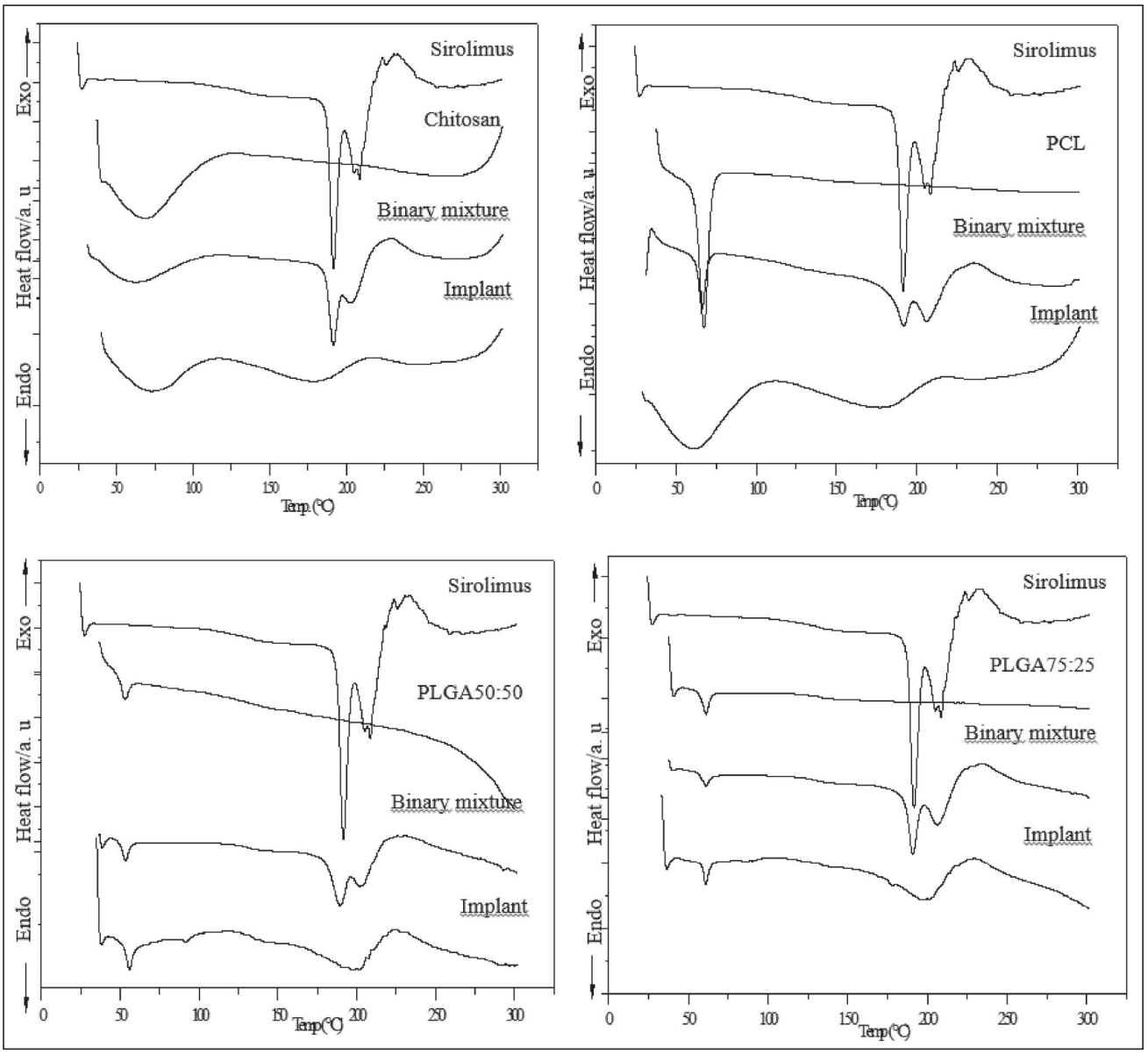

Fig. 5: DSC curves obtained at 20 °C min-1 under a dynamic nitrogen atmosphere for SRL, chitosan, PCL, PLGA 50:50, PLGA 75:25; binary mixtures (1: 1) and lyophilized implants.

dosage form that presents SRL as amorphous powder. Implants made of the polymers chitosan and PCL may accelerate the degradation of SRL when these polymers are dissolved in methanol at 50 °C, since the implants exhibited increased formation of the degradation product, in solution, as evidenced by HPLC analysis. The most appropriate formulation for the implant was found to be PLGA 75:25, but additional studies are necessary to evaluate the maintenance of the antiproliferative activities of SRL as amorphous powder.

3. Experimental

3.1. Materials

Sirolimus (SRL) was purchased from Cristália (lot 09763/2009), poly(lactic-co-glycolic acid) PLGA 50:50 and PLGA 75:25 were purchased from Evonik (lots R1204000505 and 1036455) and polycaprolactone (PCL) and chitosan were purchased from Aldrich (lots MKBH7023V and MKBF1336V, respectively).

3.2. Implant preparation

Implants were produced using the technique described by Fialho and Silva-Cunha (2005), where the polymers chitosan, PCL, PLGA 50:50 and PLGA 75:25 were dissolved in a minimum volume of acetonitrile together with the drug SRL for 10 minutes using an ultrasonic water bath at a temperature of 40 °C to aid in the solubilization. The solution was dissolved in a minimum volume of water and the resulting solution was frozen in liquid nitrogen. The samples were subsequently lyophilized to remove the solvent. SRL drug was used at a final concentration of 25 % (w/w) in the implant.

3.3. Thermal analysis (TG and DSC)

The TG/DTG curves were performed using thermobalance DTG 60 (Shimadzu, Japan). Three milligrams of each sample were placed in an aluminum crucible and heated from room temperature up to 700 °C at a rate of 10 or 20 °C min^{-1} under a nitrogen flow rate of 50 mL min^{-1}. DSC curves were obtained using DSC-60 (Shimadzu, Japan). One milligram of each sample was placed in aluminum crucible, partially closed, from room temperature up to 450 °C at a rate of 10 °C min^{-1} under a nitrogen flow rate of 50 mL min^{-1}. The DSC equipment was calibrated with indium (156.6 °C melting point; ΔH_{fus} = 28.5 J g^{-1}) and lead (327.5 °C melting point). The assessment of drug purity by DSC was made by van't Hoff equation at the drug melting point, using the *Purity Determination Program version 2.20* (Shimadzu software).

3.4. X-ray diffraction (XRD)

Analysis of the drug by XRD was performed using a diffractometer XRD-7000 (Shimadzu, Japan), provided with a copper filament and a graphite monochromator and radiation $K_{\alpha 1 Cu}$ with λ= 1,54056 Å, at an operating voltage at 40 kV and 30 mA. The sample port was subjected to rotation at 30 rpm to prevent preferential orienta-

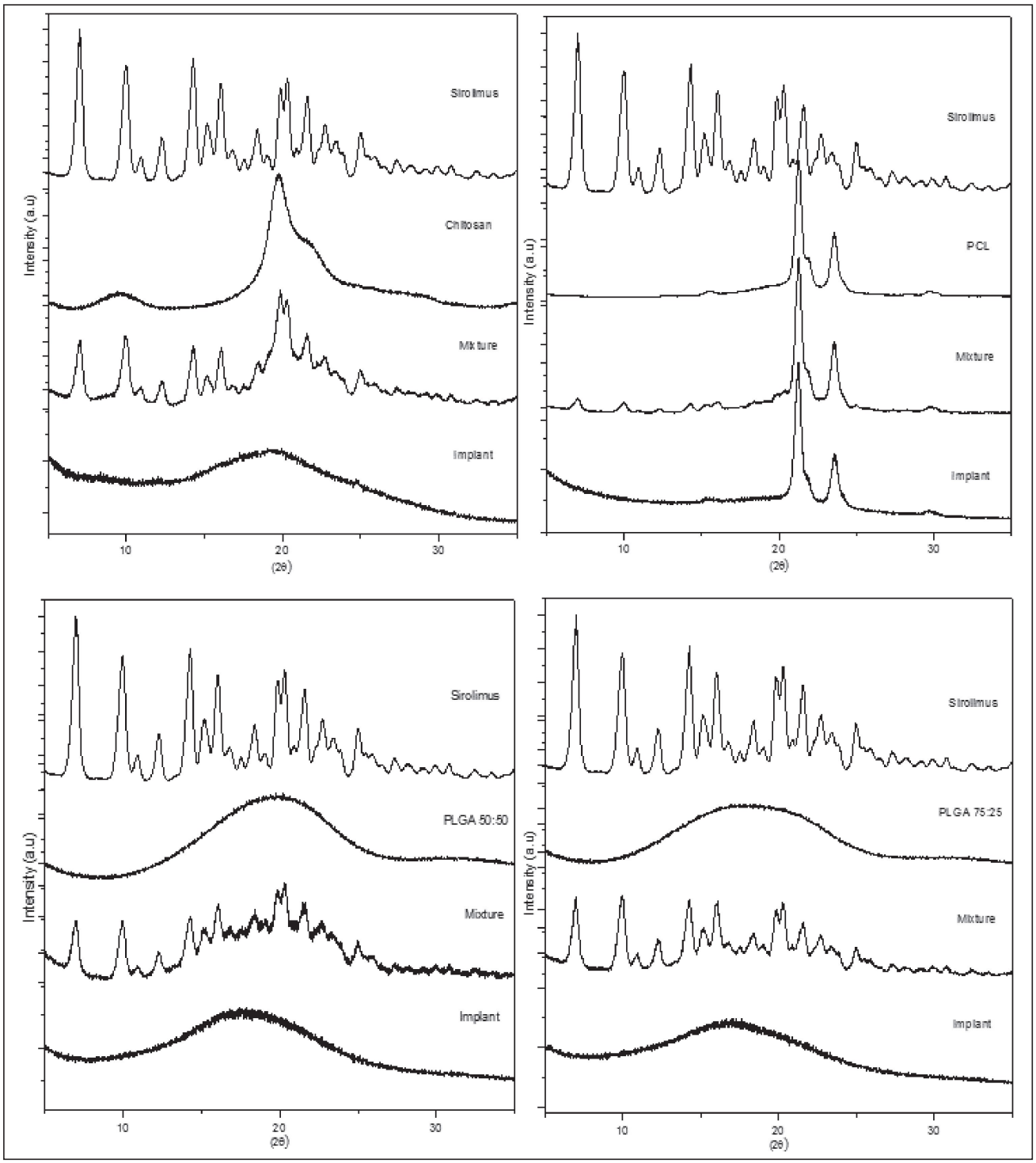

Fig. 6: XRD results of SRL; polymers, binary mixtures (1:1) and lyophilized implants.

tion and minimize roughness effects. Measurements were performed on the parallel optical policapilar and the data was obtained in the range of 4 – 70° (2θ), with steps of 0.01°/20 with a constant time of 5 s per increment. The SRL sample was submitted to analysis according to the Rietveld algorithm for precise determination of the network parameters.

2.5. High performance liquid chromatography (HPLC)

Chromatographic studies were conducted to determine the drug content of lyophilized implants; each implant was prepared with a final drug concentration of 0.05 mg mL^{-1}. A chromatograph (Waters) with an auto-injector, a diode-array detector UV/DAD and a column oven were used in the analysis. The chromatographic conditions used included: C18 column (250 x 4.6 mm, 5 µm, Varian); mobile phase containing methanol–water (80:20); flow at 1.5 mL min^{-1}; injection volume of 20 µL; UV detection at l = 278 nm; and oven temperature at 50 °C.

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Conflicts of interest: None declared.

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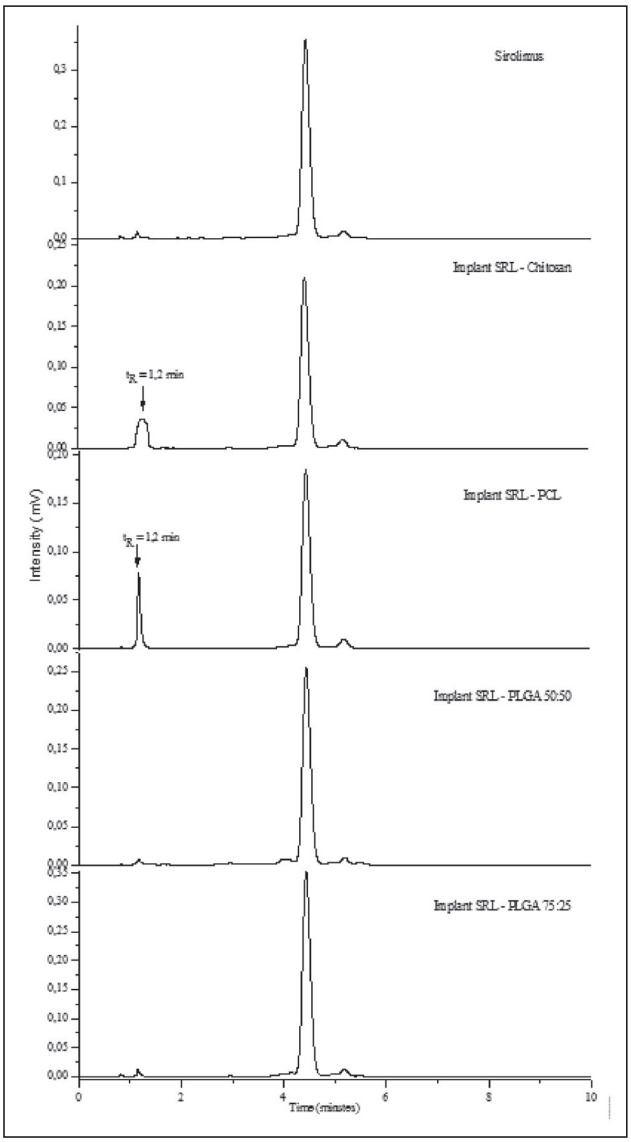

Fig. 7: Chromatograms of SRL and implants: SRL – Chitosan; SRL – PCL; SRL – PLGA 50:50; SRL – PLGA 75:25.

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