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Research paper

Design of pH-sensitive microspheres for the colonic delivery of the immunosuppressive drug tacrolimus

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Abstract

Recently, tacrolimus was shown to be effective in mitigating inflammatory bowel disease (IBD). In the treatment of IBD, oral drug delivery using pH-dependent polymers is one of the most successful therapeutic strategies. Eudragit P-4135F, a pH-sensitive polymer for colonic delivery was used to prepare tacrolimus microparticles using an oil/oil emulsification or an oil/water emulsification technique combined with a solvent extraction or evaporation step. Although the pH-dependent drug release was similar for all types of microspheres, it was generally found that encapsulation rates of oil/water systems (extraction 38.8 ± 9.4%; evaporation 56.3 ± 1.9%) were superior to the oil/oil emulsification (4.8 ± 0.4%). Eudragit P-4135F was found to limit drug leakage at pH 6.8 to levels lower than 10% within 6 h. At pH 7.4, almost immediate release (within 30 min) was observed. From differential scanning calorimetry and X-ray analyses, the drug inside the polymeric microsphere matrix was concluded to be present in a molecular dispersion. Generally, all formulations proved their applicability in vitro as a promising device for pH-dependent colonic delivery of tacrolimus, however, the oil/water technique was found to be superior to the oil/oil approach and among them solvent evaporation seemed to be more advisable, due to the higher encapsulation rate.

Keywords: Microspheres; FK506; Tacrolimus; Colon delivery; pH-dependent; Eudragit

1. Introduction

The usual treatment of inflammatory bowel disease (IBD) consists of the frequent intake of anti-inflammatory drugs at high doses in order to induce remission of the active disease. To avoid the absorption of these drugs from the small intestine, provoking significant adverse events, several strategies have been followed. These include the development of prodrugs that deliver drugs specifically to the large bowel after cleaving the active part from the hydrophilic carrier by specific bacterial enzymes in the colon [1–3] and the development of solid dosage forms that release the drug in the colon in response to the physiological environment [4–6]. Sustained drug release devices, e.g. pellets, capsules, or tablets, delivering the drug specifically in the colon for a longer time period have been developed. However, their efficiency seems to be decreased in many cases due to diarrhea, a symptom of IBD that enhances the elimination and reduces the possible drug release time [7,8]. As reported in previous work, drug carrier systems with a size larger than 200 μm are subjected to the diarrhea symptoms, resulting in a decreased gastrointestinal transit time and therefore to a distinct decrease in efficiency [7,8]. In this context the size reduction of the drug carrier downwards to micron size seems to be an interesting option.

Recently, tacrolimus, initially used as an immunosuppressive drug to inhibit transplantion rejection, was successfully introduced to the treatment of IBD as clinical studies reported from the use in refractory ulcerative colitis [9,10]. Unfortunately, since the immunosuppressive mechanism of action is not selective, and tacrolimus is known for its distinct nephrotoxicity, adverse effects may be expected when the drug is not delivered locally. Therefore, the use of a system providing local delivery is strongly advised in order to benefit from the excellent pharmacological effect of tacrolimus. Most of the commercialized systems for local drug delivery to the lower intestine after oral administration are based on the change of pH during the gastrointestinal passage. The pH-sensitive polymers such as methacrylate/methacryl acid Eudragit® S and L dissolve in aqueous

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media at pH 6 and 7, respectively, which may be equivalent to a drug release to the distal ileum. In cases of ulcerative colitis which mainly affects distal parts of the colon, an early drug loss towards non-inflamed tissue of the upper colon may lower the efficiency and risk distinct adverse effects. Recently, a new polymer has been developed for use in colon delivery specified for particularities in luminal pH based on the pathophysiological characteristics of IBD, namely Eudragit® P-4135F. It has been used for film coating purposes on pellets, tablets and the preparation of microspheres [11–13].

A colon specific drug delivery by microsphere formulations has been described for several anti-inflammatory drugs [14–16]. In those microsphere preparations the pH-sensitive polymers Eudragit® S and L were employed. Usually, the preparation methods consisted of an oil/oil emulsification process where Eudragit® S or L are dissolved in an acetone/2-propanol mixtures and emulsified in liquid paraffin [12,15,16].

Since this oil/oil emulsification is mainly applicable to more hydrophilic drugs, alternatives for this preparation method might be interesting for lipophilic compounds, such as tacrolimus. In this work we describe the preparation of tacrolimus pH-sensitive microspheres by different oil/water emulsification techniques including solvent evaporation or extraction. The characteristics of the developed microspheres were compared with the results of an oil/oil emulsification method, which was used as a standard.

2. Materials and methods

2.1. Materials

Eudragit® P-4135F was a kind gift from Roehm Pharma Polymers, Tokyo, Japan, Tacrolimus (FK506, highly lipophilic macrolide drug; solubility in distilled water at 293 K: 482 ± 122 μg/l ($n = 5$); soluble in apolar solvents, ethanol and acetone) was received as a gift from Fujisawa (Osaka, Japan). Polyvinyl alcohol (Mw 20 000 Da, 80% hydrolyzed) was purchased from Sigma (Deisenhofen, Germany). All other chemicals were obtained from Nacalai Tesque Inc. (Kyoto, Japan) and were of analytical grade.

2.2. Methods

2.2.1. Preparation of microspheres

The preparation of microspheres was either based on an oil/water emulsification—solvent evaporation or solvent extraction method. The usually employed oil/oil emulsification process is given as a standard in the preparation of tacrolimus microspheres. For all different techniques a fixed amount of polymer (200 mg) and drug (20 mg) were used.

2.2.1.1. Oil/oil emulsification. The polymer and tacrolimus were dissolved in a mixture of 3 ml acetone and 2 ml ethanol. This solution was poured into 80 ml of liquid paraffin containing 1% (w/w) Span 80 and an oil/oil emulsion was formed by stirring with a three-blade propeller at 800 rev./min. The emulsion was stirred under vacuum until solvents were removed. Microspheres were collected by filtration and washing steps were performed with *n*-hexane before redispersion in distilled water followed by lyophilization.

2.2.1.2. Oil/water emulsification—solvent evaporation. The polymer was dissolved in 3 ml methylene chloride together with tacrolimus. This solution was poured into 75 ml of 1% (w/w) polyvinyl alcohol and an oil/water emulsion was formed by extensive stirring with a three-blade propeller at 500 rev./min. The system was kept under agigation until methylene chloride was evaporated. After decantation, the microspheres were filtered (HVLP filter, Millipore, pore size 0.45 μm), washed extensively with distilled water and lyophilized overnight.

2.2.1.3. Oil/water emulsification—solvent extraction. The polymer was dissolved in 4 ml ethyl acetate together with tacrolimus. This solution was poured into 20 ml of 1% (w/w) polyvinyl alcohol and an oil/water emulsion was formed by extensive stirring with a three-blade propeller at 400 rev./min. The system was stirred at 500 rev./min for 3 min and then poured into 200 ml of 0.1% polyvinyl alcohol solution, while stirring was maintained for another 3 min. Thereafter, the microspheres were filtered (HVLP filter, Millipore, pore size 0.45 μm), washed extensively with distilled water and lyophilized overnight.

2.2.2. Scanning electron microscopy

The external and internal morphology of microspheres was analyzed by scanning electron microscopy (SEM). The microspheres were fixed on supports with carbon-glue, and coated with gold using a gold sputter module in a high-vacuum evaporator. Samples were then observed with the SEM (JEOL JSM-T330A scanning microscope, Tokyo, Japan) at 10 kV.

2.2.3. Particle size analysis

All microsphere batches were analyzed for their size distribution using a LDSA 2400A particle size analyzer (Tohnichi Computer Co., Ltd, Tokyo, Japan). Volume distribution was plotted using a computer program supplied by the manufacturer.

2.2.4. Differential scanning calorimetry

Differential scanning calorimetric (DSC) curves were recorded on a scanning calorimeter equipped with a thermal analysis data system (Seiko Instruments DSC 6200, Tokyo, Japan). Samples (7 mg) were heated in sealed aluminum pans from 25 to 200 °C at a scanning rate of 10 °C/min under nitrogen purge, with an empty aluminum pan as reference.

2.2.5. X-ray diffractometry

Samples were studied in a wide angle X-ray diffractometer (Rigaku Geigerflex, Rigaku Denki Co., Ltd, Tokyo, Japan). The instrument was operated in the step scan mode from 5 to 40° 2θ, in increments of 0.02° 2θ.

2.2.6. Determination of drug content

The drug loading efficiency in the microparticles was determined by high performance liquid chromatography (HPLC) after extraction from the particles. Microspheres were dissolved in 600 μl of an acetone–ethanol mixture (2:1). Six milliliters of acetonitrile were added to this solution and vortexed for 5 min. The samples were centrifuged at 10 000*g* for 20 min and the supernatant was diluted 1:10 before injection into the HPLC system. A previously published HPLC method [17] was adapted to our requirements and system setup was as follows: RP-18 column (Finepak SIL C18-5, JASCO Co., Japan); eluent: acetonitrile:water:phosphoric acid (700:299:1); flow rate 1.5 ml/min. Tacrolimus was detected by UV absorbance at 210 nm, samples of 50 μl were injected into the column.

2.2.7. In vitro drug release

The in vitro drug release was analyzed by the use of a paddle apparatus (USP XXIII). Drug-loaded microparticles were suspended in 100 ml phosphate buffer systems of different pH. The dissolution medium was kept under stirring at 100 rev./min. All the experiments were carried out at 37 °C for 6 h. Aliquots of the dissolution medium (300 μl) were withdrawn at predetermined time intervals. Drug concentrations were directly analyzed by the HPLC method described before.

3. Results and discussion

Eudragit® P-4135F belongs to the pH-sensitive Eudragit® group of polyacrylates, such as S100 and L100, exhibiting a dissolution threshold pH slightly above 7.2 [18]. The characteristic polymer dissolution pattern is shown in Fig. 1. These properties are based on its structure, synthesized by co-polymerization of methacrylic acid (10%), methyl acrylate (65%) and methyl methacrylate (25%). As reported earlier, one major advantage compared to the previously employed Eudragit® S100 and L100 is its solubility in methylene chloride [19]. For this reason alternative microsphere preparation methods can be applied replacing the complicated oil/oil emulsification process used in prior formulations [12,15,16]. This may avoid the use of an external oily phase as well as a long-term solvent evaporation under vacuum and the washing steps producing a distinct quantity of additional solvent waste. One proposed alternative is the oil/water emulsification which has been extensively applied to the development of microspheres.

As a standard procedure, microspheres were prepared by the oil/oil emulsification based on the technique reported earlier [12,15,16]. That the particles do not appear perfectly spherical might be due to the high viscosity of both, internal and external phase (Fig. 2). The microsphere surface was smooth, but exhibited pores of a varying diameter. Since tacrolimus displays a certain solubility in the external paraffin phase, the encapsulation rate was found to be very low (Table 1). In feasibility studies, the fluorescent dye, 6-coumarin, showed a similar behavior resulting in a distinct dissolution inside the external oil phase (data not shown). One can, therefore, conclude that this method might not be suitable for the encapsulation of a rather lipophilic drug into microspheres based on the pH-sensitive Eudragit polymers since high drug load might not be achieved. Earlier reports regarding the microencapsulation by pH-sensitive Eudragit® polymers describe, to our knowledge, exclusively the incorporation of more hydrophilic compounds.

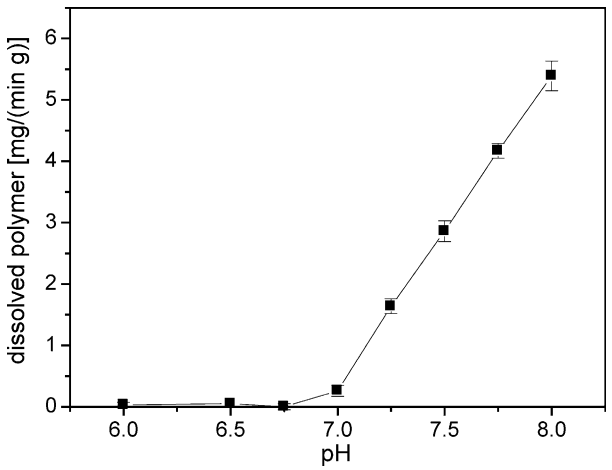

Fig. 1. Dissolution profile of Eudragit® P-4581F for a pH range from pH 6 to 8. All results are shown as mean ± SD.

Despite the low encapsulation rate and the large pores, which were detected at the particle surface, the drug release was strongly pH-dependent in all experiments. When microspheres were incubated in phosphate buffer of pH 1.2 and 6.8, only a very low drug leakage was observed (Fig. 3a). After 6 h of incubation all values of the released tacrolimus amount recovered from the supernatant were below 10% of the initial drug load. Such results are comparable with results from film coated pellets which displayed a similarly efficient drug retention [11], although the diameter of these microspheres was found to be even lower than the film thickness of the reported pellets. At a pH of 7.4, the polymer dissolved rapidly and the microsphere disintegration resulted in an immediate drug release.

In the case of the oil/oil method, a slight retention of drug release at pH 7.4 was observed in comparison with the oil/water methods (Fig. 3b and c). This may have two reasons, such as a lower drug concentration near the particle surface, as well as the protecting layer of the Span 80, which might not be completely removed by the different washing steps. Thus, the surfactant still orients its lipophilic ends towards the external phase. This may reduce the easy

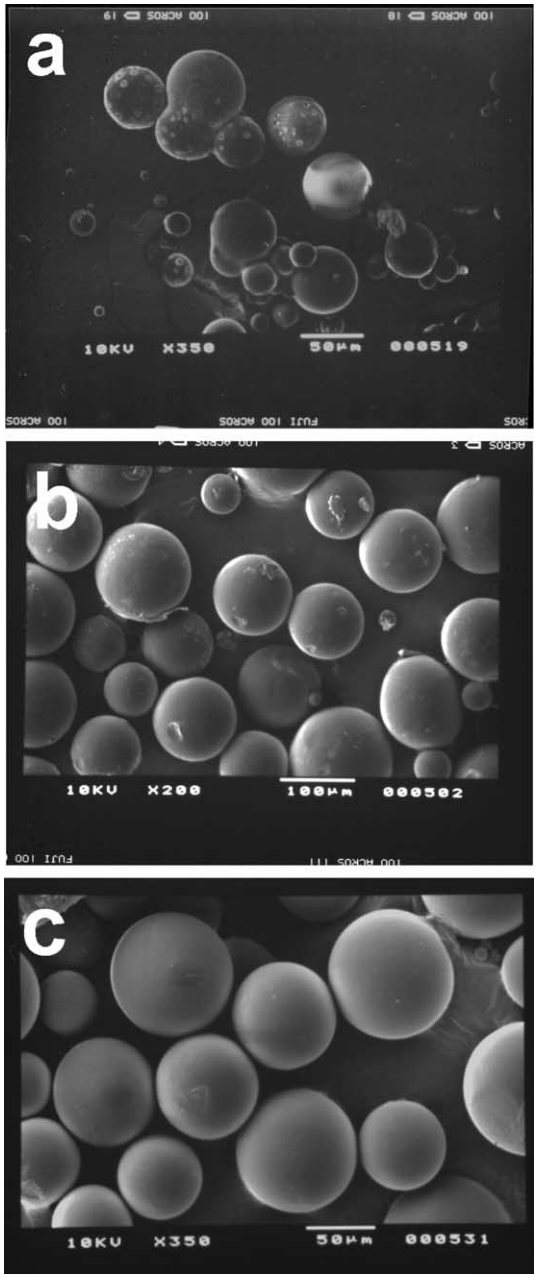

Fig. 2. SEM images of microspheres prepared with Eudragit® P-4581F using either an oil/oil (a) or an oil/water emulsification process including a solvent evaporation (b) or solvent extraction step (c).

Table 1
Characteristics of Eudragit® P-4581F microspheres prepared by the different methods

	Oil/oil	Oil/water evaporation	Oil/water extraction
Diameter (μm)	113.8 ± 14.8	157.8 ± 11.9	133.8 ± 14.8
Yield (%)	98.5 ± 10.1	82.6 ± 3.4	91.5 ± 6.2
Encapsulation rate (%)	4.8 ± 0.4	56.3 ± 1.9	38.8 ± 9.4

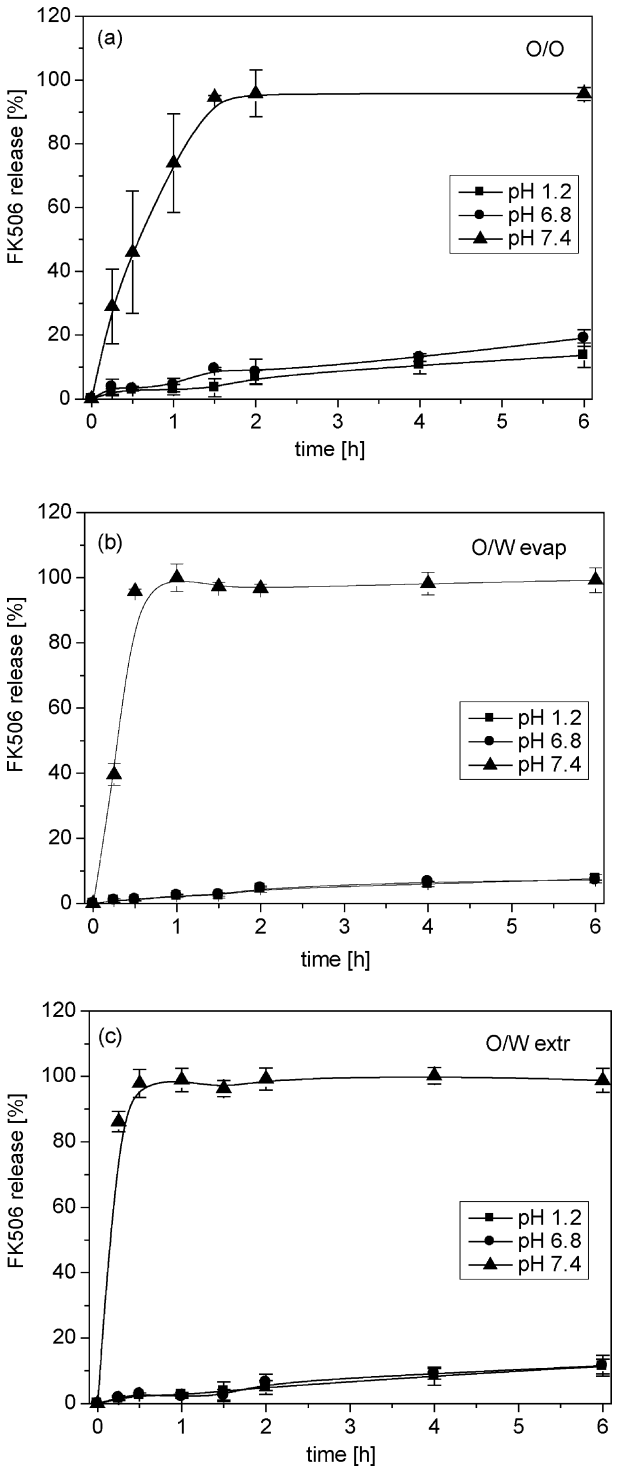

Fig. 3. Cumulated tacrolimus (FK506) release versus time of different Eudragit® P-4581F microsphere formulations (O/O emulsification (a) or O/W process/solvent evaporation (b) or O/W process/extraction step (c)) in phosphate buffer systems of pH 1.2, 6.8, and 7.4 ($n = 3$). All results are shown as mean ± SD.

contact of the aqueous phosphate buffer system to the particle surface and the fast dissolution of the polymer.

When employing oil/water emulsification, microsphere solidification was performed either by solvent evaporation

or solvent extraction. The particle size distribution was found to be only slightly different for both methods, however, the encapsulation rates between the two methods differed distinctly (Table 1). In contrast to observations for the entrapment of hydrophilic compounds, more lipophilic drugs exhibit higher incorporation efficiency through the use of solvent evaporation compared to solvent extraction. A long-term contact of the organic polymer phase with the external aqueous phase prior to particle solidification, mainly found in the case of a solvent evaporation process, might lead to an enhanced drug loss by the longer diffusion time period. In such a case a faster solidification seems to be desirable, which can be achieved by solvent extraction. For lipophilic drugs, however, other factors play dominant roles.

Although a nearly immediate particle solidification during the solvent extraction can be achieved, lower encapsulation rates were obtained. Ethyl acetate diffuses into the external aqueous phase during the solvent diffusion due to its miscibility with the external aqueous phase. Indeed, the drug has a good solubility in the organic phase, which enhanced significantly its transport towards the aqueous phase during solvent diffusion. Thus, the organic solvent 'extracted' the drug out of the particle matrix during the polymer precipitation. This phenomenon has been also observed during nanoparticle preparation by using a nanoprecipitation method. In this case, the use of water miscible solvents caused significant drug loss towards the aqueous phase during particle solidification [20,21]. The mechanism is furthermore enhanced by the increased solubilization of tacrolimus in the external aqueous phase by the presence of polyvinyl alcohol.

The drug release was found to show generally similar behavior at the respective pHs (Fig. 3b and c). Tacrolimus was retained efficiently inside the microspheres when tested in in vitro buffer systems at pH 1.2 and 6.8. Again, around 90% of the initial drug load was maintained inside the particle matrix after 6 h of incubation. A comparatively fast release was observed at pH 7.4, which delivered about 100% of the incorporated drug within 60 min.

When comparing evaporation and extraction process, a slightly faster drug release occurred after solvent extraction at pH 7.4. Also the leakage from the particle matrix at pH 6.8 and below was slightly enhanced. During the solvent extraction step, ethyl acetate diffuses into the external aqueous phase subsequently leading to the solidification of the microspheres. Simultaneously, the solvent also extracts a certain amount of the drug which is in favor of a drug location near the particle surface.

Moreover, the differences in the particle diameter may also have an influence on the drug release as the microspheres after solvent extraction are smaller, exhibiting a larger surface for an enlarged drug diffusion. Calculations based on the mean diameter demonstrated that the smaller microspheres prepared by solvent extraction exhibit an overall 18% larger surface compared the only slightly bigger ones after the evaporation process. Also the drug

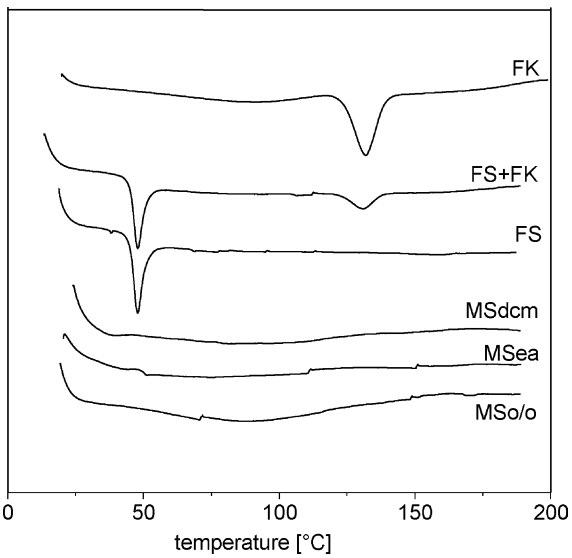

Fig. 4. DSC thermogram of tacrolimus (FK), physical mixture of tacrolimus and Eudragit® P-4581F (FS + FK), Eudragit® P-4581F alone (FS), and microsphere formulations of O/W emulsification solvent evaporation (MSdcm), O/W emulsification solvent extraction (MSea), and O/O emulsification (MSo/o).

leakage from oil/oil emulsification microspheres was observed to be higher than from oil/water microspheres while their mean diameter was distinctly lower. Therefore, the conclusion that the drug release at pH 7.4 as well as the drug leakage at pH 6.8 is dependent on the particle surface area may also appear appropriate.

The drug entrapped in the different MS formulations was found to be stable for at least 3 months at room temperature in a desiccator and under light exclusion. Using DSC, transition temperatures of tacrolimus and Eudragit P-4135F were observed at 48 and 132 °C (Fig. 4). Both peaks were also distinctly visible in their physical mixture of 1:12 which corresponds to their respective mass ratio in the microsphere formulation. However, such peaks were found neither in the blank nor in the drug-loaded microspheres.

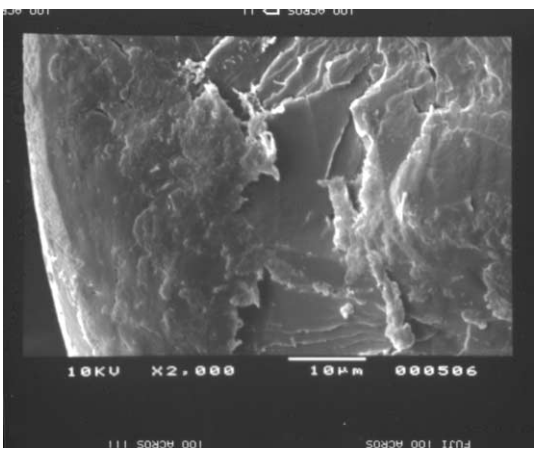

Fig. 5. SEM image of a microsphere cross-section prepared with Eudragit® P-4581F using oil/water emulsification process including a solvent evaporation step.

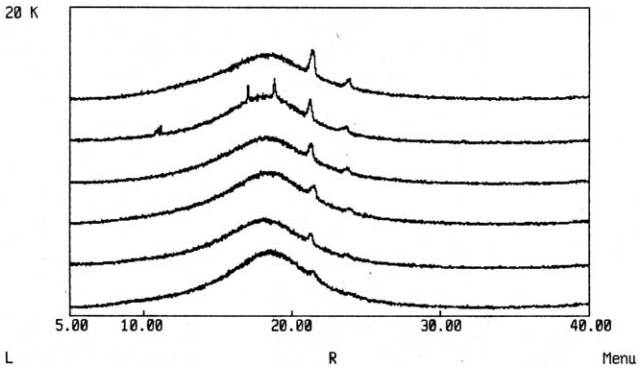

Fig. 6. X-ray analysis of Eudragit® P-4581F, tacrolimus, physical mixture of tacrolimus and Eudragit® P-4581F, microsphere formulations of O/W emulsification solvent evaporation, O/W emulsification solvent extraction, and O/O emulsification (graphs are shown in the mentioned order from top to bottom).

It might be reasonable that the incorporated drug is present in a molecular dispersed form inside the particle matrix. Also in SEM analysis of microsphere cross-sections no indications for drug crystals were found (Fig. 5).

Moreover, X-ray analysis supported this hypothesis that tacrolimus was not present in a crystal state inside the particle matrix (Fig. 6). Only the reference of free drug crystals showed characteristic peaks at around 17 and 19° θ, while the polymer, blank as well as drug loaded microspheres did not show any signal. This molecular dispersion of tacrolimus may favor efficient drug retention during the release experiments at lower pH. The observed drug retention for other drugs with different solubility properties was found to be distinctly less efficient [19]. Lipophilic interactions of FK506 with the polymer seem to be a reason for retaining the drug inside the microcarrier at pH 6.8 and below. However, due to the low drug/polymer ratio in the microspheres infrared analyses did not show clear proof for this type of interaction.

Subsequently, the above-mentioned pores may have only a small effect on the drug release, since the drug is molecularly dispersed inside the particle matrix. Compared to the incorporation of hydrophilic compounds, a lower drug leakage might be expected since the drug cannot be reached by microchannels which can enhance an early drug loss for hydrophilic compounds. In general, the efficiency of such a matrix system might be even enhanced compared to coating approaches.

4. Conclusions

A colon specific microparticulate drug delivery system for tacrolimus was developed by two different emulsification methods (oil/oil and oil/water). A relatively higher drug load can be achieved by the oil/water methods. However, a pH-dependent release can be provided by all different systems. At pH 6.8, the main drug load is retained inside the polymer matrix, and at pH 7.4, a fast dissolution of the carrier occurs. SEM, DSC, and X-ray diffraction permitted a structural analysis where a molecular dispersion of the drug inside the polymeric microsphere matrix was observed which allowed such efficient drug retention of the incorporated drug. Generally, all formulations demonstrated their applicability in vitro as a promising device for pH-dependent colon delivery of tacrolimus, however, the oil/water technique was found to be superior to the oil/oil approach.

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