CHAPTER 2. MATERIALS AND METHODS

1. Materials

Drugs: micronized carbamazepine (CBZ), ibuprofen (BASF AG, Ludwigshafen, Germany), indomethacin, cyclosporine A (Fluka Chemie AG, Buchs, Switzerland), lidocaine base (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 17β-estradiol hemihydrate (Schering AG, Berlin, Germany), heparin sodium (Carl Roth GmbH, Karlsruhe, Germany), leuprolide acetate (Lipotec, SA, Barcelona, Spain).

Polymers:

Biodegradable polymer. Poly(D,L-lactide- co-glycolide) 50:50 (PLGA, Resomer® RG 503H, Mw 34,000; Resomer® RG 502H, Mw 11,600) (Boehringer Ingelheim Pharma KG, Ingelheim, Germany).

Enteric polymers. Eudragit® L100-55 (poly(methacrylic acid-co-ethyl acrylate) 1:1), Eudragit® L100 (poly(methacrylic acid-co-methyl methacrylate) 1:1), Eudragit® S100 (poly(methacrylic acid-co-methyl methacrylate) 1:2), (Degussa AG, Darmstadt, Germany), hydroxypropyl methylcellulose phthalate (HPMCP HP-55S), hydroxypropyl methylcellulose acetate succinate (HPMCAS AS-MF) (Shin-Etsu Chemical Co., Tokyo, Japan), cellulose acetate phthalate (CAP) (Eastman Chemical Co., Kingsport, USA).

Water-insoluble non-enteric polymers. Ethylcellulose (Ethocel® standard 7 FP, Dow Chemical, Midland, MI, USA), Eudragit® RL PO (poly(ethyl acrylate-co-methyl methacrylate-co-trimethyl-ammonioethyl methacrylate chloride) 1:2:0.2) (Röhm GmbH, Darmstadt, Germany).

Water soluble polymers. Hydroxypropyl methylcellulose (HPMC, Methocel K15M) (Colorcon Ltd., Orpington, UK), hydroxypropyl cellulose (HPC, Klucel HF), hydroxyethyl cellulose (HEC, Natrosol 250 HX) (Hercules Inc., Wilmington, USA), polyvinyl alcohol (PVA, Mowiol 40-88) (Clariant GmbH, Frankfurt, Germany), polyvinylpyrrolidone (PVP, Kollidon® 12 PF, Kollidon® 17 PF), Poloxamer 407 (block copolymer of polyoxyethylene and polyoxyprolylene) (Poloxamer 188, Poloxamer 407), polyethylene glycol (PEG 1500, Lutrol® E 1500) (BASF AG, Ludwigshafen, Germany).

Organic solvents: *N*-methyl-2-pyrrolidone (NMP, Pharmasolve) (ISP GmbH, Köln, Germany), 2-pyrrolidone (Merk-Schuchardt, Hohenbrunn, Germany), triethyl citrate

(Morflex Inc., Greensboro, USA), triacetin (Fluka Chemie GmbH, Steinheim, Germany), dimethyl sulfoxide (DMSO), acetic acid, 1,4-dioxane (Merck KGaA, Darmstadt, Germany), ethyl acetate, polyethylene glycol 400 (PEG 400), triethylamine (Merk-Schuchardt, Hohenbrunn, Germany), sodium chloride, hexane, acetonitrile, chloroform (Carl Roth GmbH, Karlsruhe, Germany)

Oils: Sesame oil, soybean oil (Croda GmbH, Nettetal, Germany), medium chain triglyceride (MCT, Miglyol 812) (Sasol GmbH, Witten, Germany), Gelucire 35/10 and Gelucire 44/14 (Gattefossé, Cedex, France).

Surfactants: Polyethylene sorbitan monooleate (Tween 80), sorbitan monooleate (Span 80) (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany), sodium lauryl sulfate (SDS) (Henkel KGaA, Düsseldorf, Germany).

Other chemicals: Oleylamine (Fluka Chemie GmbH, Steinheim, Germany), methylene blue (Azure II; Merck KGaA, Darmstadt, Germany), carbamazepine-10,11-epoxide (CBZ-E), 5,5-diphenylhydantoin (phenytoin) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 9 polystyrene standard kits with peak average molecular weight (M_p) in the range of 580-299,400 Da (Polymer Laboratories GmbH, Darmstadt, Germany), sodium chloride (Carl Roth GmbH, Karlsruhe, Germany).

2. Methods

2.1. Enteric microparticles

2.1.1. Preparation of enteric microparticles

An aqueous polymer phase was prepared by dissolving HPMC, Poloxamer 407, HPC, HEC or PVA in water, or HPMC in pH 7.4 phosphate buffer (KH_2PO_4 50 mM, NaOH 39.1 mM) or 0.1 N HCl solution (pH 1.2) (Table 1) and the organic polymer phase by dissolving the enteric polymers (Eudragit® L100-55, Eudragit® L100, Eudragit® S100, HPMCP HP-55S, HPMCAS-MF) in ethanol, cellulose acetate phthalate in acetone/ethanol (1:1, v/v) and Eudragit® L100-55 in isopropanol or acetone (Table 2).

The enteric microparticles were formed by dropping 15 g aqueous phase into 10 g organic phase containing the enteric polymer (20% w/w based on polymer solution) with/without drug (carbamazepine, lidocaine, cyclosporine A, indomethacin, ibuprofen, 17β-estradiol hemihydrate) (10-20% w/w based on polymer and drug) under magnetic stirring (Janke & Kunkel GmbH & Co. KG, Staufen, Germany) at 800 rpm for 5 min**.** The viscous microparticle suspension was diluted with 50 ml water and the microparticles were observed and photographed with a polarized light microscope (Carl Zeiss Jena GmbH, Jena, Germany), collected by centrifugation (3000 rpm, 10 min) (Biofuge 22R, Heraeus Sepatech GmbH, Osterode, Germany), vacuum-dried (12 h) (Heraeus Holding GmbH, Hanau, Germany) and stored in desiccators.

2.1.2. Viscosity of the aqueous polymer phase

The viscosity of the aqueous polymer phase was determined with a rotational viscometer (Rheostress RS 100, HAAKE Mess-Technik GmbH, Karlsruhe, Germany) in a controlled rate mode (D = 30 s^{-1}) at 25°C utilizing a plate-cone-geometry (20 mm/4°, 60 mm/1°) (n = 3).

2.1.3. Yield of enteric microparticles and encapsulation efficiency of carbamazepine

15 g 1%w/w HPMC solution in 0.1 N HCl or pH 7.4 phosphate buffer was added dropwise into an ethanolic solution containing 20% w/w Eudragit® L100-55 or HPMCP HP-55S. The obtained suspensions were diluted with 50 ml water and centrifuged (3000 rpm, 10 min) (Biofuge 13/Haemo, Heraeus Instruments, Osterode, Germany). The collected enteric microparticles were vacuum-dried. The yield of the enteric microparicles was calculated as the weight ratio of the amount of microparticles to the amount of polymer used.

The carbamazepine loading was determined by dissolving 10 mg microparticles in 100 ml pH 6.8 phosphate buffer followed by UV-spectrophotometric assay at 287 nm (Shimadzu Scientific Instrument, Columbia, MD, USA) (n = 3). The enteric polymeric carrier did not interfere at this wavelength. The drug encapsulation efficiency was the ratio of the actual drug loading to the theoretical loading expressed in percentage.

2.1.4. Particle size analysis

The size of the microparticles was measured by laser diffractometry (LS 230, Beckman Coulter GmbH, Krefeld, Germany) and calculated on the basis of the volume distribution.

2.1.5. Phase separation of enteric polymers and precipitation of drugs

The phase separation of enteric polymer solutions and precipitation of drugs were evaluated by the addition of 1% w/w HPMC aqueous solution to 10 g ethanolic solutions containing 20% w/w enteric polymers (Eudragit® L100-55, Eudragit® L100, Eudragit® S100, HPMCAS, HPMCP), or 20% w/w Eudragit® L100-55 in 8 g isopropanol or acetone, or 8 g ethanol containing 10-30% w/w drug (carbamazepine, indomethacin, ibuprofen) (based on enteric polymer and drug) under magnetic stirring at 800 rpm. The phase separation point and precipitation point were the points at which the solution started to become turbid.

2.1.6. Determination of coacervation region

Phase diagrams were generated to investigate the coacervation condition. HPMC (1% w/w) in water was added dropwise into an ethanolic solution (10 g) of Eudragit® L100-55 or HPMCP (10%, 15%, 20%, 25%, 30% and 40% w/w, based on polymer and ethanol) with/without 20% w/w carbamazepine under magnetic stirring at 800 rpm. The phase separation and precipitation of the polymer were identified at different stages with a polarized light microscope (Carl Zeiss Jena GmbH, Jena, Germany). The composition of the mixtures used to generate the phase diagrams was calculated based on the weight ratio of 1% w/w HPMC aqueous solution, ethanol (96% v/v) and enteric polymer and/or carbamazepine.

2.1.7. Scanning electron microscopy

The microparticles were spread and fixed on a sample holder with a double-sided tape and were then coated for 230 s with gold-palladium (SCD 040, Bal-Tec GmbH, Witten, Germany) under an argon atmosphere. The surface morphology of the microparticles was examined with a scanning electron microscope (SEM) (S-4000, Hitachi High-Technologies Europe GmbH, Krefeld, Germany) using secondary electron imaging at 10 kV.

2.1.8. Differential scanning calorimetry

Thermograms of drugs (carbamazepine, ibuprofen, indomethacin), enteric polymer (Eudragit® L100-55), physical mixture of drug (10-20% w/w) and enteric polymer and drug loaded enteric microparticles (10-20% w/w) were obtained using a differential scanning calorimeter (Mettler DSC 821e, Mettler, Toledo, Giessen, Germany). Drug physical state in the enteric carrier was investigated by determining the presence of drug melting point. The temperature calibration was accomplished with the melting transition of indium. Samples of 4-7 mg were sealed in aluminum pans. The heating rate and cooling rate was 10°C/min and 30°C/min, respectively. All tests were run under a nitrogen atmosphere.

2.1.9. Powder X-ray diffraction

The physical state of the carbamazepine was determined by powder X-ray diffraction. The measurements were conducted with a Philips PW 1830 X-ray generator with a copper anode (Cu Kα radiation, λ = 1.5418 Å, 40 kV, 20 mA) fixed with a Philips PW 1710 diffractometer (Philips Industrial & Electro-acoustic Systems Division, Almelo, The Netherlands). The radiation scattered in the crystalline regions of the samples was measured with a vertical goniometer. The scanning rate was 0.02° s^{-1} over a range of 4-40°.

2.1.10. Compatibility of drugs and enteric polymer

Thin, drug-loaded polymeric films were prepared by casting ethanolic polymer solutions (Eudragit® L100-55, 20% w/w) containing drugs (carbamazepine, lidocaine, cyclosporine A, ibuprofen, indomethacin, estradiol; 10-30% w/w based on polymer and drug) on Teflon plates, followed by drying for 48 h at room temperature and 24 h vacuum-drying. The dried films were removed from the Teflon plates and stored for 24 h in a desiccator until constant weight. A transparent film qualitatively indicated the compatibility of the drug with the polymer. The freshly prepared films and films stored at room temperature for 1.5 years were observed under a polarized light microscope (Carl Zeiss Jena GmbH, Jena, Germany) to investigate possible crystallization of the drugs in the films. A non-crystalline state was identified by the absence of birefringence of drug crystals.

2.1.11. Determination of enteric polymer and drugs in polymer-rich/-poor regions

The amount of enteric polymer (Eudragit® L100-55) and drugs (carbamazepine, ibuprofen) in the polymer-rich and -poor regions was determined. 1% w/w HPMC aqueous solution was dropped into 10 g ethanolic solution of Eudragit® L100-55 (20% w/w) containing 20% w/w carbamazepine or ibuprofen. A 4 g sample was withdrawn at water/ethanol ratios (w/w) of 0.9, 1.2, 1.9, 3.9 and 5.5, photographed under a polarized light microscope and centrifuged at 13000 rpm (Biofuge 13/Haemo, Heraeus Instruments, Osterode, Germany) for 20 min. The content of Eudragit® L100-55 in the polymer-rich and -poor regions was determined gravimetrically by oven-drying 1 g samples at 105 °C to a constant weight. The amount of drugs (carbamazepine, ibuprofen) in the two regions was quantified by dissolving 0.1 g samples in 100 g 1 N NaOH solution, followed by UV-spectrophotometric analysis (carbamazepine: λ = 287 nm; ibuprofen: λ = 221 nm; UV-2101 PC, Shimadzu Scientific Instrument, Columbia, MD, USA).

2.1.12. Solubility of carbamazepine and ibuprofen in water/ethanol mixtures

The solubility of carbamazepine and ibuprofen in water/ethanol (ratios of 0.05, 0.32, 1.27, 1.90 and 5.07 w/w) was determined. 0.5 g drug (carbamazepine or ibuprofen) was placed into a 10 ml glass tube filled with 5 ml water/ethanol, which was shaken overnight at room temperature. The concentration of drugs was measured UV-spectrophotometrically after proper dilution in water.

2.1.13. Partition of carbamazepine between polymer rich/poor regions in coacervate

To clarify the mechanism of encapsulation of carbamazepine in enteric microparticles, drug partition coefficient between polymer rich/poor regions was determined. Blank coacervate was first prepared by addition of 10 g (or 15 g) 1% w/w HPMC solution to 10 g ethanolic solution of Eudragit® L100-55 (20% w/w) under a magnetic stirring at 800 rpm. 0.22 g (or 0.11 g) carbamazepine was either dissolved in polymer solution and formed coacervate or in the preformed coacervate. The complete dissolution of the drug was ensured by observation under polarized light microscope (Carl Zeiss Jena GmbH, Jena, Germany). The drug content in both regions after 1, 2 and 17 h stirring (200 rpm) at room temperature was determined by separating the two regions by centrifugation (13000 rpm, 20 min) (Biofuge 13/Haemo, Heraeus

Instruments, Osterode, Germany), dissolving the two regions (0.2 g) in 0.1 N NaOH and determining the drug content by UV-spectrophotometric analysis at 287 nm. The partition coefficient was the ratio of drug concentrations between polymer rich and poor regions.

2.1.14. Wettability

The compacts (diameter: 13 mm; weight: 500 mg) of Eudragit® L100-55 powder, carbamazepine, a physical mixture of 20% w/w carbamazepine and Eudragit® L100-55 and 20% w/w drug-loaded microparticles were prepared manually with a hydraulic press (SPECAC Ltd., Orpington, England) at a compression force of 1200 kg. A drop of pH 6.8 phosphate buffer (KH_2PO_4 102.1 mM, Na_2HPO_4 97.9 mM**)** was supplied from a microsyringe onto the surface of the compacts and observed under a microscope. The contact angle between the baseline of the drop and the tangent at the drop boundary was measured using a goniometer after 1 min (Krüss GmbH, Hamburg, Germany).

2.1.15. In vitro drug release studies

Sink conditions: The in vitro dissolution/release of carbamazepine from pure drug, physical mixture, freshly prepared and microparticles stored for 5 months (20% w/w loading) was determined with a USP XXV rotating paddle method [900 ml 0.1 N HCl or pH 6.8 phosphate buffer; 50 rpm; 37°C; n = 3] (VanKel® 700, Vankel Industries, Edison, NJ, USA). The solubility of carbamazepine in pH 6.8 phosphate buffer is 126.7 µg/ml. A sample equivalent to 10 mg drug (< 10% of drug solubility in 900 ml medium) was used to maintain sink conditions. At predetermined time intervals, samples were withdrawn (3 ml, not replaced) and assayed UV-spectrophotometrically at 287 nm.

Non-sink conditions: Carbamazepine-loaded enteric microparticles (10% w/w, 150 mg; 20% w/w, 75 mg) and physical mixtures of the same composition were dispersed in 10 g pH 6.8 phosphate buffer in 20 ml plastic syringes (B. Braun Melsungen AG, Melsungen, Germany), coupled with a filter (0.2 µm, Schleicher & Schuell MicroScience GmBH, Dassel, Germany) and shaken in a horizontal shaker (37°C, 80 rpm, GFT, Gesellschaft Für Labortechnik GmbH, Berlin, Germany). Subsequently, 0.5 g samples were filtered through the filter at the predetermined time points and properly diluted with pH 6.8 phosphate buffer before UV measurement. The drug crystals in the release medium were observed under a polarized light microscope (Carl Zeiss Jena GmbH, Jena, Germany) after 1 and 24 h incubation.

2.1.16. Stability of the microparticles

Carbamazepine-loaded enteric microparticles were stored for 5 months in a desiccator at room temperature. The microparticles were characterized by powder X-ray diffraction (Philips Industrial, Electro-acoustic Systems Division, Almelo, The Netherlands) and release study in pH 6.8 phosphate buffer to evaluate the physical stability of the drug.

2.1.17. In vivo study

2.1.17.1. Animal experiments

Male and adult New Zealand rabbits (2.69 ± 0.06 kg, n = 8) were randomly divided into two groups to investigate 20% w/w carbamazepine-loaded enteric microparticles (group A) and physical mixture (group B). The rabbits were fasted but had free access to water overnight. Each rabbit received an equivalent dose of 100 mg carbamazepine filled in the hard gelatin capsules orally, followed by 10 ml water. After dose administration, the rabbits were kept in cages and had free access to food and water after 6 h. Serial blood samples (1.0 ml) were withdrawn from the marginal ear vein into a vial containing sodium citrate (50 µl) at a predose (-30 min) and postdoses (1, 2, 3, 4, 6, 8, 10 and 24 h), which were gently mixed and centrifuged at 5000 rpm for 10 min (Biofuge 22R, Heraeus Sepatech GmbH, Osterode, Germany) within 1 h of collection. The obtained plasma was stored at –20°C until analysis.

2.1.17.2. Assay of drug and its metabolite in plasma

Carbamazepine and its major active metabolite carbamazepine-10,11-epoxide were determined in the blood plasma by HPLC (Nagasawa et al., 2002). Briefly, 0.8 ml acetonitrile containing phenytoin (1 µg/ml) as internal standard was added to 0.2 ml plasma to precipitate proteins. After 30 sec vortexing, the mixture was centrifuged at 17,000 rpm for 10 min (Biofuge 22R, Heraeus Sepatech GmbH, Osterode, Germany). 20 µl supernatant was injected into a HPLC apparatus (SCL-10A vp, Shimadzu Co., Tokyo, Japan) equipped with a diode array detector (SPD-M10Avp, Shimadzu Co., Tokyo, Japan). The HPLC analysis conditions were as follows: C_{18} column, 150 × 4.6 mm i.d., Eurospher-100 (Knauer GmbH, Berlin, Germany); mobile phase, acetonitrile-methanol-pH 7.0 phosphate buffer (0.33 mM) (18:18:64, v/v); column temperature, 40°C; flow rate, 1.0 ml/min; wavelength, 210 nm.

2.1.17.3. Pharmacokinetic analysis

The maximum plasma level (C_{max}) and the time to reach C_{max} (t_{max}) of the drug and its metabolite were obtained directly from the actual observed data. The area under the curve from 0 to 24 h ($AUC_{0\rightarrow24h}$) was calculated by means of linear trapezoidal rule.

2.2. In-situ forming microparticles (ISM)

2.2.1. Parenteral ISM

2.2.1.1. Reduction of heparin particle size

Ball milling. Heparin alone or blends with RG 502H powder were micronized with a ball milling (MM2000, Retsch GmbH, Haan, Germany). The resulting drug powder (10%, w/w) was suspended in RG 502H solutions (40% w/w in NMP or 20% w/w in TEC). The milled blends of heparin (10% w/w) and RG 502H were dispersed in NMP or TEC. Heparin was suspended in the polymer solutions with the dissolution of RG 502H,

Wetting method. A heparin aqueous solution containing 10% v/v ethanol as wetting agent was mixed with RG 502H powder (10% heparin w/w, based on polymer and drug), following by vacuum-drying (Heraeus Holding GmbH, Hanau, Germany) to remove water and ethanol. The dry powder was dispersed in NMP (40% w/w) and TEC (20% w/w), respectively.

Colyophilization method. A series of solutions (0.5 ml) containing 20 mg of heparin and 20 mg of amphiphilic polymers (PEG 1500, Poloxamer 188, Poloxamer 407, PVP 12) or PVP 17 (10, 20, 30 mg) were frozen (-70°C) and freeze-dried (Gama 2-20, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) (-25°C, 24 h), following by a secondary drying (15°C, 12 h). By dispersing the co-lyophilized powder of heparin and the amphiphilic polymers into 40% w/w RG 502H in NMP or 20% w/w in TEC solutions, the heparin particles were homogeneously dispersed in the polymer solutions after the dissolution of the amphiphilic polymers.

2.2.1.2. Preparation of parenteral ISM

An oil-in-oil (O/O) ISM emulsion was prepared by emulsifying polymer phase (0.4 g, 40% RG 502H in NMP or 20% RG 502H in TEC) containing 10% w/w heparin from the three methods into 0.8 g sesame oil with 2% Span 80. Emulsification was performed in a two-syringe (2 ml) (B. Braun Melsungen AG, Melsungen, Germany)

system coupled by a connector with mixing forth and back 50 cycles at a speed of 1 cycle/sec.

2.2.1.3. In vitro drug release

The obtained O/O emulsions were injected into 100 ml pH 7.4 phosphate buffer (KH_2PO_4 50 mM, NaOH 39 mM) containing 0.1% Tween 80 and shaken in a horizontal shaker (37°C, 80 rpm) (GFT, Gesellschaft Für Labortechnik GmbH, Berlin, Germany). 1 ml supernatant was withdrawn at appropriate intervals and replaced with fresh buffer. The amount of heparin released was determined with a modified Azure II colorimetric method (Jiao et al., 2002A). A stock solution of Azure II (methylene blue, 1.0 mg/ml) was prepared by dissolving in water and diluted to 0.01 mg/ml before use. Each aqueous sample (1 ml) was reacted with 9 ml of the Azure II solution (0.01 mg/ml) at room temperature and assayed spectrophotometrically at 530 nm (Shimadzu Scientific Instrument, Columbia, MD, USA) (n = 3).

2.2.1.4. Recovery of heparin

Heparin was dissolved in water or pH 7.4 phosphate buffer containing 0.1% Tween 80 and determined by the modified Azure II method. 0.8 g sesame oil (2% Span 80) and blank O/O ISM formulations (0.4 g polymer solution and 0.8 g oil phase) were introduced into 100 ml the heparin solutions (n = 3), and incubated at 37°C for 10 days. The heparin concentrations were measured and compared to the original values.

2.2.2. Oral in-situ forming microparticles

2.2.2.1. Preparation of oral ISM

Polymer solutions. RG 502H was dissolved in triethyl citrate (20% w/w) or *N*-methyl-2-pyrrolidone (40%, w/w) in the absence or presence of Tween 80 (5% w/w), oleylamine (3-10% w/w) and drug (10% w/w based on polymer and drug). Eudragit® RL and Eudragit® RS were dissolved in 96% v/v ethanol (30% w/w). Blends of RG 502H and Eudragit® RL/RS at ratio of 2:1, 1:1, and 1:2 were dissolved in dimethyl sulfoxide containing 10% w/w heparin and 10% w/w polyvinyl pyrrolidone (PVP 17).

Formulation preparation. The polymer solutions in the absence or presence of drug and other excipients were emulsified into a molten Gelucire 44/14 (phase ratio 1:2) at 60°C by using an Ultra Turrax (Ultra Turrax T25, Janke & Kunkel GmbH & Co KG,

Staufen, Germany) at 10,000 rpm for 1 min, cooled down in an ice bath and stored in a refrigerator.

2.2.2.2. Particle size distribution and surface zeta potential

The chilled semisolid formulations were molten and photographed with a microscope (Carl Zeiss Jena GmbH, Jena, Germany) to observe the particle size distribution of internal polymer droplets in the external Gelucire 44/14 phase.

After dispersing the formulations in 0.1 N HCl or pH 6.8 phosphate buffer, the mean particle diameter, polydispersity index and zeta potential were measured by photon correlation spectroscopy (PCS) (Zetasizer 4, Malvern Instruments, Malvern, UK). Zeta potential measurements were carried out in the original dispersion media (0.1 N HCl or pH 6.8 phosphate buffer). The size of microparticles was measured by laser diffractometry (LS 230, Beckman Coulter GmbH, Krefeld, Germany) and calculated on the basis of the volume distribution.

2.2.2.3. In vitro drug release

5 mg carbamazepine or indomethacin dissolved in 0.2 g Eudragit® RS or RL ethanolic solutions (30% w/w), and emulsified into 0.4 g Gelucire 44/14 at 60°C by Ultra Turrax (Ultra Turrax T25, Janke & Kunkel GmbH & Co KG, Staufen, Germany) at 10,000 rmp for 1 min and stored at 2-8°C. In vitro drug release in pH 6.8 phosphate buffer (indomethacin) and 0.3% SDS water solution (carbamazepine) was carried out by shaken in a horizontal shaker (GFT, Gesellschaft Für Labortechnik GmbH, Berlin, Germany) (37°C, 80 rpm) in 100 ml buffer. At predetermined time intervals, 1 ml samples were withdrawn and analyzed UV-spectrophotometrically (indomethacin: λ = 265 nm; carbamazepine: λ = 287 nm).

2.3. PLGA stability in in-situ forming systems

2.3.1. Water uptake of organic solvents during storage at 75 % relative humidity

The water uptake of the organic solvents (NMP, 2-pyrrolidone, DMSO, triacetin, triethyl citrate, PEG 400), which were used as solvents for PLGA, was determined by placing open glass vials filled with 5 g solvents over a saturated sodium chloride solution in a desiccator (75% relative humidity) (n = 3). The water uptake of the solvents was measured gravimetrically over a 2 week period. The initial and final water content of the solvents was measured by Karl Fischer titration (section 2.3.3).

2.3.2. Preparation of PLGA solutions, suspensions and lyophilized sponges

PLGA solutions were prepared by dissolving 1 g PLGA powder in 4 g solvent (*N*-methyl-2-pyrrolidone, 2-pyrrolidone, DMSO, triacetin, triethyl citrate, PEG 400 and ethyl acetate) with/without leuprolide acetate (10% w/w based on polymer and drug). The water content of the polymer solutions was determined by Karl Fischer titration (section 2.4). The water content of PLGA solutions in NMP, 2-pyrrolidone, triacetin and triethyl citrate was adjusted to 0.7% and 1.2% w/w in order to evaluate the effect of water content on polymer degradation.

PLGA suspensions were prepared by suspending 1 g PLGA powder in 4 g sesame oil, soybean oil, medium chain triglyceride, deionized water and saturated sodium chloride solution.

PLGA sponges were prepared by dissolving PLGA (20% w/w based on polymer and solvent) in acetic acid or 1,4-dioxane with/without leuprolide acetate or lidocaine base (10% w/w based on polymer and drug). The polymer solutions were then lyophilized (Christ Alpha I-5, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at -25°C for 24 h (primary drying) and 15°C for 12 h (secondary drying) to obtain the porous PLGA sponges.

The resulting PLGA solutions, suspensions and sponges were sealed in 10 ml glass bottles and stored in desiccators at 4°C, 25°C, 40°C and 60°C.

2.3.3. Determination of the water content

The water content of PLGA powder, leuprolide acetate, solvents, polymer solutions and oils was determined by Karl Fischer titration (Titrator TR151, Schott-Geräte GmbH, Hofheim, Germany). 0.01 g deionised water, 0.8 g PLGA powder, 1 g leuprolide solution in NMP, 10 g solvents or oils, or 1-2 g polymer solutions were placed in a titration vessel containing 40 ml Karl Fischer reagent A (Merck KGaA, Darmstadt, Germany) and titrated with Karl Fischer reagent B. The water content of the samples was calculated based on the consumption of Karl Fischer reagent B by the samples and the deionised water (n = 3).

2.3.4. Thermal analysis

The glass transition temperature (T_g) of PLGA after incubation in oils (MCT, sesame oil, soybean oil) or aqueous solutions (deionized water, saturated sodium chloride solution) was determined by differential scanning calorimetry (STAR® software, DSC 821, Mettler Toledo AG, Gießen, Germany) (n = 2). The temperature calibration was done with the melting transition of indium. The samples were prepared by mixing PLGA powder and oils or aqueous solutions at a mass ratio of 50/50. 7-10 mg of the mixture was sealed in aluminum pans. The heating rate was 10°C/min from 20°C to 70°C, the cooling rate was 30°C/min. The T_g of PLGA was determined in the second run in order to eliminate the thermal history of the polymer (Hausberger and DeLuca, 1995).

2.3.5. Degradation studies of PLGA followed by gel permeation chromatography

PLGA, PLGA solutions, dried PLGA powders (PLGA was separated from the oily suspension through washing with hexane, and from the aqueous suspensions by filtration and vacuum-drying) and sponges were dissolved in chloroform (2 mg/ml). Leuprolide acetate (insoluble in chloroform)-containing PLGA/chloroform solutions were filtered through a 0.2 µm membrane filter (Schleicher & Schuell MicroScience GmbH, Dassel, Germany) and then injected into a HPLC apparatus (SCL-10A, Shimadzu, Tokyo, Japan). The conditions were as follows: column, PLgel 5 µm MIXED-D, 7.5 × 300 mm (Polymer Laboratories Ltd, Church Stretton, Shropshire, UK); injection volume, 50 µl; mobile phase, chloroform containing 0.1% v/v triethlylamine; flow rate, 1 ml/min; column temperature, 40°C; detector, refractive

index. The weight average molecular weight (Mw) and polydispersity index (PI) were calculated by Cirrus^{TM} GPC software (Polymer Laboratories Ltd, Church Stretton, Shropshire, UK) using 9 polystyrene standards with a peak average molecular weight from 580 to 299,400 (Polymer Laboratories GmbH, Darmstadt, Germany). The relative molecular weight (relative Mw) was expressed as percentage of the Mw of the sample to the initial Mw of the polymer.

2.3.6. RP-HPLC assay for stability studies of leuprolide acetate

The stability of leuprolide acetate was determined by RP-HPLC. Leuprolide acetate-containing polymer solutions (25 mg) and sponges (5 mg) were dissolved in 1 ml chloroform, vortexed with 8 ml pH 4.0 acetate buffer (0.186 M) for 1 min and shaken overnight. After centrifugation (Biofuge 22R, Heraeus Sepatech GmbH, Osterode, Germany) at 3000 rpm for 10 min, 50 µl supernatant was injected into a HPLC apparatus (SCL-10A vp, Shimadzu, Tokyo, Japan). The conditions for analysis were as follows: column, C_{18}, 4 × 150 mm (Eurospher-100, Knauer GmbH, Berlin, Germany); mobile phase, acetonitrile-pH 7.0 phosphate buffer (0.33 M) (30:70 v/v); flow rate, 1.2 ml/min; column temperature, 30°C; wavelength, 279 nm. The aggregation and degradation of leuprolide acetate were calculated from the percentage of peak areas.