Sustained release injectables formed *in-situ* for veterinary use

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Abbreviations

Å	angstrom
AI	active ingredient
AIDS	acquired immunodeficiency syndrome
AUC	area under the plasma concentration-time curve
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
CH	Switzerland
cm	centimeter
C_{max}	maximal concentration
d	day
D	Germany
Da	dalton
DMSO	dimethyl sulfoxide
DNA	desoxy-ribonucleic acid
HPLC	high pressure liquid chromatography
ICH	International Conference on Harmonization
i.m.	intramuscular
i.v.	intravenous
EDTA	ethylene diamine tetraacetic acid
EMEA	European Agency for the Evaluation of Medicinal Products
F	France
FDA	Food and Drug Administration
FSH	follicle stimulating hormone
FT	Fourier transform
f_1	difference factor
f_2	similarity factor
g	gram
G	gauge
GPC	gel permeation chromatography
GnRH	gonadotropin releasing hormone
h	hour
hGH	human growth hormone
IR	infrared
k	reaction rate

kg	kilogram
kJ/mol	kilojoule per mole
log P	partition coefficient
max	maximum
mg	milligram
min	minute
ml	milliliter
µl	microliter
mm	millimeter
µm	micrometer
mol	mole
mPa*s	millipascal second
MS	mass spectroscopy
mW	milliwatt
MW	molecular weight
N	newton
NIH	National Institutes of Health
NMP	N-methyl-2-pyrrolidone
NIR	near infrared
ng	nanogram
nm	nanometer
O/W	oil-in-water
Pa*s	pascal second
PEG	polyethylene glycol
PLA	poly-lactic acid
PLG	poly-glycolic acid
PLGA	poly-(lactic-co-glycolic) acid
psi	pounds per square inch
PVA	polyvinyl alcohol
PVP	polyvinyl pyrrolidone
rpm	revolutions per minute
s	second
s.c.	subcutaneous
S.D.	standard deviation
sh	shoulder
t	time
THF	tetrahydrofuran

TM	trademark
T_{max}	time to reach maximum plasma concentration
TNF	tumor necrosing factor
USP	The United States Pharmacopoeia
UV	ultraviolet light
V	volt
v/v	by volume
w/v	weight by volume
w/w	by weight
℃	degree Celsius
%	percent

Objectives

The aim of this work was to explore sustained release injectables formed *in-situ* by precipitation of polymer and to develop a parenteral formulation for dogs with an extended drug release over several months. In order to investigate this technology for lipophilic drugs, the active ingredient selected for this exploration was the highly potent, lipophilic anti-infective milbemycine derivative with the internal name-code NOA449851. The new formulation is expected to be attractive to patients and veterinarians and competitive on the market. It therefore must show advantages over existing products. These include predictable and controlled drug release over a longer time period, with preferably no or only a limited burst effect, simple and cost effective manufacturing method, good stability of the formulation during storage and excellent tolerability for the patient.

Summary

Repetitive oral administration of tablets to companion animals is particularly challenging and there is a continuing need for alternative options such as long acting injections or implants. Therefore, properties of sustained release injectables formed *in-situ* for use in dogs were investigated. These formulations comprise a biocompatible solvent in which the biodegradable PLA/PLGA polymers and the lipophilic anti-infective NOA449851, derivative of milbemycin against the parasite *Dirofilaria immitis* are dissolved. These formulations coagulate into solid implants on contact with aqueous fluids after i.m. or s.c. injection, thereby releasing the incorporated drug slowly over a period of weeks to months. This technology has several attractive features such as simplicity of concept, ease of manufacturing as well as use of FDA approved polymers.

Dissolution tests were performed to investigate *in-vitro* drug release characteristics from injectable formulations varying in polymer type, polymer concentration, active ingredient concentration and solvent composition. At high drug loads, release properties were independent of polymer type. However, in case of very low drug loads, drug release was controlled by polymer properties. Major releasing mechanism was found to be drug diffusion and therefore was influenced by drug concentration. Significant reduction of initial burst was observed when polymer concentration was increased. Also the solvent composition influenced *in-vitro* drug release. Especially a significant reduction of the initial burst was observed when a fraction of the main solvent triacetin was substituted with hydrophilic co-solvents such as ethanol absolute or anhydrous glycerol, while a lipophilic co-solvent such as Miglyol 812 did increase the initial drug release. Solvent composition, depending on its affinity to the dissolution medium, influences the rate of fluid-convection, the hardening process of the polymers, the internal structure of the implant and therefore its drug release rate.

Raman and IR spectroscopy revealed that the active ingredient was incorporated in the amorphous conformation in all investigated batches. No evidence of any interaction between the active ingredient and the polymeric matrix could be detected.

Tolerability and pharmacokinetic properties of six sustained release injectables formed *in-situ*, varying in polymer concentration and solvent composition were explored after subcutaneous administration to Beagle dogs. The high viscosity of the formulations and consequently the poor syringeability turned out to be a critical issue. Viscosity of the

formulations was decreased by reducing the polymer concentration and by varying the composition of the solvent mixture. All investigated formulations were very good tolerated by the animals. In agreement with *in-vitro* investigations, reduction of polymer concentration gave rise to increased initial drug release. Presence of hydrophilic co-solvents reduced maximum drug concentration in dog plasma profiles. The active ingredient NOA449851 was detectable in blood of experimental animals over 450 days after subcutaneous injection of sustained release formulations. However, very high inter-animal variations were found for some formulations and important differences in AUC values were calculated, despite the same amount of drug injected to each dog. These differences could be explained by possible encapsulation of the subcutaneous implant with connective tissue.

The degree of correlation between the *in-vitro* dissolution parameters and the *in-vivo* pharmacokinetic data was investigated. C_{max} was positively correlated to cumulative *in-vitro* drug release at T_{max}, however not in a significant manner. In general, for this type of dosage form and drug, no satisfactory IVIVC are observed. The model used for *in-vitro* drug release testing neglect probably some crucial aspects of physiological conditions governing *in-vivo* release and cannot replace biological systems.

Stability studies were performed for three sustained release injectables formed *in-situ* during six months storage at the four selected temperatures 5°C, 25°C, 30°C and 40°C. The formulations were based on PLA polymers, active ingredient NOA449851, solvent triacetin and in case of one formulation, co-solvent ethanol absolute. An HPLC-method was utilized for determination of the active ingredient content. No differences between the three formulations were observed. The content of active ingredient slightly decreased with time and temperature. Molecular weights of PLA polymers were determined with GPC. Decrease in molecular weight was significantly increased with storage temperature and time. These results are in agreement with the findings of Wang et al. [Wang et al., 2003]. No significant influence of co-solvent ethanol absolute on the PLA stability could be measured. However, presence of active ingredient seemed to decrease hydrolysis process of PLA polymer, probably by competitively attracting water molecules responsible for polymer degradation. NIR data analysis of solvent triacetin showed spectral changes for wavelengths at 1900 nm. These spectral changes were consistent in every analyzed spectra set as solvent triacetin was in excess in all investigated samples. Influence of solvent effect could not be removed by study design, as no specific wavelength could be attributed to PLA polymers.

Surprisingly, *in-vitro* drug releases from a formulation tested directly after manufacturing and after six months storage at 40°C were found to be similar, despite the important reduction of the molecular weight of the PLA polymers. This confirms a drug release mechanism mainly controlled by drug diffusion through the matrix and not erosion controlled.

Microspheres and sustained release injectables formed *in-situ* are both technologies intended for parenteral application, planned to achieve a long lasting drug release. In both technologies, the sustained effect is caused by biodegradable PLA/PLGA polymer matrix in which the active ingredient is embedded. In order to investigate the influence of the preparation method of the polymer matrix on the release of the drug substance, microparticles batches were prepared for comparison with regards to *in-vitro* release properties. For all tested microsphere batches, drug release was independent on type of biodegradable polymer. A bigger fraction of active ingredient was released from the microparticles at high drug loads. In every investigated case, drug release from sustained release injectables formed *in-situ* was faster and to a much larger extent than from related microparticles. As possible explanation of the slower release from microparticles may be the denser packing of the polymer matrix compared to the *in-situ* formed implants. The microspheres polymer matrix is solidified before injection by applying a much more efficient solvent extraction procedure then the implants which only solidifies slowly at the site of injection. For that reason, diffusion controlled drug release is slower from the more densely packed microsphere matrix.

Sustained release injectables formed *in-situ* showed, under *in-vitro* as well as *in-vivo* conditions, a prolonged active ingredient release, confirming that this drug delivery technology is a suitable approach to achieve a controlled long term release of the lipophilic anti infective NOA449851. This technology fulfills, for this particular compound, some basic requirements such as a good tolerability, controlled release of the active ingredient over a long period of time as well as an acceptable stability of formulation during storage for several months at low temperature conditions. Release properties of the active ingredient could be modified by changing composition of the formulation and possible detrimental burst effects could be suppressed by careful selection of polymer concentration and solvent mixture. Especially, the latter finding, the suppression of a burst effect can be considered as a significant improvement of the *in-situ* implant technology. It is to be expected that in the future, development of new implantable systems will, increasingly, help reducing cost for drug therapy, potentate medical treatments and, simultaneously enhance patient compliance.

Chapter 1

General Introduction

Today's veterinary needs and expectations reflect more than ever requirements typical for current human medication. Therefore, veterinary pharmaceutical companies are investigating ways to maximize effectiveness and convenience for the administration of compounds to animals. To meet future market expectations, requirements for formulation development continue to increase: the new product should be innovative and of superior efficacy. Major driving forces for the development of innovative veterinary controlled release products include the reduction of frequency of administration, duration of medical treatment and stress for the animals. As a consequence, an increased ease of use by the veterinarians and the pet's owner as well as a decreased treatment costs should be typical for these products. These factors have stimulated the development of extended or modified releasing drug delivery systems for use in both companion and farm animals [Bowersock, 1999; Matschke et al., 2002, Medlicott et al., 2004; Sun et al., 2004; Ahmed et al., 2002; Rathbone et al., 2002].

While oral drug delivery continues to be the primary route of administration, the parenteral route offers an interesting alternative when oral administration is difficult or useless. The development of new injectable drug delivery systems has received extensive attention over the past few decades. This interest has been fostered by the potential advantages these technologies may provide including ease of application, decrease of overall drug dose associated with possible reduction of local or systemic side effects, as well as prolonged delivery periods at desired releasing rates. The minimization of dosing frequency enhances patient compliance and comfort. Injectable drug delivery systems capable of releasing an active ingredient in a controlled manner for a desired period has therefore a high priority. A zero order release kinetic is often the preferred delivery profile which assures a constant blood level of the drug for a given period. Additionally, biodegradable systems allowing the administration without the need for a subsequent medical procedure to remove the device contribute to higher patient compliance. However, these innovative therapies are developed at the expense of increased complexity, leading often to issues such as high development

and production costs [Chien et al., 1981; Yewey et al., 1997; Arnold, 1988; Rathbone et al., 2002]. Therefore, such developments are only pursued when the benefits outweigh the increase of development investments and risks. Controlled release parenteral dosage forms may be difficult to develop because of the prerequisite to understand and investigate innovative formulation manufacturing and packaging, product stability, injectability, injection site absorption, tissue drug residues, tissues irritation, extended pharmacokinetic profiles as well as efficacy over long period of time [Medlicott et al., 2004; Packhaeuser et al., 2004; Ahmed et al., 2002; Arnold, 1988; Chien, 1981; Dash et al., 1998; Sun et al., 2004].

Delivery systems such as emulsions, liposomes, microspheres and implants are successful in certain parenteral applications as modified release formulations [Rothen-Weinhold et al., 2000; Winzenburg et al., 2004; Packhaeuser et al., 2004]. Since drug release from emulsions or liposomes lasts for a limited period of only a few weeks, injectables *in-situ* solidifying drug depots are under development as alternative systems with more extended drug delivery periods. These innovative formulations are made of biodegradable polymers which can be dissolved in suitable solvents and injected into the body as a liquid. Upon administration and dilution with tissue water, the polymers solidify to form a depot. They can be classified in four groups based on the mechanism of solidification *in-vivo*: the thermoplastic pastes, the *in-situ* cross-linked systems, the *in-situ* solidifying organogels and the *in-situ* solidifying system by precipitation [Hatefi et al., 2002; Sinha et al., 1998].

1. Sustained release injectables formed *in-situ* by precipitation

Parenteral depot system can minimize side effects by achieving infusion-like plasma level time profiles, especially for drugs with narrow therapeutic indices. An overall dose reduction, resulting from the constant blood level as well as the enhancement of patient compliance by reducing the frequency of application are further potential benefits.

The sustained release injectable formed *in-situ* by precipitation is a drug delivery technology which combines a biodegradable polymer with a biocompatible solvent, resulting in a solution that can be injected using standard syringes and needles. For controlled release applications, an active ingredient can be either dissolved or suspended in the injectable formulation. When the system comes in contact with physiological fluids, the water-insoluble polymers precipitate as the solvent diffuses into the surrounding tissues. As a result, a biodegradable polymeric implant matrix is formed and encapsulates the dissolved or

suspended drug. During precipitation of the polymer, the incorporated drug is entrapped within the solidified matrix and is then slowly released [Yewey et al., 1997].

Since this technology has been patented [Dunn et al., 1990], numerous *in-vitro* release experiments and *in-vivo* trials were performed to understand the drug release mechanisms of the sustained release injectable formed *in-situ* and to improve the prediction of the drug release kinetics. A summary of the most relevant publications investigating this technology is listed in Tables 1.1 and 1.2. The different polymer types, solvents and active ingredients used as well as their concentration in the formulations are mentioned. In Table 1.1, the active ingredients used are all water soluble substances whereby the selected drug of the experiment listed in Table 1.2 were only slightly water soluble, very slightly water soluble or water insoluble compounds, according to the usual classification of pharmacopoeias.

Table 1.1: List of publications on the sustained release injectables formed *in-situ* drug delivery technology for water soluble active ingredients.

Reference	Polymer composition	Solvent(s)	Active ingredient
Radomsky et al., 1993	PLA; 50/50 PLGA; polylactidecaprolactone (40-55 %)	NMP	ganirelix acetate (10-15 %)
Shah et al., 1993	50/50 PLGA (1-20 %)	triacetin; triethyl citrate	myoglobin (0.40-0.45 %)
Lambert et al., 1995	PLA; PGA; 85/15 PLGA; 50/50 PLGA (10-40 %)	NMP; DMSO	BSA (1 %)
Eliaz et al., 1997	75/25 PLGA (10-20 %)	glycofurol	BSA; sp55-TNF-receptor (3-10 %)
Yewey et al., 1997	PLA; PLG (5-45 %)	NMP; DMSO	pellucida antigen protein (0.2 %); FSH (1 %); GnRH antagonist (1 %); BSA (0.01-20 %); lysozyme (5 %); trypsin (5 %); horse radish peroxidase (5 %); bovine insuline (1 %); myoglobin (10 %)
McHugh et al., 1999	PLGA (50 %)	NMP; triacetin; ethyl benzoate	lysozyme (10 %)
Brodbeck et al., 1999 (1)	50/50 PLGA (50 %)	ethyl benzoate; NMP; triacetin	lysozyme (10 %)
Jain et al., 1999 (2)	PLA (20 %)	NMP	isoniazide (10 %)
Graham et al., 1999	PLGA (32.3-50 %)	NMP; triacetin	lysozyme (10 %)

Table 1.1 (Continued):

Reference	Polymer composition	Solvent(s)	Active ingredient
Brodbeck et al., 1999 (2)	50/50 PLGA	NMP; triacetin; ethyl benzoate; benzyl benzoate	hGH (5-10 %); lysozyme (5-10 %)
Ravivarapu et al., 2000 (1)	75/25 PLGA (40-50 %)	NMP	leuprolide acetate (3-6 %)
Ravivarapu et al., 2000 (2)	50/50 PLGA; 75/25 PLGA (28-45 %)	NMP	leuprolide acetate (3 %)
Ravivarapu et al., 2000 (3)	75/25 PLGA (45 %)	NMP	leuprolide acetate (3-6 %)
Eliaz et al., 2000 (1)	75/25 PLGA (10-20 %)	glycofurol	BSA; sp55-TNF-receptor (3-10 %)
Eliaz et al., 2000 (2)	50/50 PLGA; 75/25 PLGA (10-20 %)	glycofurol	BSA; sp55-TNF-receptor (1.5-30 %)
DesNoyer et al., 2001	PLA; polycaprolactone (13.5-45 %)	ethyl benzoate	lysozyme (10 %)
Kranz et al., 2001	PLA; PLGA (10-40 %)	NMP, DMSO, 2-pyrrolidone	bupivacaine hydrochloride; buserelin acetate (2 mg/ml)
Eliaz et al., 2002	75/25 PLGA (10 %)	glycofurol	plasmid DNA (500 µg/ml)
DesNoyer et al., 2003	PLA; poly(ethylene oxide); poly(propylene oxide) (10.8-18 %)	NMP	lysozyme (10 %)
Pechenov et al., 2004	50/50 PLGA (50 %)	acetonitrile	crystalline amylase (10-300 mg/ml)

Table 1.2: List of publications on the sustained release injectables formed *in-situ* drug delivery technology using slightly water soluble (*), very slightly water soluble(**) or water insoluble(***) compounds.

Reference	Polymer composition	Solvent(s)	Active ingredient
Shah et al., 1993	50/50 PLGA (1-20 %)	triacetin; triethyl citrate	hydrochlorothiazide(***) (0.4 %); theophyllin(*) (1 %); cytochrome C(*) (0.4 %)
Shively et al., 1995	PLA; 85/15 PLGA; 75/25 PLGA; 65/35 PLGA; 50/50 PLGA (35-57 %)	NMP; DMSO; ethyl acetate	naltrexone base(***) (5 %)
Chandrashekar et al., 1996	50/50 PLGA; 70/30 PLGA; 80/20 PLGA; 90/10 PLGA (5-20 %)	triacetin	diclofenac sodium(*) (5 %)
Dunn et al., 1996	85/15 PLGA; polylactide-co-caprolactone (70-50 %)	DMSO	cisplatine(**) (8 %)
Singh et al., 1997 (1)	50/50 PLGA; 70/30 PLGA; 80/20 PLGA; 90/10 PLGA (5-25 %)	triacetin	plumbagin(*) (5 %)
Singh et al., 1997 (2)	50/50 PLGA (5-25 %)	triacetin	methotrexate(***) (5 %)
Dernell et al., 1998	PLGA; polylactide-co-caprolactone	DMSO; NMP	cisplatine(**) (8 %)
Jain et al., 2000 (1)	PLGA (12.5 %)	triacetin	cytochrome C(*) (0.08 %)
Matschke, 2002	PLA; 85/15 PLGA; 75/25 PLGA (2.5-15 %)	NMP; triacetin	NOA440094(*) (7-37%)

The drug release mechanism from this polymeric system is complex. Just after injection the solvent diffuses into the medium as the polymer matrix begins to solidify. The release of active ingredient is presumably due to diffusion of the drug out of the polymer matrix as well as erosion of the solid implant surface of the matrix, depending on the properties of polymer and drug substance [Lambert et al., 1995; Yewey et al., 1997; Eliaz et al., 2000 (1); Brodbeck et al., 1999 (2)].

The release rate from sustained release injectables formed *in-situ* is affected by the properties of the drug substance and a large number of parameters of the formulation. These parameters include type, concentration and molecular weight of the polymer used to form the matrix of the implant, type and amount of the solvent as well as the presence of additives like surfactants or co-solvents. Amount and physicochemical characteristics such as hydrophilic or lipophilic properties, water miscibility or molecular weight of the active ingredient also have an impact on the characteristics of the drug delivery device [Yewey et al., 1997; Graham et al., 1999; Eliaz et al., 2000 (1), (2); Matschke, 2002].

1.1. The polymers

Candidates of biodegradable polymers for the sustained release injectable formed *in-situ* technology include homopolymers of poly(DL-lactide) (PLA) and co-polymers of poly(DL-lactide-co-glycolide) (PLGA) [Yewey et al., 1997; Jain, 2000; Shively et al., 1995; Singh et al., 1997 (1); Lambert et al., 1995]. PLA and PLGA were two of the first polymers used and established in parenteral controlled-release technology. They have been utilized in biomedical applications such as sutures and orthopedic devices prior to their use in drug delivery [Middleton et al., 2000]. Safety, excellent tolerability and biocompatibility of these biodegradable polymers as well as their ease of availability made them ideal candidates for parenteral controlled release excipients. PLA/PLGA polymers are already FDA approved for parenteral application for specific products and widely used as polymeric matrix for implants or microspheres [Heller, 1993; Sinha et al., 1998; Chien et al., 1981; Dash et al., 1998; Shah et al., 1992; Vert et al., 1998]. Some marketed sustained release products based on PLA/PLGA polymers and formulated as solid implant, injectable implant or microspheres are listed in Table 1.3. All active ingredients of the sustained release products mentioned in Table 1.3 are water soluble compounds.

Table 1.3: List of various commercial sustained release products based on PLA/PLGA polymers.

Product	Distributor	Form	Active ingredient
Atridox	Atrix Laboratories	injectable implant	doxycycline hyclate
Decapeptyl® Depot	Ferring	microspheres	triptorelin acetate
Decapeptyl® Gyn	Ferring	microspheres	triptorelin acetate
Eligard™	Atrix Laboratories	injectable implant	leuprolide acetate
Enantone® Gyn	Takeda Pharma	microspheres	leuprorelin acetate
Lucrin Depot®	Abbott	microspheres	leuprorelin acetate
Nutropin Depot®	Genentech	microspheres	human growth hormone
Parlodel® LAR	Sandoz	microspheres	bromocriptin mesilate
Profact ® Depot	Aventis Pharma	implant	buserelin acetate
Risperdal Consta®	Janssen-Cilag	microspheres	risperidon
Sandostatin® LAR Depot	Novartis Pharma	microspheres	octreotide acetate
Trelstar® Depot	Debiopharm	microspheres	triptorelin pamoate
Trenantone®	Takeda Pharma	microspheres	leuprorelin acetate
Uropeptyl® Depot	Uropharm	microspheres	triptorelin acetate
Zoladex®	AstraZeneca	implant	goserelin acetate
Zoladex® GYN	AstraZeneca	implant	goserelin acetate

For the synthesis of both polylactide and polyglycolide, an intermediate cyclic diester is prepared prior to polymerization from the lactic acid and glycolic acid, respectively. These cyclic dimers are called lactide and glycolide. Poly(lactide) exists in two stereo-isomers, charcaterised by a D or L for dexorotary or levorotary or by DL for the racemic mixture. Ring-opening polymerization of the lactide and/or glycolide is the most frequently used method for synthesis of PLA/PLGA with a molecular weight greater than 10'000 Da. By varying the synthesis conditions, it is possible to obtain PLA and PLGA polymers of various lactide/glycolide ratios and molecular weights [Middleton et al., 2000; Wang et al., 2000; Hollinger et al., 1986]. The various steps of the synthesis of poly(lactide) and poly(lactide-co-glycolide) polymers are illustrated in Figure 1.1.

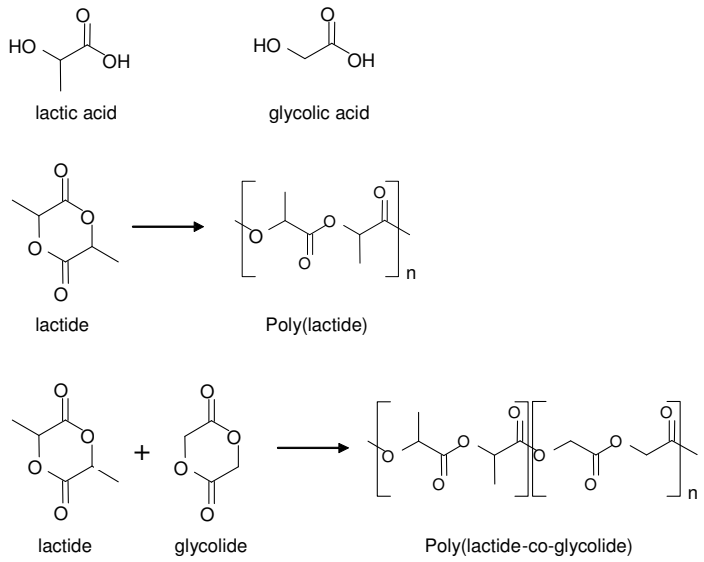


Figure 1.1: Synthesis of poly(lactide) and poly(lactide-co-glycolide) from the lactic acid and the glycolic acid.

The principal mode of degradation for PLA and PLGA polymers *in-vitro* as well as *in-vivo* is hydrolysis [Middleton et al., 2000; Yewey et al., 1997; Gunatillake et al., 2003; Jain, 2000; Hollinger et al., 1986]. The degradation proceeds first by diffusion of water into the polymeric matrix, followed by random hydrolysis and fragmentation of the material. The products of hydrolysis are then either metabolized or excreted. Poly(lactide) undergoes hydrolytic de-esterification into lactic acid which is transformed to pyruvate by lactate dehydrogenase. Pyruvate is decarboxylated into acetyl co-enzyme A which is incorporated into the tricarboxylic acid cycle to form carbon dioxide and water as schematized in the Figure 1.2. Poly(glycolide) is degraded by hydrolysis to glycolic acid. Glycolic acid may be excreted directly via urine or may react to form glycine. In the body, glycine can be used to synthesize serine and subsequently transformed into pyruvic acid where it enters the tricarboxylic acid cycle as depicted in the Figure 1.2 [Middleton et al., 2000; Athanasiou et al., 1998; Hollinger et al., 1986].

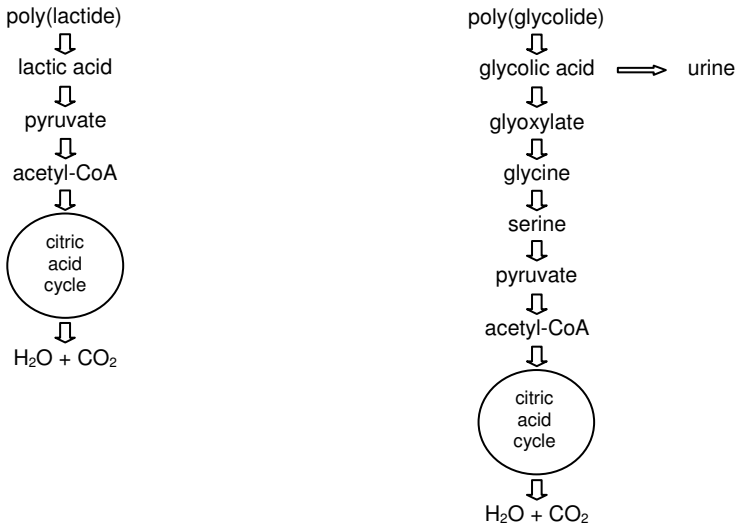


Figure 1.2: Biodegradation pathways of poly(lactide) and poly(glycolide).

The rate of hydrolysis of PLA/PLGA polymers is affected by the size and hydrophilicity of the polymer bulk, by the crystallinity and molecular weight of the polymers and by the pH and temperature of the environment. In general, degradation time is shorter for lower molecular weight polymers, more hydrophilic or amorphous polymers and therefore for co-polymers with higher numbers of glycolic units. Increase of lactic acid content in the matrix decreases the degradation rate by reducing water uptake and making the ester bond less accessible to water, due to hindrance by the bulky methyl groups. Although there is no linear relationship between co-polymer composition and degradation rate, the degradation time can theoretically be varied from one month up to several years [Göpferich, 1996; Middleton et al.,

2000; Wang et al., 1997; Athanasiou et al., 1998]. More specifically, the time for complete resorption of the polymers was reported to be between 12 and 16 months for the amorphous poly-DL-lactic acid, between 5 and 6 months for 85/15 PLGA polymer and between 4 and 5 months for 75/25 PLGA polymer [Middleton et al., 2000; Gunatillake et al., 2003].

As already mentioned, the degradation products of the PLA/PLGA polymers are acidic compounds. Due to their inability to diffuse away from the bulky device, they lead to a local acidic environment in the interior of the polymeric implant [Wang et al., 1997; Middleton et al., 2000]. This lowering of the environmental pH can be detrimental for stability of active ingredients with a pH driven degradation process.

Because these polymers are hydrolytically unstable, the presence of moisture can lead to their degradation during processing or storage. Passive hydrolysis is by far the most important degradation mechanism for synthetic polymers, since for most of them no specific enzymes exist [Göpferich, 1996]. In theory, the reduction of sensitivity for hydrolysis of the polymer bonds can be achieved by simply eliminating moisture. As the materials are hygroscopic by nature, keeping the polymers free of water is difficult. Polymer drying may be accomplished by vacuum drying or drying in a resorption circulating air dryer. The drying process should occur at room temperature to avoid thermal degradation, despite the consequently higher percentage of residual moisture and lower efficacy of the procedure [Middleton et al., 2000].

Numerous investigations on the biocompatibility of the polymers *in-vivo* have been performed, with mostly very favorable results. The PLA/PLGA do not invoke any inflammatory or toxic response, and the degradation products are metabolized in the body leaving no trace. Despite the good biocompatibility of the implanted PLA/PLGA polymeric system, the formation of a fibrous capsule surrounding the system is often described. This tissue response is not inert and is expected to modulate the *in-vivo* release of the implanted systems [Medlicott et al., 2004; Royals et al., 1999; Dernell et al., 1998; Dunn et al., 1996; Athanasiou et al., 1998].

Synthetic polymers such as PLA/PLGA offer in general significant advantages over materials from natural origin. They can be tailored to give a wider range of characteristics and have more predictable lot-to-lot uniformity than natural polymers. A more reliable source of raw materials is obtained with synthetic polymers, with little concerns regarding immunogenicity [Middleton et al., 2000]. Despite these considerations, natural excipients such as albumin, collagen, gelatin and alginate have been suggested as suitable polymers for the formulation

matrix of the injectable depot formulations [Dunn et al., 1990]. These substances are more critical with regard to possible contamination with viruses (AIDS, hepatitis) and prions (BSE). Further alternatives to the PLA and PLGA polymers for the matrix of the sustained release injectables formed *in-situ* consist in excipients such as chitosan, poly(anhydrides), poly(orthoester), poly(amides) and the poly(ester) polycaprolactone [Heller, 1993; Dunn et al., 1990; Gunatillake et al., 2003; Uhrich et al., 1999, Davis, 2000]. Although all these options may be usable and have potential applicability for future drug delivery systems, the most serious candidates for parenteral application remain the PLA/PLGA polymer which have been selected to form the implant matrix in the present study [Burgess et al., 2004; Middleton et al., 2000].

1.2. The solvent

The injectable *in-situ* forming drug delivery system requires pharmaceutically acceptable solvents that are able to form concentrated polymer solutions in order to achieve high drug entrapment and suitable drug release profiles [Shively et al., 1995]. The vehicle not only acts as a solvent but also serves as a plasticizer for the polymers. The gel matrix is not just a precipitated polymer but a polymer matrix with a rubbery state depending on the concentration of the vehicles entrapped into the polymer on injection [Singh et al., 1997 (1)]. The choice of an appropriate solvent is also an essential factor, as this excipient plays a determinant role during the implant forming process and therefore highly influences the drug release rate [Graham et al., 1999].

The solvent for the biodegradable polymer implant should be well tolerated and biocompatible. It should cause neither pain following administration, nor tissues irritation or necrosis at the site of injection. The solvent should be water miscible to a major extent and diffuse into the body fluids and, reciprocally, allow water permeation into the polymer solution and cause it to coagulate or solidify [Dunn et al., 1990; Kranz et al., 2001].

Among the numerous pre-clinical trials reported in the literature (see Tables 1.1 and 1.2), mostly tested on mice, rats or dogs, the solvents used were often NMP (N-methyl-2-pyrrolidone) [Radomsky et al., 1993; Shively et al., 1995; Yewey et al., 1997; Brodbeck et al., 1999 (2); Jain et al., 1999; Ravivarapu et al., 2000 (1), (2), (3); Kranz et al., 2001] and DMSO (dimethylsulfoxide) [Shively et al., 1995; Dunn et al., 1996; Yewey et al., 1997; Dernell et al., 1998; Kranz et al., 2001]. Both solvents NMP and DMSO allow the formation of highly concentrated polymer solutions up to 70 % in weight [Dunn et al., 1996]. Biocompatibility and

tolerability of placebo biodegradable *in-situ* forming injectable systems with NMP and DMSO as solvents were studied in rhesus monkeys [Royals et al., 1999]. A mild fibroplasias was observed, resulting in bands of connective tissue with inflammatory cells circumscribing the polymer implants. This effect can be related to the toxicity of the solvents. There are extensive toxicity data for oral, intraperitoneal and intravenous administration of these solvents [Fiedler Lexikon, 2002; Martindale, 1993; Bertholom et al., 2000; Wells et al., 1988]. In spite of these positive findings in monkeys, in general parenteral injection of pure solvents (i.e. not diluted with isotonic aqueous media) is not very well accepted and a continuous debate on the use of these vehicles in animals and men is ongoing. Further, the use of the organic solvent acetonitrile allows the formation of a concentrated PLGA solution up to 50 % [Pechenov et al., 2004], but considering its toxicity, parenteral application is excluded [Fiedler Lexikon, 2002; Martindale, 1993].

As listed in Tables 1.1 and 1.2, further solvents which have been used in this approach for *in-vitro* experiments or for *in-vivo* trials include triacetin [Shah et al., 1993; Chandrashekar et al., 1996; Singh et al., 1997 (1), (2); Brodbeck et al., 1999 (1), (2); Graham et al., 1999; Jain et al., 2000 (1)], triethyl citrate [Shah et al., 1993], ethyl acetate [Shively et al., 1995], glycofurol [Eliaz et al., 1997; Eliaz et al., 2000 (1), (2); Eliaz et al., 2002], ethyl benzoate [Brodbeck et al., 1999 (1), (2); DesNoyer et al., 2001], benzyl benzoate [Brodbeck et al., 1999 (2)] and 2-pyrrolidone [Kranz et al., 2001]. In comparison to the formulations based on NMP, DMSO and acetonitrile, the injectables with these alternative solvents contain a reduced concentration of polymers as the solubility of the PLA/PLGA in these solvents is relatively low. However, some of these solvents may be less toxic and better tolerated [Fiedler Lexikon, 2002; The Merck Index, 1996; Bertholom et al., 2000; Bleiberg et al., 1993].

1.3. The active ingredient

A wide range of active ingredients can be used for the sustained release injectable formed *in-situ* technology as the drug can be either suspended or dissolved in the formulation. The physical state of the active ingredient in the formulation depends on the physicochemical properties of the active ingredient and on the solvent used. For example, water soluble compounds such as leuprolide acetate or BSA were dispersed in formulations based on the solvent NMP [Ravivarapu et al., 2000 (1), (2), (3); Lambert et al., 1995], while the active ingredients bupivacain hydrochloride and buserelin acetate, both also hydrophilic, were dissolved in the same solvent NMP [Kranz et al., 2001]. The slightly water soluble anticancer drug cisplatine as well as the water soluble protein BSA were both suspended in DMSO

formulations [Dunn et al., 1996; Lambert et al., 1995]. Using the solvent triacetin, active ingredients such as the antihypertensive hydrochlorothiazide [Shah et al., 1993], the antitumor agent plumbagin [Singh et al., 1997 (1)], or the protein cytochrome C [Jain et al., 2000 (1)] were dissolved in the formulations. Contrarily, diclofenac sodium and methotrexate were dispersed in the formulations with triacetin as sole solvent. The addition of an appropriate co-solvent can solubilise an active ingredient which was previously in suspension, influencing the release mechanism and therefore the drug release rate. This was the case for the practically water insoluble drugs diclofenac sodium [Chandrashekar et al., 1996] and methotrexate [Singh et al., 1997 (2)], both actives being in suspension in the solvent triacetin, and in solution with the use of the hydrophilic co-solvent propyleneglycol.

The lower dose limit of active ingredient incorporated into the formulation depends on the activity of the drug and the length of time needed for treatment. The upper limit is defined by the technical feasibility of the viscosity for injection through a syringe needle and by the solubility of the active compound in the solvent, when a solution is preferred to a suspension. In the different trials reported in the literature (see Tables 1.1 and 1.2), the drug concentration was in general between 5 % and 10 %. However, the concentrations varied in a range from 0.01 % [Yewey et al., 1997] to 37 % [Matschke, 2002].

In any case, the active ingredient must be highly potent so that the necessary dose for the extended treatment period can be incorporated in the smallest possible injectable volume of the formulation. The substance should preferably have a broad therapeutic index and cause no side effects or tissues irritation at the injection site. Obviously, the disease indication of the drug must be in agreement with a long term treatment. The physicochemical properties of the active ingredient which influence the drug release profiles from the injectable depot are mainly the molecular weight, the structure and the hydrophilicity/lipophilicity of the substance [Shah et al., 1993; Hatefi et al., 2002, Burgess et al., 2002]. In the various studies reported in the literature, the substances selected to be incorporated into sustained released injectables formed *in-situ* included natural substances like the enzymes lysozyme [McHugh et al., 1999; Brodbeck et al., 1999 (1), (2); Graham et al., 1999; DesNoyer et al., 2001] and crystalline amylase [Pechenov et al., 2004] or model proteins such as myoglobin [Shah et al., 1993], cytochrome C [Shah et al., 1993; Jain et al., 2000 (1)], BSA [Yewey et al., 1997; Eliaz et al., 2000 (1), (2)], pellucida antigen protein [Yewey et al., 1997] or fluorescein isothiocyanate-labeled bovine serum albumin [Lambert et al., 1995]. The sustained release of a plasmid DNA was also studied [Eliaz et al., 2002]. The use of the following hormones has been widely investigated: natural hGH [Brodbeck et al., 1999 (2)], FSH [Yewey et al., 1997] and GnRH [Yewey et al., 1997] or the synthetic LHRH-analog, leuprolide acetate [Ravivarapu et

al., 2000 (1), (2), (3)]. Some hormone-antagonists have also been tested, like ganirelix acetate, a GnRH-antagonist [Radomsky et al., 1993] and the substance buserelin acetate [Kranz et al., 2001]. Cancer treatment has been explored, using the substances cisplatin [Dunn et al., 1996; Dernell et al., 1998], plumbagin [Singh et al., 1997 (1)] and methotrexate [Singh et al., 1997 (2)]. Other substance models with a large range of physicochemical properties and for various indications have also been investigated such as hydrochlorothiazide [Shah et al., 1993], theophyllin [Shah et al., 1993], naltrexone base [Shively et al., 1995], diclofenac sodium [Chandrashekar et al., 1996], sp55-TNF-receptor [Eliaz et al., 1997; Eliaz et al., 2000 (1), (2)], isoniazide [Jain et al., 1999 (2)] and bupivacain hydrochloride [Kranz et al., 2001].

Most of the studied active ingredients are water soluble and are therefore subject to fast metabolism and elimination processes (Table 1.1) Only few investigated substances, are slightly soluble in water practically insoluble in water or insoluble in water (Table 1.2) with a respective slower elimination metabolism. The use of lipophilic compounds may amplify the sustained released effect of PLA/PLGA delivery systems.

A derivative of the lipophilic anti-infective milbemycine, the NOA449851, was selected as active ingredient for the development and exploration of the sustained release injectables formed *in-situ*. It is a potent water insoluble drug which has a prophylactic protective activity against the parasite *Dirofilaria immitis* in dogs, when a blood level of 10 ng/ml is achieved (unpublished internal data of Novartis Animal Health Inc.). These characteristics of the drug substance can be ideally combined with the *in-situ* formed sustained release injectable technology.

2. General issues

In the numerous trials reported in the literature, the major problems concerning the sustained release injectables formed *in-situ* are recurrent. Until now, the release mechanism has not been entirely clarified due to its high complexity and of the numerous parameters which simultaneously influence it. These include polymer type and polymer concentration, solvent type and amount as well as concentration and physicochemical properties of the active ingredient, especially regarding solubility in water. The influence of these parameters seems not to be constant and sometimes even contradictory. In addition, the drug releasing mechanism from the matrix is a consequence of concomitant processes such as solidification kinetics of the polymers, precipitation kinetics of the active ingredient, diffusion of the active

ingredient through the polymer-matrix along a concentration gradient, dynamic of solvents and water flows, erosion of biodegradable polymers by hydrolysis, bulk water uptake, swelling of polymers as well as release through pores formed in the matrix.

The drug release rate from a polymeric matrix may also depend on interactions between active ingredient and polymer. High interactions resulting from e.g. hydrogen bonds between drug and polymer would lead to reduced release rate of the drug substance from the matrix. Investigations for detection of polymer/drug interactions can be performed using vibrational methods such as Raman and IR spectroscopy [Geze et al., 1999; Breitenbach et al., 1999]. Additionally, Raman spectroscopy allows the characterization of polymorphic forms or physical state of the drug within a matrix. These parameters affect the solubility properties and dissolution kinetics, and therefore influence the drug release rate of substances from a polymeric matrix [Bolton et al., 1984; Taylor et al., 1997; Broman et al., 2001].

An initial burst release is an undesired effect which should be controlled to avoid the loss of an important amount of active ingredient during the first days of the treatment and to prevent possible toxic effects caused by high peak levels of the drug. The latter factor is more relevant to achieve a therapy which is safe for the patient. The released drug dose should not give rise to a critical high blood levels to prevent systemic or local side effects. Since the injectable implant system is administered as a liquid, it is reasonable to assume that there is a lag time between injection and formation of the solid implant. Especially during this time period, at which no encapsulating matrix exists, the initial burst of drug may very well exceed the therapeutic plasma concentration [Dernell et al., 1998].

The high viscosity of the sustained release injectable formed *in-situ* system is an important issue of this technology, since it directly determines the syringeability. The viscosity of the final formulation should be low enough to allow application with standard syringes and needle diameters. The thinner needle diameters are, the more tolerable are the injections, leading to better acceptance by patients. The viscosity of the formulation can be adjusted in a limited range by varying in the amount of polymer and of active ingredient as well as by use of solvent type in the formulation. The toxicity problems generated by solvents such as NMP or DMSO stimulates the use of alternative solvents which are better tolerated such as triacetin, possibly in combination with co-solvents, despite a higher viscosity of the formulation.

The sustained release injectables formed *in-situ* are formulations intended for parenteral application and must therefore be sterile. As terminal sterilization commonly leads to a higher

sterility assurance level compared to aseptic manufacturing, different processes have been evaluated in the literature. Sterilization by autoclaving is not recommended due to the sensitivity of the polymers to heat and moisture. The effects of γ-irradiation on the physicochemical properties of biodegradable drug delivery systems have been controversially described in the literature [Mohr et al., 1999; Volland et al.,1994; Dunn et al., 1996; Burgess et al., 2002; Martínez-Sancho et al., 2004; Athanasiou et al., 1998; Davis, 2000]. It is now generally accepted that γ-irradiation causes a radiation dose-dependent decrease in polymer molecular mass by radiolytic chain scission. Therefore, the sterilization conditions as well as the radiation need to be carefully adjusted for the final dosage form.

3. Aim of the work

The aim of this work was to explore *in-situ* solidifying systems by precipitation of the polymers with the goal to develop a parenteral formulation for dogs for a drug with extended drug release over several months. In order to investigate this technology for lipophilic drugs, the active ingredient selected for this exploration was the highly potent, lipophilic anti-infective milbemycine derivative with the internal name-code NOA449851. The new formulation is expected to be attractive to patients and veterinarians, and competitive on the market. It therefore must show advantages over existing products. These include a predictable and controlled drug release over a longer time period, with preferably no or only a limited burst effect, a simple and cost effective manufacturing method and an excellent tolerability for the patient.

Chapter 2

Characterization of the Active Ingredient NOA449851

1. Objective and introduction

The objective of the present study was to develop a sustained release injectable formed *in-situ* based on PLA/PLGA biodegradable polymers. The active ingredient selected as marker for this purpose was a derivative of the anti-infective Milbemycin. The drug release from the novel formulation depends highly on the physico-chemical characteristics of the substance model. Therefore, the active ingredient was characterized with regard to solubility in different solvents and to conformation properties using vibrational spectroscopy and X-ray diffraction analytical method.

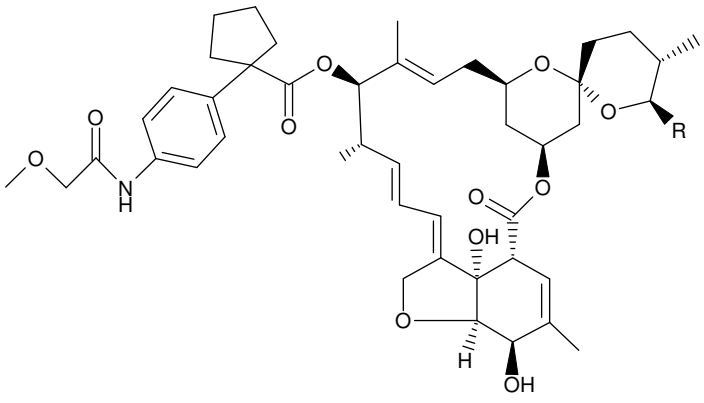
First, the solubility of the active ingredient in various organic solvents was determined. All investigated excipients can be used either as solvent or as co-solvent in sustained release injectables formed *in-situ*, since they are physiologically well tolerated and already approved for parenteral application by the regulatory authorities.

Various solid samples of the active ingredient were prepared by recrystallisation from different solvents and analyzed using IR and Raman spectroscopy. Further IR spectral analysis was performed on two liquid samples of the active ingredient. Special attention was paid to detect different morphological conformations of the active ingredient as well as its ability to interact with the solvents. In addition, the X-ray patterns of two solid samples were obtained for analysis of the cristallinity parameters of the different conformations.

2. Materials and methods

2.1. Materials

The drug substance NOA449851 was manufactured at and obtained from CarboGen Laboratories, Aarau, CH. The selected active ingredient NOA449851 has two homologues, S-1238 and S-1262, in a ratio of 80 % for S-1238 to 20 % for S-1262. The empirical formula are $C_{47}H_{63}NO_{11}$ and $C_{46}H_{61}NO_{11}$, respectively. These compounds have a molecular weight of 818.03 and 804.00, respectively. The chemical structure of the active ingredient is illustrated in Figure 2.1.



S-1238: R = C_2H_5
S-1262: R = CH_3

Figure 2.1: Chemical structure of the active ingredient NOA449851. S-1238 represents 80 % while S-1262 represents only 20 % of the active ingredient.

The solvents dichloromethane, triacetin, NMP, glycerol formal, anhydrous glycerol, propylenglycol and PEG 300 were purchased from Fluka AG, Buchs, CH. Ethanol absolute, methanol, water for chromatography and DMSO were obtained from Merck Inc., Darmstadt, D. Miglyol 812 was provided by Hänseler AG, Herisau, CH. All solvents were of reagent analytical grade.

2.2. Methods

2.2.1. HPLC-method

An HPLC-method was used for quantitative analysis of the active ingredient NOA449851 in solutions. For this purpose, a LC HP 1100 (Hewlett Packard, Basel, CH) apparatus was employed. The column (250 mm length and 4.6 mm internal diameter) was packed with Nucleosil 5 µm, C18 (Macherey-Nagel, Düren, D) and maintained at a temperature of 37°C. The elution medium consisted of a mixture of water for chromatography and methanol. The composition of the mobile phase permanently changed in relation to the time of 30 min. The flow rate was 1.3 ml/min during the whole analyze-run. A volume of 10 µl of the sample solution was injected per run with an auto-sampler. The samples were detected and analyzed with UV-light at a wavelength of 240 nm.

The tailing factor determined on the peaks of both homologues S-1238 and S-1262 was between 0.5 and 1.5. The resolution between the two peaks of the NOA449851 components was over 2.5. As reference, a solution of 0.040 % (w/v) NOA449851 in methanol was used.

2.2.2. Solubility of NOA449851 in various organic solvents

The solubility of the active ingredient NOA449851 was determined for the solvents NMP, ethanol absolute, glycerol formal, triacetin, PEG 300, Miglyol 812, propylenglycol, anhydrous glycerol and water for chromatography.

An excess of active ingredient was equilibrated in the corresponding solvent by stirring over night at room temperature (Magnetic stirrer, Ikamag, RET S8^{TM}, Ikawerke, Stauffen, D). After centrifugation (Centrifuge 5415, Eppendorf, Netheler + Hinz GmbH, 2000 Hamburg, D) and appropriate dilution, the concentration of NOA449851 in the supernatant solution was determined with the HPLC-method described in Section 2.2.1.

2.2.3. Preparation of solid samples for spectroscopic and X-ray analysis

Different solid samples of the active ingredient NOA449851 were prepared for subsequent analysis with vibrational spectroscopy and X-ray diffraction method.

Sample A represented the active ingredient as received from CarboGen: the substance was obtained by solving the active ingredient in ethyl acetate and by gently adding heptane until recristallisation. Samples B to G were obtained after solubilizing the active ingredient in various solvents and after evaporation at reduced pressure (Salvis Trockenschrank, Typ KVTS11, Reussbühl, CH). The solvents used were dichloromethane (sample B), NMP (sample C), triacetin (sample D), ethanol absolute (sample E), Miglyol 812 (sample F) and glycerol formal (sample G). An overview of the different solid samples and the applied analytical methods is listed in Table 2.1.

Table 2.1: Overview of the NOA449851 solid samples used for IR spectroscopy, Raman spectroscopy and X-ray analysis.

Sample	Solvent for recristallisation	IR	Raman	X-ray
A	ethyl acetate/heptan (CarboGen)	yes	yes	yes
B	dichloromethan	yes	yes	yes
C	NMP	yes	yes	no
D	triacetin	yes	yes	no
E	ethanol absolute	yes	yes	no
F	Miglyol 812	yes	yes	no
G	glycerol formal	yes	yes	no

2.2.4. IR spectroscopy

IR spectroscopy is widely used for determination of organic structures as well as for investigations of hydrogen bonding. Therefore, IR spectroscopy was applied to the solid samples A to G prepared in Section 2.2.3. Additionally, two saturated solutions of the active ingredient NOA449851 in DMSO and dichloromethane were analyzed. Bands were assigned according to their localization and intensity.

IR spectra of solid samples were run on a Perkin Elmer i-Series FTIR microscope coupled with a spectrum 2000 spectrometer over a wavenumber range from 4000 cm^{-1} to 700 cm^{-1} with a resolution of 4 cm^{-1}.

IR spectra of solution samples were run on a Bruker IFS 66 FTIR spectrometer over a wavenumber range from 4000 cm^{-1} to 700 cm^{-1} with a resolution of 2 cm^{-1}.

2.2.5. Raman spectroscopy

Raman spectroscopy is a useful tool for the analytical assessment of molecular structure of drugs. Complementary to the infrared spectroscopy, it is particularly appropriate for the detection of different polymorphic forms. Therefore, Raman spectroscopy was performed on the solid active ingredient samples A to G prepared in Section 2.2.3.

The FT Raman spectra were recorded with a Bruker RFS 100 FT Raman spectrometer equipped with a liquid nitrogen cooled germanium detector. The resolution was 4 cm^{-1} and 250 scans were accumulated by using a laser output of 600 mW. The spectrum was corrected for instrumental response.

2.2.6. X-ray powder diffraction

The active ingredient samples A and B were analyzed with X-ray diffraction method. The cristallinity of the active ingredient in both samples was investigated qualitatively by wide-angle X-ray diffraction with a Philips PW1710 X-ray diffractometer. X-ray diffraction spectra were recorded on a diffractometer equipped with a $Cu_{k\alpha}$ source and a diffracted beam monochromator. The diffractograms were taken from $2\theta = 0° - 50°$ with a step size of 0.02° and a time interval of 2.4 seconds.

3. Results

3.1. Solubility of NOA449851 in various organic solvents

The maximal solubility [% (w/v)] of the active ingredient NOA449851 in various organic solvents was determined with the HPLC-method described in Section 2.2.1. A relative low solubility was found for the active ingredient NOA449851 in water (< 0.001 %), anhydrous glycerol (0.05 %), propylenglycol (3.02 %), Miglyol 812 (5.60 %) and PEG 300 (19.04 %). However, higher solubility for NOA449851 was measured in triacetin (35.22 %), glycerol formal (37.14 %), in ethanol absolute (50.93 %) and in NMP (53.18 %). All results are depicted in Figure 2.2.

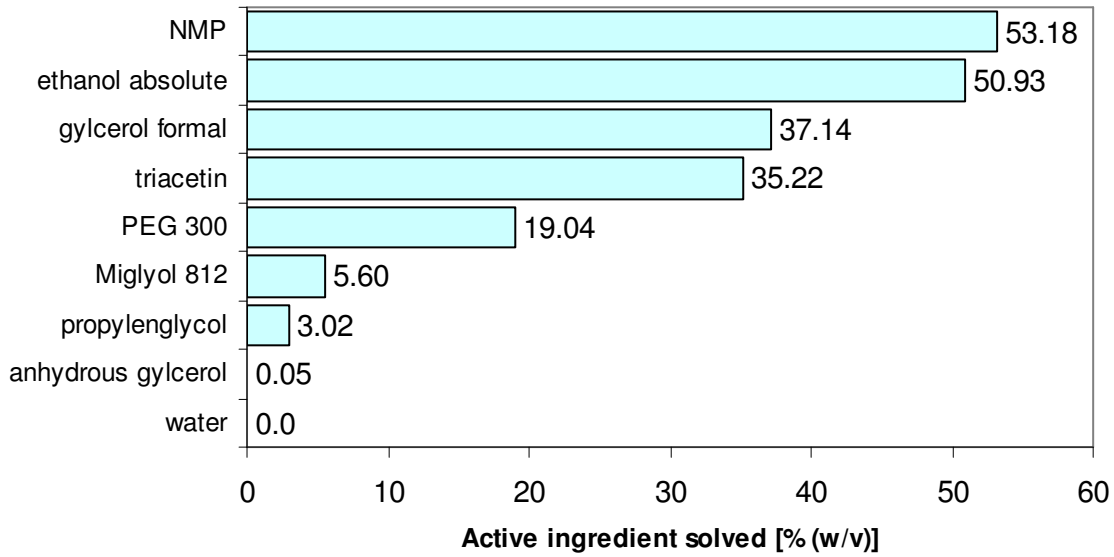


Figure 2.2: Maximal solubility [% (w/v)] of the active ingredient NOA449851 in various organic solvents.

3.2. IR spectroscopy

IR spectra were taken from the different solid samples of the active ingredient NOA449851 prepared in Section 2.2.3 as well as from two solutions in dichloromethane and DMSO, respectively. The IR spectra of the solid samples A and B and of both liquid samples are represented in Figure 2.3. The assignments of the specific IR bands are listed in Table 2.2.

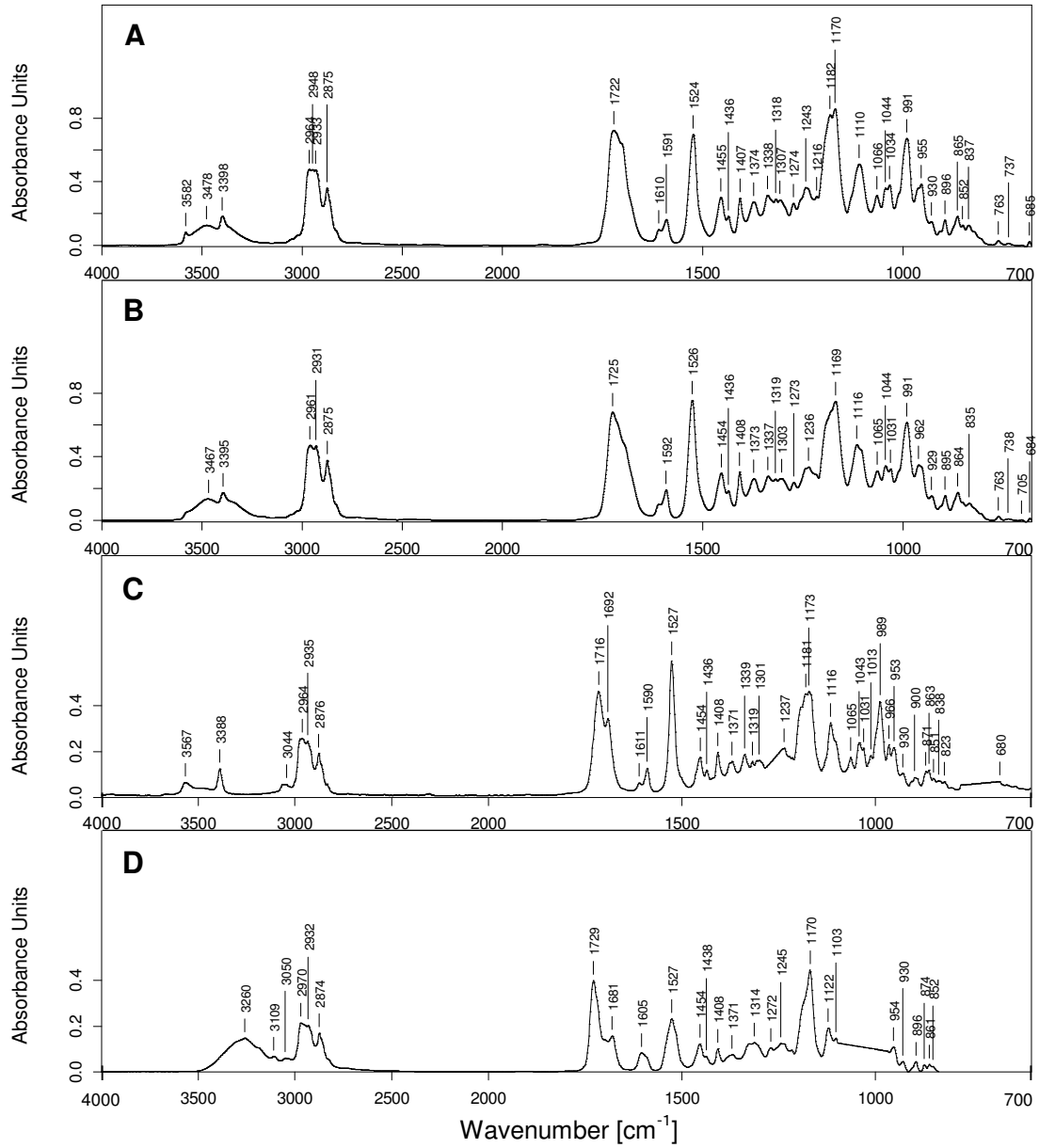


Figure 2.3: IR spectra of the active ingredient NOA449851 plotted in the region from 700 cm^{-1} to 4000 cm^{-1} as solid samples A (A) and B (B) as well as in solution in dichloromethane (C) and DMSO (D).

Table 2.2: Assignments of IR bands of the active ingredient NOA449851 from the spectra illustrated in Figure 2.3.

Sample A [cm^{-1}]	**Sample B** [cm^{-1}]	**NOA449851 dissolved in dichloro-methane** [cm^{-1}]	**NOA449851 dissolved in DMSO** [cm^{-1}]	**Assignment of the bands**
3582		3567		-OH stretch
3478	3467		~3260	-OH stretch
3398	3395	3388		-NH stretch
3100 ~ 3000	3100 ~ 3000		3109, 3050	aromatic CH stretch
2948, 2933	2961	2964	2970	C-H stretch
	2875	2876	2874	C-H stretch
1722	1725	1716	1729	C=O stretch ester
~1700 (sh)	~1700 (sh)	1692	1681	C=O stretch amide
1610, 1591	1610, 1592	1590	1605	benzene ring stretch
1524	1526	1527	1527	amide II of sec amide + benzene ring stretch
1455	1454	1454	1454	C-H bend
1407	1408	1408	1408	benzene ring stretch
1374	1373	1371	1371	CH bend of CH_3 groups
1170	1169	1173	1170	C-O-C asymmetric stretch C-O of ester
1110	1116	1116	1103	C-O stretch of CH_2-O-CH_3
1034	1044	1043		mainly C-O stretch
991	991	989		
896	895	900	896	
955	962	966	954	CH o.p. bend of trans-disubst C=C
865	864	863	861	aromatic CH o.p. bend

Figure 2.4 represents the IR spectra of samples A and B in the region between 3000 cm^{-1} and 3700 cm^{-1}. The spectra exhibit important differences in this region which is specific for changes involving hydrogen bonding. The spectra of the sample A exhibit sharp signals at 3581 cm^{-1} and 3398 cm^{-1}, corresponding to a OH-stretch and a NH-stretch, respectively. The spectra of sample B showed only a slight shoulder at 3580 cm^{-1} and also a weaker peak at 3398 cm^{-1}. The IR patterns of the samples C to G were similar to the spectra of the sample B and were therefore not shown.

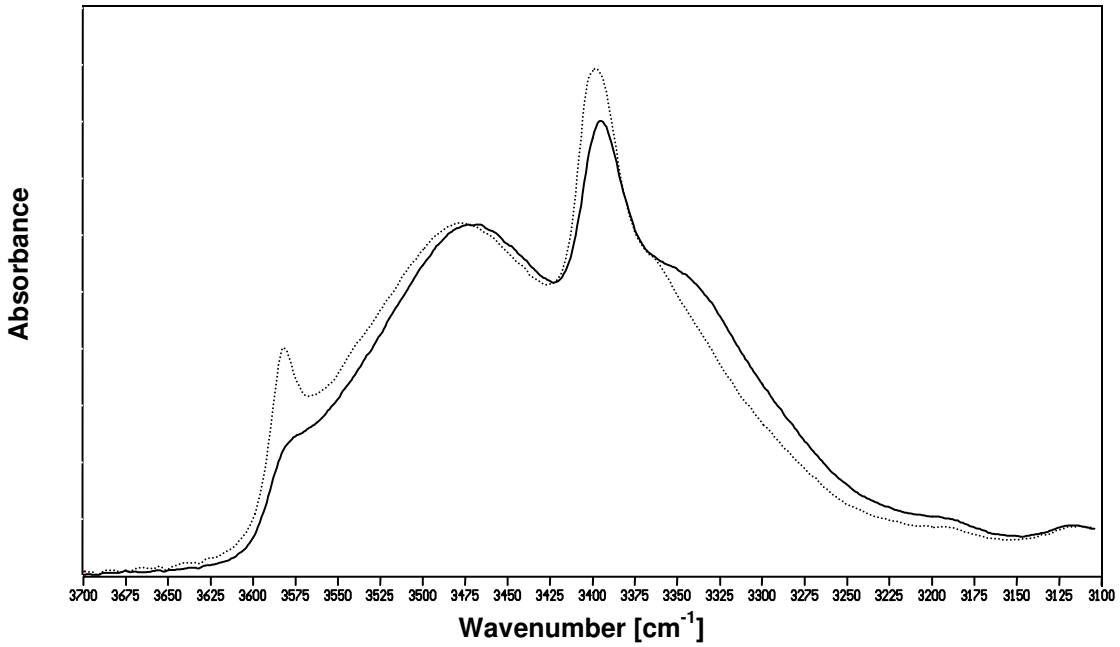


Figure 2.4: IR spectra of the active ingredient sample A, recristallized in ethyl acetate/heptane (.....) and B, recristallized in dichloromethane (__), plotted in the region 3000 cm^{-1} to 3700 cm^{-1}.

3.3. Raman spectroscopy

Raman spectra were taken from the solid samples A to G prepared as described in Section 2.2.3. The Raman spectra of sample A, obtained by recrystallisation in ethyl acetate/heptane and of sample B, obtained by recrystallisation in dichloromethane are illustrated in Figure 2.5. Two conformations can be distinguished, mainly concerning the shift of the band from 1528 cm^{-1} (sample A) to 1520 cm^{-1} (sample B). Changes in this region correspond to different conformations of the amid II and of the benzene ring of the side chain of the molecule. The ratio of the bands at 1500 cm^{-1} - 1400 cm^{-1} also significantly changed. The Raman spectra of solid samples C to G were identical to the spectra of sample B and are therefore not shown.

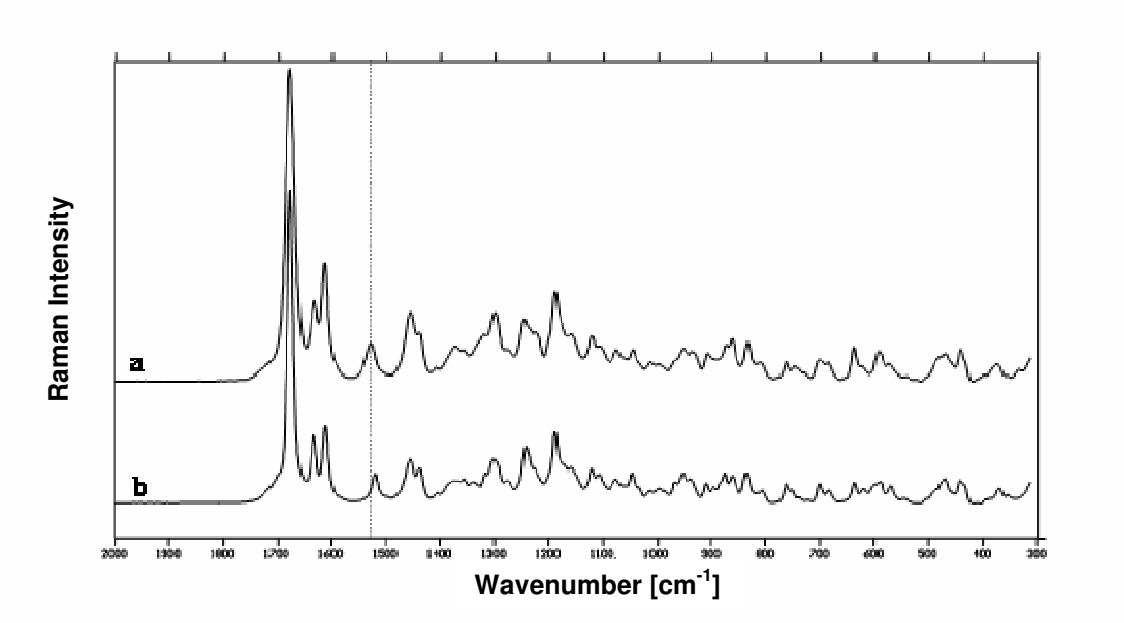


Figure 2.5: Raman spectra of sample A (a) and sample B (b) of the active ingredient NOA449851 plotted in the region between 300 cm^{-1} and 2000 cm^{-1}.

3.4. X-ray analysis

The morphology of the active ingredient in samples A and B was checked by X-ray diffraction. The diffractogram of sample A obtained by recrystallisation in ethyl acetate/heptane is illustrated in Figure 2.6 and shows sharp diffraction maxima. This indicates crystal conformation of active ingredient in sample A.

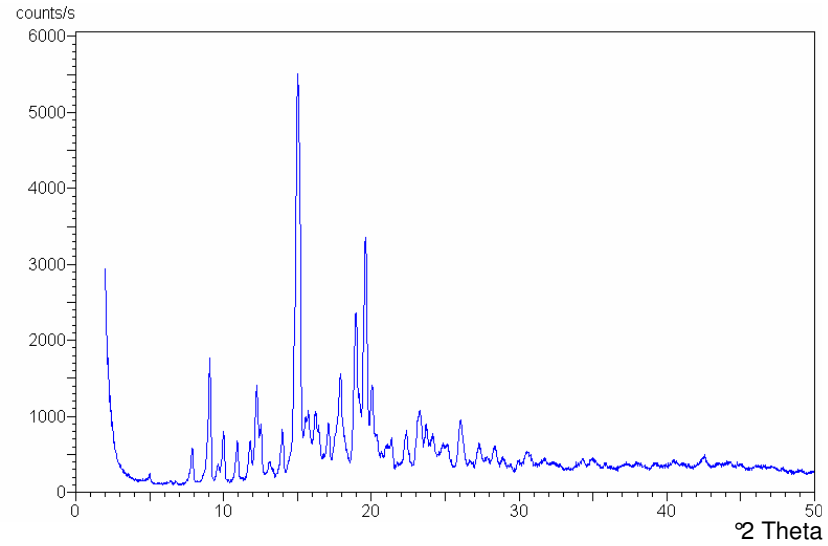


Figure 2.6: X-ray diffractogram of sample A, obtained after recrystallization in ethyl acetate/heptane. The sharp diffraction maxima indicates that the substance is in crystalline form.

The diffractogram of the solid sample B which was obtained after rectisallization in dichloromethane is illustrated in Figure 2.7. The broad diffusion band observed in the spectra is typical for amorphous compound.

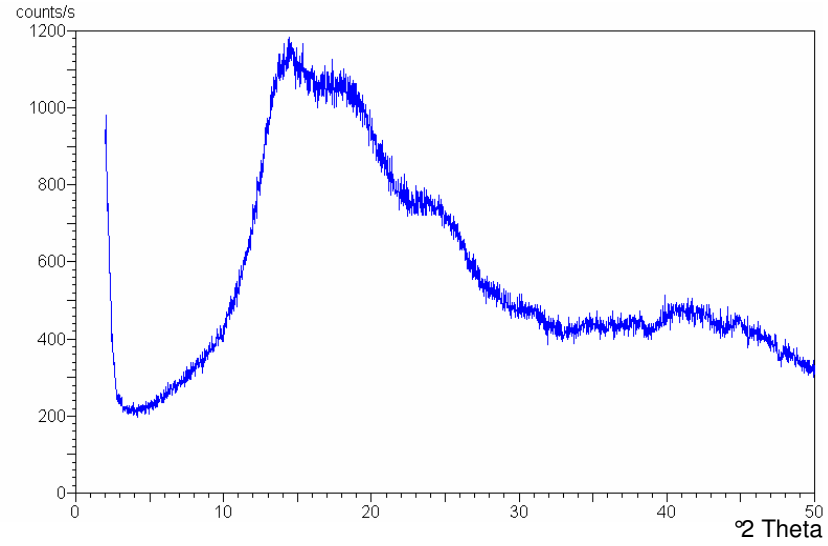


Figure 2.7: X-ray diffractogram of the solid sample B, obtained after rectristallization in dichlorometane. The broad diffusion band indicates that the substance is amorphous.

4. Discussion

4.1. Solubility of the drug substance in various solvents

The development of sustained release injectables formed *in-situ* based on PLA/PLGA polymers requires previous physico-chemical characterisation of the active ingredient. Therefore, the solubility properties of the drug substance NOA449851 in organic solvents were tested. All solvents investigated were appropriate candidates for parenteral application. Glycerol formal, NMP and triacetin are strong solvents for the PLA/PLGA polymers and could therefore be used as main solvent for the sustained release injectables formed *in-situ* [Matschke, 2002]. These three excipients are also very good solvents for the active ingredient NOA449851 as the maximum concentration for the active substance was 35.22 % in triacetin, 37.14 % in glycerol formal and 53.18 % in NMP.

A solubility of less than 5 % of NOA449851 was observed in hydrophilic solvents such as anhydrous glycerol (0.05 %), propylenglycol (3.0 %) and water for chromatography (< 0.001 %). Better solubility for the active ingredient was found in the solvents PEG 300 (19.04 %) and ethanol absolute (50.93 %). Miglyol 812, a mixture of medium-chain triglycerides, only solves 5.6 % of the active ingredient. These excipients are not able to solve enough PLA/PLGA polymers to form a matrix after precipitation [Matschke, 2002]. However, they could be added to the formulations as hydrophilic, respectively lipophilic co-solvents and act as release modifying factors or viscosity enhancing excipients.

4.2. Morphology conformation

A number of techniques has been employed to characterize the active ingredient, including infrared spectroscopy, Raman spectroscopy and X-ray crystallography.

Raman and IR spectroscopy depend on chemical composition but are also sensitive to configuration, polymorphism and to the presence of crystalline domains. Data from Raman spectroscopy is complementary to that from IR spectroscopy in the sense that both give information on the vibrational structure of a molecule, however, they are based on different features. IR spectroscopy is useful for the analysis of strongly polar bonds while Raman is more useful for analysis of non-polar or symmetrical bonds. Raman spectroscopy has been shown to be more sensitive to the detection of different polymorphs than IR spectroscopy, whereas, when polymorphic changes involve hydrogen bonding, IR spectra exhibit larger

differences than Raman spectra [Deeley et al., 1991; Tudor et al., 1991; Sloane, 1971]. IR and Raman spectra are complex with a large number of bands in the fingerprint region (1500 cm^{-1} - 500 cm^{-1}). Such detailed spectra are not unexpected since the active ingredient contains a large number of different functional groups, as illustrated in Figure 2.1.

The IR spectrum illustrated in Figure 2.4 shows that the active ingredient is able to form hydrogen bonds, depending on its conformation. This is an important information for further investigations of the active ingredient interaction in the different polymer matrix.

The Raman spectra as illustrated in Figure 2.5 clearly show two conformations of the active ingredient. Sample A which is the active ingredient as received from CarboGen is distinguished from samples B to G (recrystallisation in diverse solvents) by a shift from 1528 cm^{-1} to 1520 cm^{-1}. This region represents the amid II and the benzene ring of the side chain of the molecule as previously determined with IR spectra which is more specific for functional group determination. There are, additionally to this shift, other changes concerning band ratio intensity. Raman confirms the IR assumptions that there are two conformations of active ingredient in the solid state.

In further investigations using X-ray diffraction analysis, the crystalline parameters of both conformations were checked by analyzing the solid samples A and B. The patterns of the diffractogram (Figure 2.6) exhibited well-defined bands for sample A which was in the crystalline form. However, sample B was in the amorphous form as indicated by the broad diffusion bands (Figure 2.7). It was therefore conclude that all samples with the same Raman spectra as sample B were also in the amorphous conformation. This was the case for all solid samples recrystallized in the various solvent listed in Table 2.1.

This set of analyses indicated that sample A was crystalline and sample B was amorphous. As the Raman spectra of sample C, D, E, F and G were identical to the spectra of sample B, it can be concluded that the conformation of the active ingredient of the five samples was also amorphous.

5. Conclusion

Prior to the development of new sustained release injectables formed *in-situ*, the selected active ingredient NOA449851 was characterized with regard to solubility parameters in various organic solvents. A high solubility for the active ingredient was found in NMP, glycerol formal and triacetin, which are also strong solvents for the PLA/PLGA polymers. The solubility of NOA449851 was also investigated in other excipients which could be used in the formulation as co-solvents. Low solubility was observed in the lipophilic solvent Miglyol 812, as well as for hydrophilic solvents such as propylenglycol, anhydrous glycerol and water for chromatography. However, NOA449851 was highly soluble in ethanol absolute and in PEG 300.

In addition, morphological conformation of the active ingredient NOA449851 was investigated using Raman and Infrared spectroscopy, as well as X-ray diffraction. Two conformations of the active ingredient in the solid state could be distinguished. The crystalline compound as received from CarboGen and the amorphous compound, after recrystallized in any of the solvents investigated. The conformation of the active ingredient in the solid state may influence its solubility and therefore its release rate from the injectable implant.

Chapter 3

In-vitro investigations of sustained release injectables formed *in-situ*

1. Introduction and objectives

Dissolution testing has emerged as a highly valuable *in-vitro* test to characterize drug product performance. It is an important tool to guide drug product development and to select appropriate formulations for *in-vivo* tests. In order to investigate *in-vitro* release of the active ingredient NOA449851 from different sustained release injectables formed *in-situ*, several formulations varying in type of solvent, type of polymer, amount of solvent and of drug substance were manufactured and injected in a dissolution test apparatus. The release kinetics of the drug from these polymer based formulations were followed over a period of 40 days.

In addition, interactions between PLA polymers, active ingredient, solvents and water were evaluated through the construction of ternary phase diagrams. Compositions at precipitation were recorded in function of the components concentration and of the solvent mixture used.

Finally, vibrational spectroscopy was used to investigate *in-vitro* solidified implants. Raman spectroscopy appeared to be a suitable method for analysing the samples directly from solid devices without any special preparation. The analysis focused mainly on possible polymorph conformations of the active ingredient in the solid implants, depending on the solvent mixture used in the formulation. Detection of interactions between active ingredient and polymer matrix was also of interest.

2. Materials and methods

2.1. Materials

The drug substance NOA449851 was obtained from CarboGen Laboratories, Aarau, CH. The polymers PLA, 85/15 PLGA and 75/25 PLGA (inherent viscosity of 0.68 dl/g, 0.63 dl/g and 0.67 dl/g in trichloromethane at 30°C, respectively) were provided by Birmingham Polymers Inc., Birmingham, AL, USA. Triacetin, NMP, glycerol formal and anhydrous glycerol were purchased from Fluka AG, Buchs, CH. Ethanol absolute, methanol and water for chromatography were obtained from Merck Inc., Darmstadt, Germany. Miglyol 812 was provided by Hänseler AG, Herisau, CH. Solutol HS 15® was purchased from BASF, Ludwigshafen, D. All solvents were of reagent analytical grade.

2.2. Methods

2.2.1. *In-vitro* releasing profiles

2.2.1.1. Preparation of sustained release injectables formed in-situ

Polymer (PLA, 85/15 PLGA or 75/25 PLGA) and solvent (triacetin, NMP or glycerol formal) were weighed into a 50 ml beaker and stirred at 300 rpm for approximately 12 hours at a temperature of about 60°C (magnetic stirrer, Ikamag, RET S8™, Ikawerke, Stauffen, D). After complete dissolution of the polymer, the solution was cooled to room temperature and the active ingredient was added and mixed with the polymer solution. The mixture was gently stirred at 100 rpm until the active ingredient was completely dissolved and finally the formulation was drawn up in a 2 ml syringe.

In case of addition of co-solvent to the formulation (e.g. Miglyol 812, ethanol absolute, glycerol formal or anhydrous glycerol), the active ingredient was first triturated with this excipient before the mixture was added to the polymer solution.

Changes in composition of the formulations concerned type of solvent, type of polymer, drug loading, polymer loading as well as substitution of a fraction of the main solvent with co-solvents. The exact compositions (% w/w) of the sustained release injectables formed *in-situ* investigated *in-vitro* are listed in Tables 3.1 to 3.10.

Table 3.1: Composition (% w/w) of formulations using different solvents (triacetin, NMP and glycerol formal).

Component	***PLA-AI(1:1)*** **[% (w/w)]**	***NMP; PLA-AI(1:1)*** **[% (w/w)]**	***GF; PLA-AI(1:1)*** **[% (w/w)]**
NOA449851	17.00	17.00	17.00
PLA	17.00	17.00	17.00
triacetin	66.00	-	-
NMP	-	66.00	-
glycerol formal	-	-	66.00

Table 3.2: Composition (% w/w) of formulations using different types of polymer (PLA, 85/15 PLGA and 75/25 PLGA). The weight polymer to drug ratio was 1:1 in all three formulations.

Component	***PLA-AI(1:1)*** **[% (w/w)]**	***85/15 PLGA-AI(1:1)*** **[% (w/w)]**	***75/25 PLGA-AI(1:1)*** **[% (w/w)]**
NOA449851	17.00	17.00	17.00
PLA	17.00	-	-
85/15 PLGA	-	17.00	-
75/25 PLGA	-	-	17.00
triacetin	66.00	66.00	66.00

Table 3.3: Composition (% w/w) of formulations using different types of polymer (PLA, 85/15 PLGA and 75/25 PLGA). The weight polymer to drug ratio was 5:1 in all three formulations.

Component	***PLA-AI(5:1)*** **[% (w/w)]**	***85/15 PLGA-AI(5:1)*** **[% (w/w)]**	***75/25 PLGA-AI(5:1)*** **[% (w/w)]**
NOA449851	3.40	3.40	3.40
PLA	17.00	-	-
85/15 PLGA	-	17.00	-
75/25 PLGA	-	-	17.00
triacetin	79.60	79.60	79.60

Table 3.4: Composition (% w/w) of formulations using different types of polymer (PLA, 85/15 PLGA and 75/25 PLGA). The weight polymer to drug ratio was 100:1 in all three formulations.

Component	***PLA-AI(100:1)*** **[% (w/w)]**	***85/15 PLGA-AI(100:1)*** **[% (w/w)]**	***75/25 PLGA-AI(100:1)*** **[% (w/w)]**
NOA449851	0.17	0.17	0.17
PLA	17.00	-	-
85/15 PLGA	-	17.00	-
75/25 PLGA	-	-	17.00
triacetin	82.83	82.83	82.83

Table 3.5: Composition (% w/w) of formulations with different amounts of PLA polymer. The weight polymer to drug ratios were 1:1, 3:4 and 1:2.

Component	***PLA-AI(1:1)*** **[% (w/w)]**	***PLA-AI(3:4)*** **[% (w/w)]**	***PLA-AI(1:2)*** **[% (w/w)]**
NOA449851	17.00	17.00	17.00
PLA	17.00	12.75	8.50
triacetin	66.00	70.25	74.50

Table 3.6: Composition (% w/w) of the formulation with 10.00 % Miglyol 812 as lipophilic co-solvent.

Component	***Co-solvent (10 %) Miglyol, PLA-AI(1:1)*** **[% (w/w)]**
NOA449851	17.00
PLA	17.00
Miglyol 812	10.00
triacetin	56.00

Table 3.7: Composition (% w/w) of formulations with 5.00 %, respectively 10.00 % anhydrous glycerol as hydrophilic co-solvent.

Component	***Co-solvent (5 %) glycerol, PLA-AI(1:1)*** **[% (w/w)]**	***Co-solvent (10 %) glycerol, PLA-AI(1:1)*** **[% (w/w)]**
NOA449851	17.00	17.00
PLA	17.00	17.00
glycerol anhydrous	5.00	10.00
triacetin	61.00	56.00

Table 3.8: Composition (% w/w) of the formulation with 10.00 % glycerol formal as hydrophilic co-solvent.

Component	***Co-solvent (10 %) GF, PLA-AI(1:1)*** **[% (w/w)]**
NOA449851	17.00
PLA	17.00
glycerol formal	10.00
triacetin	56.00

Table 3.9: Composition (% w/w) of the formulation with 10.00 % NMP as hydrophilic co-solvent.

Component	*Co-solvent (10 %) NMP, PLA-Al(1:1)* [% (w/w)]
NOA449851	17.00
NMP	17.00
glycerol formal	10.00
triacetin	56.00

Table 3.10: Composition (% w/w) of formulations with 10.00 % ethanol absolute as hydrophilic co-solvent. The weight polymer to drug ratios were 1:1, 3:4 and 1:2.

Component	*Co-solvent (10 %) ethanol, PLA-Al(1:1)* [% (w/w)]	*Co-solvent (10 %) ethanol, PLA-Al(3:4)* [% (w/w)]	*Co-solvent (10 %) ethanol, PLA-Al(1:2)* [% (w/w)]
NOA449851	17.00	17.00	17.00
PLA	17.00	12.75	8.50
ethanol absolute	10.00	10.00	10.00
triacetin	56.00	60.25	64.50

2.2.1.2. Solubilizing effect of Solutol HS 15®on solubility of NOA449851 in water

The solubilizing effect of Solutol HS 15® on solubility of NOA449851 in water was investigated to determine an adequate composition for the dissolution medium solution. Therefore five aqueous solutions containing 0 %, 1 %, 3 %, 5 % and 10 % (w/w) Solutol HS 15® were prepared. An excess amount of NOA449851 was equilibrated in these solutions by stirring at 300 rpm over night at room temperature. After centrifugation and appropriate dilution, concentration of NOA449851 in the supernatant solutions was determined using the HPLC-method described in Chapter 2, Section 2.2.1.

2.2.1.3. Dissolution method: modified USP basket method

A Sotax AT 7 Smart™ (Sotax AG, Basel, CH) was used as the dissolution apparatus. The six vessels of the apparatus were each filled with one liter of dissolution medium which consisted of an aqueous solution containing 5 % (w/w) Solutol HS 15®. Since this excipient is semi solid at room temperature, it was first liquified in a microwave oven for five minutes and then added to demineralised water. The solution was finally homogenised using a magnetic stirrer at 300 rpm for about ten minutes. The vessels of the dissolution apparatus were

immersed in a water bath and subsequently heated to a temperature of 37°C. Copper sulfate 2 % was used as anti-microbial preservative in the water bath.

Water permeable felt bags were individually prepared and placed in each basket of the dissolution apparatus. Formulations were taken up into 2 ml syringes (Braun Inc., Melsungen, Germany) and injected through 16 G needles (Becton and Dickenson, Basel, CH) into the felt bags. The syringes were weighed before and after injection and the corresponding amount of active ingredient was calculated from the concentration of NOA449851 in the formulation. Approximately 2.5 g of each formulation, equivalent to an injection volume of 2 ml, were injected into the baskets. Each formulation was investigated for *in-vitro* dissolution test in triplicate. To keep sink conditions, dissolution medium was replaced as soon as the drug concentration reached 0.01 %.

The baskets rotated at 100 rpm over a period of 40 days. However in case of formulations "*Co-solvent (10 %) ethanol, PLA-AI(3:4*)" *and* "*Co-solvent (10 %) ethanol, PLA-AI(1:2)*", dissolution tests were continued up to 120 days.

Samples were taken initially after 2, 4, 8, 24 and 32 hours and subsequently at almost every working day until the end of the experiment. A volume of 1 ml of the dissolution medium was taken from each vessel, using a 2 ml syringe and filled into a 2 ml vial. Afterwards, about 0.5 ml of each sample were transferred into an amber coloured HPLC-vial. The NOA449851 concentration in the dissolution medium was determined immediately after sample collection, using the HPLC-method as described in Chapter 2, Section 2.2.1.

2.2.1.4. Graphical and statistical evaluation of the release profiles

The concentration of active ingredient detected in the dissolution medium was plotted against time. On the X-axis, time was represented in days, whereas on the Y-axis, cumulative percentage (% w/w) of active ingredient released from the formulation was illustrated.

The linear phase of the release profile was selected to extrapolate a straight line with, when possible, a correlation coefficient value of at least 0.98. The corresponding equation of type "y = ax + b" was calculated, where "a" is the rate of release of active ingredient per day during the linear phase (%/day), and "b", the ordinate to the origin, give indication about magnitude of the initial burst effect. An additional factor "b/a" was also calculated to qualify and compare the various kinetic profiles.

2.2.2. Ternary phase diagrams

2.2.2.1. PLA precipitation

Stock PLA polymer solutions (30.00 % w/w) were prepared with triacetin or NMP as solvent. Serial dilutions using the appropriate solvent were made to prepare different concentrations of the polymer. Additionally, PLA polymer solutions were prepared substituting a fraction of the solvent triacetin with one of the co-solvents Miglyol 812, glycerol formal or ethanol absolute. The concentration of the co-solvent represented 10.00 % (w/w) of the initial polymer solution. The vials containing the polymeric solution were placed in a water bath kept at 25°C. Demineralised water was added to 10 µl aliquots until polymer precipitation was observed. Composition at precipitation was recorded using the known initial weight of polymer, solvent and the weight of water that was added. Composition at precipitation were then plotted as ternary graphs.

2.2.2.2. NOA449851 precipitation

Similar process as described in Section 2.2.2.1 was applied to active ingredient solutions to determine the quantity of water needed to observe drug precipitation. The solvents triacetin and NMP were used for preparing active ingredient solutions of different concentrations.

2.2.3. Raman spectroscopy

Raman spectroscopy was used for analytical assessment of molecular structure of the active ingredient within the polymeric matrix. This analytic method is particularly appropriate for the detection of different polymorphic forms.

2.2.3.1. Preparation of in-vitro formed solid implants for Raman spectroscopy

Various formulations were selected for Raman spectroscopy investigations. First, the formulation "*PLA-AI(1:1)*" which contained PLA as polymer and triacetin as unique solvent was chosen. Three further formulations containing as co-solvents glycerol formal *(*"*Co-solvent (10 %) GF, PLA-AI(1:1)*"), absolute ethanol ("*Co-solvent (10 %) ethanol, PLA-AI(1:1)*") and Miglyol 812 ("*Co-solvent (10 %) Miglyol, PLA-AI(1:1)*") were selected for

spectral investigations. Exact compositions of formulations are described in Tables 3.1, 3.6, 3.8 and 3.10.

For preparation of the solid implants, 0.5 g of each formulation were injected into a 100 ml beaker containing 75 ml demineralised water. Formulations were kept for 48 hours at room temperature to allow fluid convection and formation of the solid implants.

2.2.3.2. Raman measurements

Raman spectroscopy was performed on *in-vitro* formed solid implants according to the method described in Chapter 2, Section 2.2.5.

The FT Raman spectra were recorded with a Bruker RFS 100 FT Raman spectrometer equipped with a liquid nitrogen cooled germanium detector. The resolution was 4 cm^{-1} and 250 scans were accumulated by using a laser output of 600 mW. The spectra were corrected for instrumental response.

3. Results

In-vitro release of the active ingredient NOA449851 from various sustained release injectables formed *in-situ* was studied using dissolution testing during 40 days. Composition of investigated formulations varied with respect to the type of solvent and biodegradable polymer as well as the use of different co-solvents. Different drug and polymer concentrations were also tested. In addition, ternary phase diagrams with different solvent mixtures were carried out to determine the amount of water needed to reach polymer or active ingredient precipitation. Finally, Raman spectra were taken from solid implants formed *in-vitro* to observe possible conformation changes or interactions due to the different solvent mixtures of the formulation compostition.

3.1. Solubilizing effect of Solutol HS 15® for NOA449851 in aqueous solution

Figure 3.1 illustrates solubility of NOA449851 in aqueous solutions containing Solutol HS 15®. Concentration of Solutol HS 15® in water varied from 0 % to 10 %. Solubility of the active ingredient NOA449851 in the different solutions increased with the amount of surfactant added to the water. In pure water, the amount of NOA449851 dissolved was below the detection limit of the HPLC method, whereby a concentration of 0.002 % NOA449851 was measured with 1 % of Solutol HS 15®. In solutions with 3 % and 5 % Solutol HS 15®, a concentration of 0.041 % and 0.083 %, respectively, of the active ingredient were reached. A concentration of 0.219 % NOA449851 was found in a 10 % Solutol HS 15® solution.

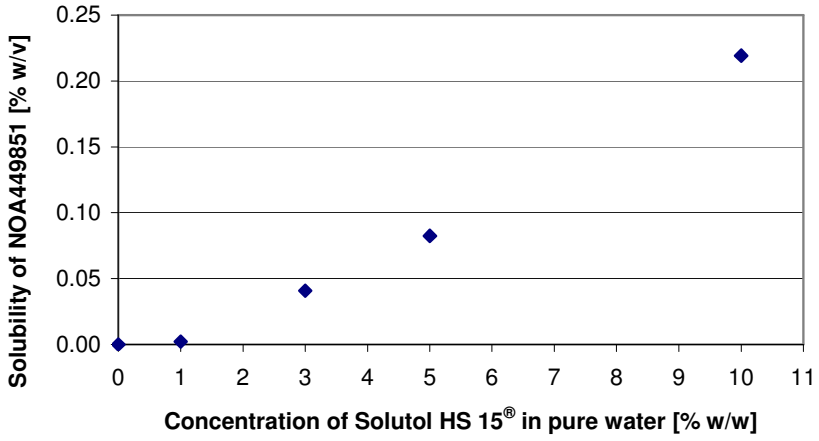


Figure 3.1: Maximal concentrations [% (w/v)] of NOA449851 in aqueous Solutol HS 15® solutions. Concentration of Solutol HS 15® varied from 0 % to 10 % (w/w).

3.2. *In-vitro* release profiles

Results of *in-vitro* release profile parameters from sustained injectables formed *in-situ* are summarised in Table 3.11. Initial burst duration (in days) as well as fraction of total drug released during this period (in % w/w) are listed. Rate of drug release during the second phase (in fraction of total per day (%/d)) was derived from the observed linear release profiles. The fraction of the total drug released after the 40 days of dissolution testing was listed (in % w/w). The equation of type “y = ax + b” extrapolated from the linear phase as well as the factor “b/a” were calculated for each tested formulation. The “b/a” factor of an ideal sustained release profile of zero order kinetic without any initial burst would converge to the value of 1. It is also indicated if the formulation was selected for further *in-vivo* tests in animals (Chapter 4).

Table 3.11: Overview of parameters describing the *in-vitro* drug release profiles of *in-situ* formed release injectables.

Formulation	Initial burst [d]	Drug release during initial burst [%]	Release rate during linear phase [%/d]	Drug release after 40 days [%]	Mathematical equation of type "y = ax + b"	Factor "b/a"	Test *in-vivo*
PLA-AI(1:1)	6	17.57	0.73	42.64	y = 0.73x + 14.29	19.5	Yes
NMP; PLA-AI(1:1)	7	35.21	0.51	52.51	y = 0.51x + 34.05	67.0	No
GF; PLA-AI(1:1)	7	24.13	0.71	49.79	y = 0.71x + 21.37	30.1	No
85/15 PLGA-AI(1:1)	6	16.55	0.72	41.90	y = 0.72x + 12.43	17.2	No
75/25 PLGA-AI(1:1)	6	13.00	0.75	39.08	y = 0.75x + 8.04	10.7	No
PLA-AI(5:1)	6	14.15	0.05	16.05	y = 0.05x + 14.22	296.2	No
85/15 PLGA-AI(5:1)	6	12.91	0.06	14.75	y = 0.06x + 14.69	268.6	No
75/25 PLGA-AI(5:1)	6	14.11	0.05	16.39	y = 0.05x + 13.16	284.9	No
PLA-AI(100:1)	6	18.03	0.06	21.69	y = 0.06x + 19.76	329.3	No
85/15 PLGA-AI(100:1)	6	18.76	0.11	25.06	y = 0.11x + 19.81	180.1	No
75/25 PLGA-AI(100:1)	6	19.59	19.59	76.54	y = 0.18x + 19.31	107.3	No
PLA-AI(1:2)	6	72.89	0.29	83.58	y = 0.29x + 72.84	248.5	No
PLA-AI(3:4)	6	60.13	0.35	72.85	y = 0.35x + 60.11	173.7	Yes
Co-solvent (10 %) Miglyol, PLA-AI(1:1)	13	38.50	0.15	42.60	y = 0.15x + 36.72	241.6	No
Co-solvent (5 %) glycerol, PLA-AI(1:1)	7	12.76	0.87	40.54	y = 0.87x + 5.65	6.5	No
Co-solvent (10 %) glycerol, PLA-AI(1:1)	7	9.82	0.84	36.65	y = 0.84x + 2.67	3.1	Yes
Co-solvent (10 %) GF, PLA-AI(1:1)	7	15.86	0.45	31.31	y = 0.45x + 13.46	30.2	Yes
Co-solvent (10 %) NMP, PLA-AI(1:1)	7	12.40	0.70	37.51	y = 0.70x + 6.85	9.8	No
Co-solvent (10 %) ethanol, PLA-AI(1:1)	1	4.46	1.25	52.22	y = 1.25x + 2.46	1.9	Yes
Co-solvent (10 %) ethanol, PLA-AI(3:4)	7	30.76	0.29	52.15*	y = 0.29x + 37.51	129.7	Yes
Co-solvent (10 %) ethanol, PLA-AI(1:2)	7	49.94	0.11	59.78*	y = 0.11x + 53.77	505.3	No

* Experiments continued for 120 days.

3.2.1. Influence of type of solvent

The excipients used to dissolve the polymer have a strong influence on the way the formulation coagulates after injection into an aqueous medium and therefore on the release characteristics of the sustained release injectables. Physico-chemical characteristics of the main solvent such as viscosity, lipophilicity and partition coefficient (octanol/water) are determinant during implant hardening process. The *in-vitro* drug release from formulations based on different solvents was compared. The selected solvents were triacetin ("*PLA-AI(1:1)"*), NMP (*"NMP; PLA-AI(1:1)"*) and glycerol formal (*"GF; PLA-AI(1:1)"*). The dynamic viscosity of triacetin is 17.4 mPa*s at 25°C and its log P-value is +0.25. NMP has a dynamic viscosity of 1.65 mPa*s and a log P-value of -0.38 [The Merck Index, 1996; Fiedler Lexikon, 2002].

All three excipients are excellent solvents for the PLA polymer [Matschke, 2002; Hatefi et al., 2002; Brodbeck et al., 1999 (2)] as well as for the drug substance NOA449851 (Chapter 2). Concentrations of PLA polymer and of active ingredient in formulations were 17.00 % (w/w). For composition details, see Table 3.1.

The release profiles from formulations based on triacetin and on NMP are illustrated in Figure 3.2. Formulation *"PLA-AI(1:1)"* released 43 % of active ingredient after 40 days of dissolution test, while formulation *"NMP; PLA-AI(1:1)"* released 53 % in the same test conditions. Formulation with NMP showed a large initial burst, corresponding to 35 % of active ingredient released during the first 7 days and a relatively slow release rate during the second phase of the kinetic profile, which represented almost 0.51 %/d. In contrast, formulation with triacetin showed a slight initial burst corresponding to 18 % of active ingredient released within the first 7 days and a faster release rate during the linear phase representing 0.73 %/d.

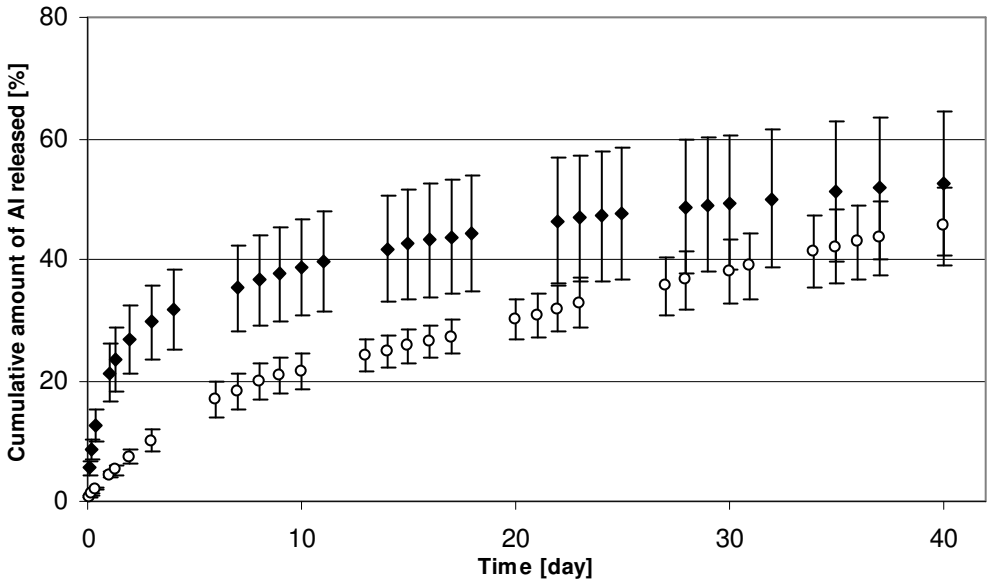


Figure 3.2: Influence of solvent type on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ,* related to time. Solvents used in the formulations were triacetin (O) and NMP (◆).

The release profile from formulation based on the solvent glycerol formal is compared in Figure 3.3 to the release profile of the triacetin based formulation. Formulation *"GF; PLA-AI(1:1)"* showed a slight increased initial release, with a fraction of 24 % of the total active ingredient released during the first 7 days of the dissolution test, while the formulation *"PLA-AI(1:1)"* released only 18 % of the active ingredient into the medium during the same period. Release rate during the linear phase was very similar for both formulations, i.e. about 0.73 %/d. The "b/a" factor was therefore higher for formulation *"GF; PLA-AI(1:1)"* than for formulation *"PLA-AI(1:1)"*, with values of 30.1 and 19.5, respectively .

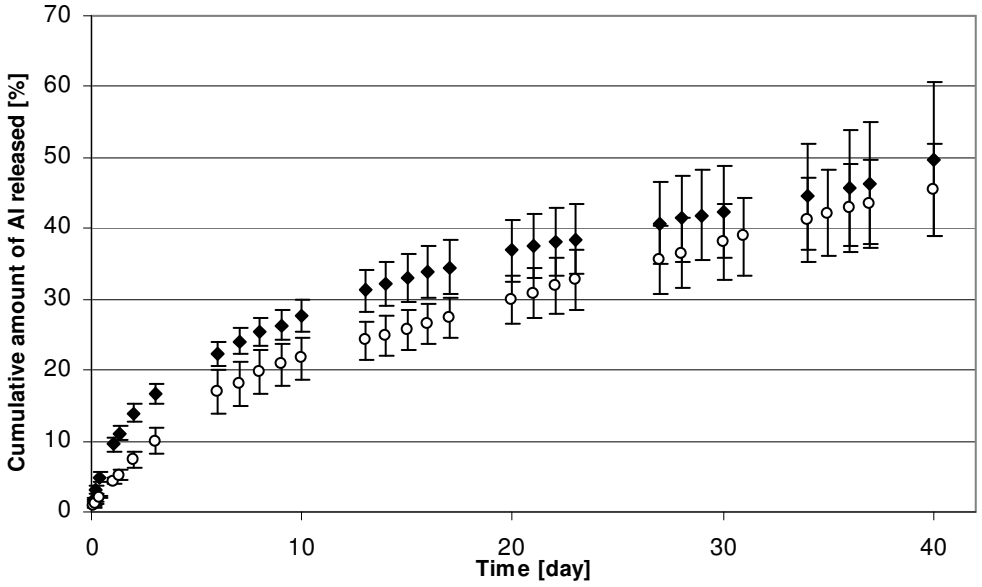


Figure 3.3: Influence of solvent type on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ,* related to time. Solvents used in the formulations were triacetin (○) and glycerol formal (◆).

3.2.2. Influence of type of polymer

In order to study the influence of polymers with different biodegradation properties on the *in-vitro* release of NOA449851 from sustained release injectables formed *in-situ*, formulations containing PLA *("PLA-AI(1:1)")*, 85/15 PLGA *("85/15 PLGA-AI(1:1)")* or 75/25 PLGA polymers *("75/25 PLGA-AI(1:1)")* were investigated. Three batches were prepared changing only the type of polymer. All formulations contained 17.00 % NOA449851 and 66.00 % triacetin, with a weight polymer to drug ratio of 1:1. For composition details, see Table 3.2.

Release curves of the active ingredient from the three formulations based on three types of polymer did not show any significant difference. Formulation "*PLA-AI(1:1)"* released 43 %, formulation "*85/15 PLGA-AI(1:1)*" 42 % and formulation "*75/25 PLGA-AI(1:1)"* 39 % NOA449851, respectively, during the 40 days of the dissolution test. Only small initial burst effects were observed in the three release profiles: after 6 days, 18 % of NOA449851 from PLA formulation, 17 % from 85/15 PLGA formulation and 13 % from 75/25 PLGA formulation, respectively, were released into the dissolution medium. Drug release observed during the second phase of the profile was similar for all three formulations, with a release rate of approximately 0.7 % of active ingredient per day. The three release profiles are illustrated in Figure 3.4.

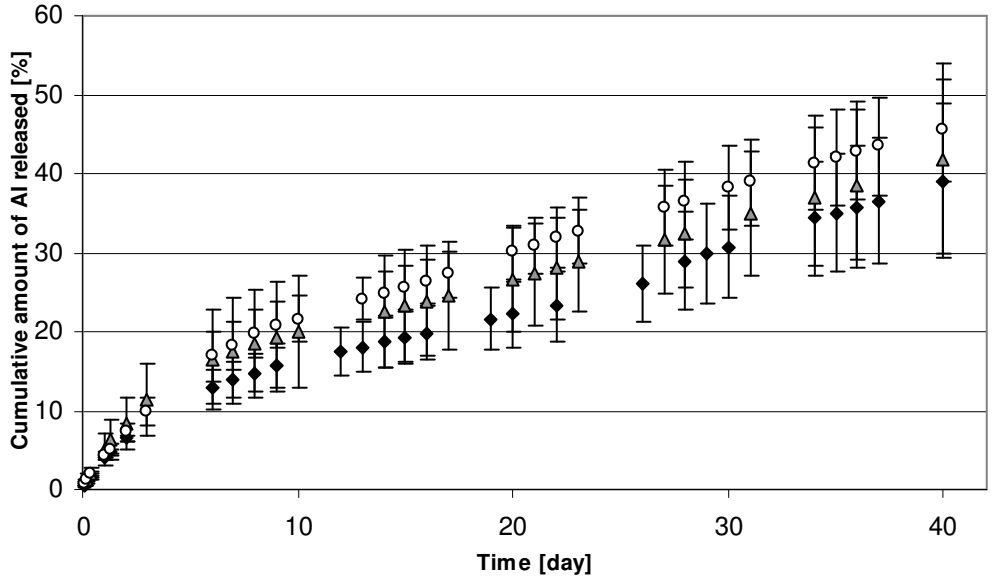


Figure 3.4: Influence of polymer type on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ,* related to time. Formulations were based on PLA (○), 85/15 PLGA (▲) and 75/25 PLGA (◆) polymers with a weight polymer to drug ratio of 1:1.

A second experiment was carried out in order to study the influence of polymers with different biodegradation properties, where the amount of active ingredient was reduced in all three formulations from 17.00 % to 3.40 %; polymer concentration was maintained at 17.00 %, which resulted in a weight polymer to drug ratio of 5:1. In all three formulations, triacetin was used as solvent. For composition details, see Table 3.3.

The release profiles of all three formulations were again very similar as showed in Figure 3.5. The drug released after 40 days dissolution test was 16 % for formulation *"PLA-AI(5:1)"*, 15 % for formulation *"85/15 PLGA(1:1)"* and 16 % for formulation "*75/25 PLGA(5:1*)". All formulations showed a substantial initial burst: within 6 days, already 14 % (*"PLA-AI(5:1)"*), 13 % (*"85/15 PLGA(5:1)"*) and 14 % (*"75/25 PLGA(5:1*)") of the active ingredient was released. Release rate of NOA449851 from the implant during the linear phase was very low for all formulations and represented about 0.05 % of active ingredient released per day. Consequently, the factors "b/a" in all three cases were very high (296, 268 and 284, respectively).

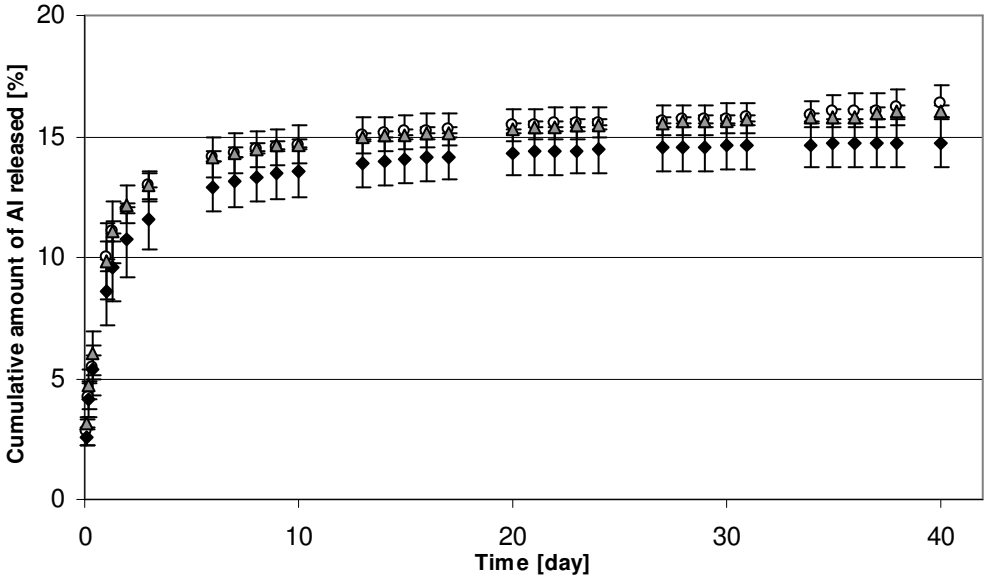


Figure 3.5: Influence of polymer type on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ,* related to time. Formulations were based on PLA (O), 85/15 PLGA (△) and 75/25 PLGA (◆) polymers with a weight polymer to drug ratio of 5:1.

A further dissolution test was carried out in order to study the influence of polymers with different biodegradation properties on the *in-vitro* drug release. This time, the polymer concentration was maintained at 17.00 % and the amount of active ingredient was reduced to 0.17 %, which resulted in a weight polymer to drug ratio of 100:1. In all three formulations, triacetin was used as solvent. For composition details, see Table 3.4.

Release profiles of the three formulations were very similar for the first 20 days of the experiment. Formulations showed an initial burst effect within 6 days representing a release of active ingredient 18 % for *"PLA-AI(100:1)"*, 19 % for *"85/15 PLGA(100:1)*" and 20 % for *"75/25 PLGA(100:1*)". Release rate during the second phase was very low for all three formulations and represented less than 0.2 % of active ingredient released per day. This lag phase ended for formulation *"75/25 PLGA(100:1*)" at day 20 and was followed by a rapid drug release which could be described with the exponential formula "$y = 5.7893 * e^{0.0613}$". For the two other formulations *"PLA-AI(100:1)"* and *"85/15 PLGA(100:1)*", the second phase lasted until the end of the dissolution experiment. After 40 days, the cumulative drug release was 22 % for formulation *"PLA-AI(100:1)"*, 25 % for formulation *"85/15 PLGA(100:1)"* and 77 % for formulation "*75/25 PLGA(100:1*)".

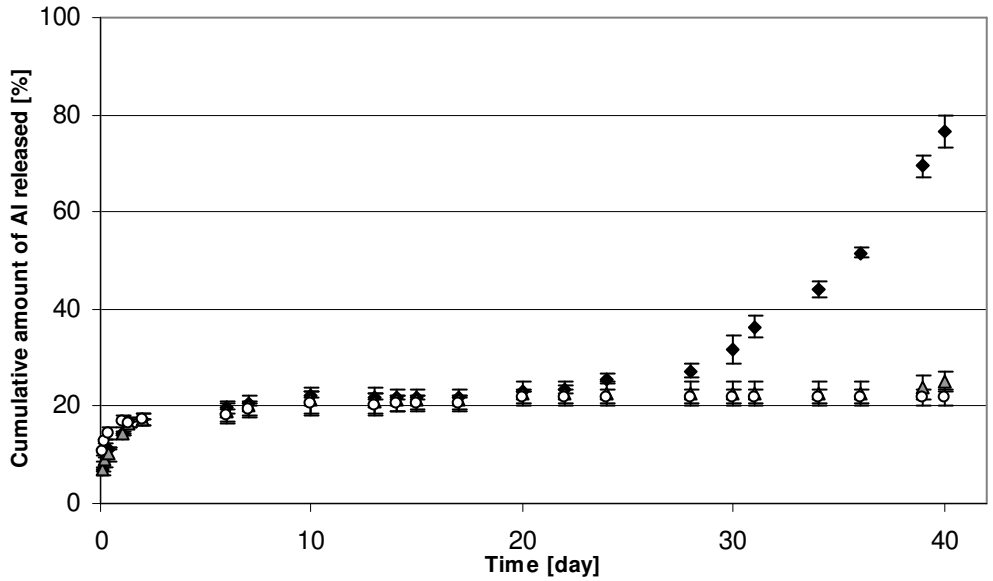


Figure 3.6: Influence of polymer type on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ,* related to time. Formulations were based on PLA (○), 85/15 PLGA (△) and 75/25 PLGA (◆) polymers with a weight polymer to drug ratio of 100:1.

3.2.3. Influence of polymer load

In order to study the influence of polymer load on the *in-vitro* release of NOA449851 from sustained release injectables formed *in-situ*, three different formulations were produced, containing, respectively 8.50 %, 12.75 % and 17.00 % PLA. The amount of active ingredient NOA449851 was kept constant at 17.00 % resulting in a weight polymer to drug ratio of 1:2, 3:4, and 3:4, respectively. The amount of triacetin was adjusted (74.50 %, 70.25 %, 66.00 %) to compensate for the increased polymer concentration. For composition details, see Table 3.5.

The amount of active ingredient released during the 40 days of the dissolution testing was 43 % for formulation "*PLA-AI(1:1)*", 73 % for formulation "*PLA-AI(3:4)*" and 84 % for formulation "*PLA-AI(1:2)*". Formulations "*PLA-AI(3:4)*" and "*PLA-AI(1:2)*" showed very strong initial burst effects, since 60 %, respectively 73 %, of the active ingredient was released within 6 days. During the same period of time, only 18 % of the NOA449851 was released from formulation *"PLA-AI(1:1)"*. The release rate during the linear phase was about 0.7 %/d for *"PLA-AI(1:1)"*, but only 0.3 %/d and 0.4 %/d for formulations *"PLA-AI(3:4)" and "PLA-AI(1:2)"*. This resulted in high "b/a" factors of 248.5 and 173.7 for these two formulations. The drug release from the three different formulations is plotted in Figure 3.7.

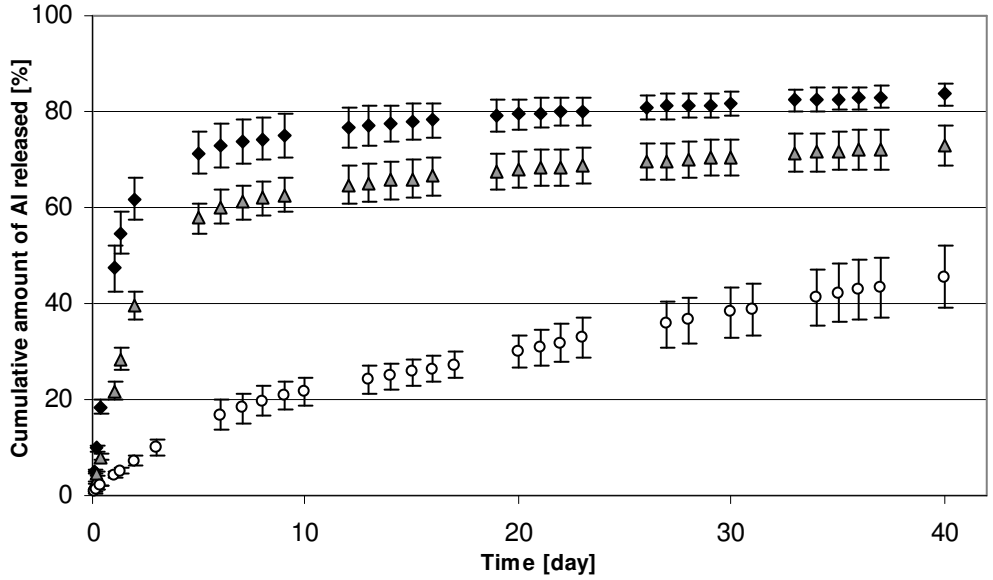


Figure 3.7: Influence of polymer load on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ,* related to time. PLA content varied from 17.00 % (O), to 12.75 % (△) and to 8.50 % (◆).

3.2.4. Influence of co-solvents

3.2.4.1. Influence of Miglyol 812 as co-solvent

The influence of substituting a fraction of triacetin with Miglyol 812 as lipophilic co-solvent on drug release from sustained release injectables formed *in-situ* was investigated. For this purpose, release from two formulations was compared. Both formulations were based on PLA polymer and triacetin. Formulation *"PLA-AI(1:1)"* consisted of PLA (17.00 %), NOA449851 (17.00 %) and triacetin (66.00 %). In formulation *"Co-solvent (10 %) Miglyol, PLA-AI(1:1)"*, 10.00 % triacetin was replaced by Miglyol 812. The amount of NOA449851 and PLA polymer in both formulations resulted in a weight polymer to drug ratio of 1:1. For composition details, see Tables 3.1 and 3.6.

Both formulations released about 43 % of the active ingredient during the 40 days of the dissolution test experiment. However, formulation *"Co-solvent (10 %) Miglyol, PLA-AI(1:1)"* showed an increased initial release, with a fraction of 39 % of active ingredient released during the first 13 days of the dissolution test. During the same time, the formulation *"PLA-AI(1:1)"* released only about half of the amount, i.e. 24 % of the active ingredient into the medium. Thus, the release rate of the second phase was lower for the formulation *"Co-solvent (10 %) Miglyol, PLA-AI(1:1)"* (0.15 %/d) than for the formulation *"PLA-AI (1:1)"* (0.73 %/d). Both release profiles are represented in Figure 3.8.

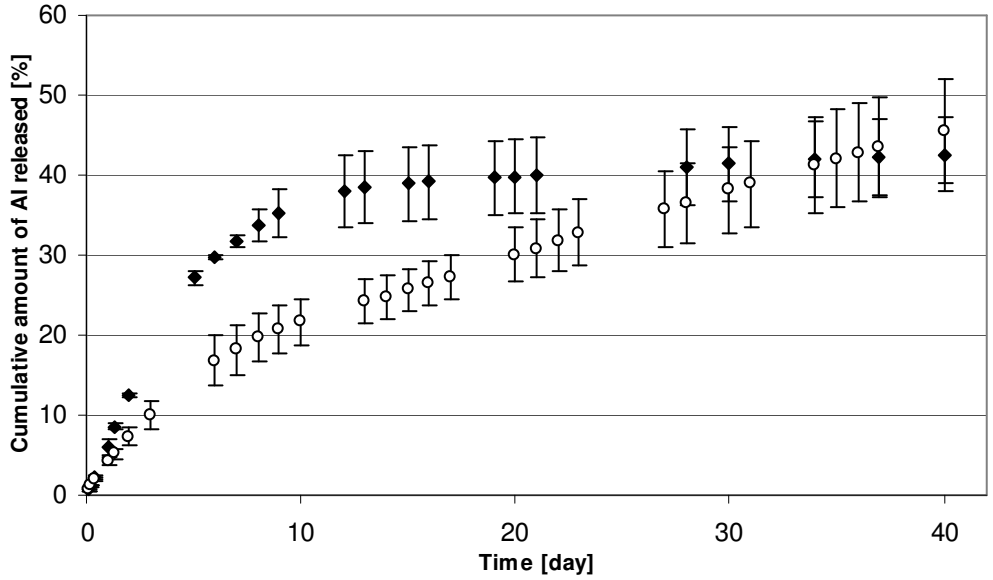


Figure 3.8: Influence of co-solvent Miglyol 812 on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ,* related to time. Formulation *"PLA-AI(1:1)"* (○) had triacetin as sole solvent, formulation *"Co-solvent (10 %) Miglyol, PLA-AI(1:1)"* (◆) contained triacetin as well as 10.00 % Miglyol 812.

3.2.4.2. Influence of anhydrous glycerol as co-solvent

The influence of anhydrous glycerol used as hydrophilic co-solvent in the formulation on the drug release of NOA449851 from sustained release injectables formed *in-situ* was studied. Neither the PLA polymer, nor the active ingredient NOA449851 were soluble in the pure excipient. Formulation *"PLA-AI(1:1)"* consisted of 17.00 % PLA as biodegradable polymer, 17.00 % of NOA449851 as active ingredient and 66.00 % of triacetin as solvent. In formulations *"Co-solvent (5 %) glycerol, PLA-AI(1:1)"* and *"Co-solvent (10 %) glycerol, PLA-AI(1:1)",* 5 % respectively 10 % triacetin was replaced with anhydrous glycerol. As anhydrous glycerol was not completely miscible with triacetin, a very viscous emulsion was formed. For composition details, see Tables 3.1 and 3.7.

The three release profiles are represented in Figure 3.9 where it can be seen that the burst effect was reduced with increased amount of co-solvent anhydrous glycerol. In formulation *"PLA-AI(1:1)"*, which did not contain co-solvent, 18 % of the active ingredient was released during the first 7 days, while 13 % and 10 % of drug were released from formulations *"Co-solvent (5 %) glycerol, PLA-AI(1:1)"* and *"Co-solvent (10 %) glycerol, PLA-AI(1:1)"* respectively. The release rate during the linear phase was very similar for all three formulations, i.e. about 0.7 to 0.8 %/d. The "b/a" factor decreased when the amount of anhydrous glycerol was increased, with values of 19.5, 6.5 and 3.1, respectively.

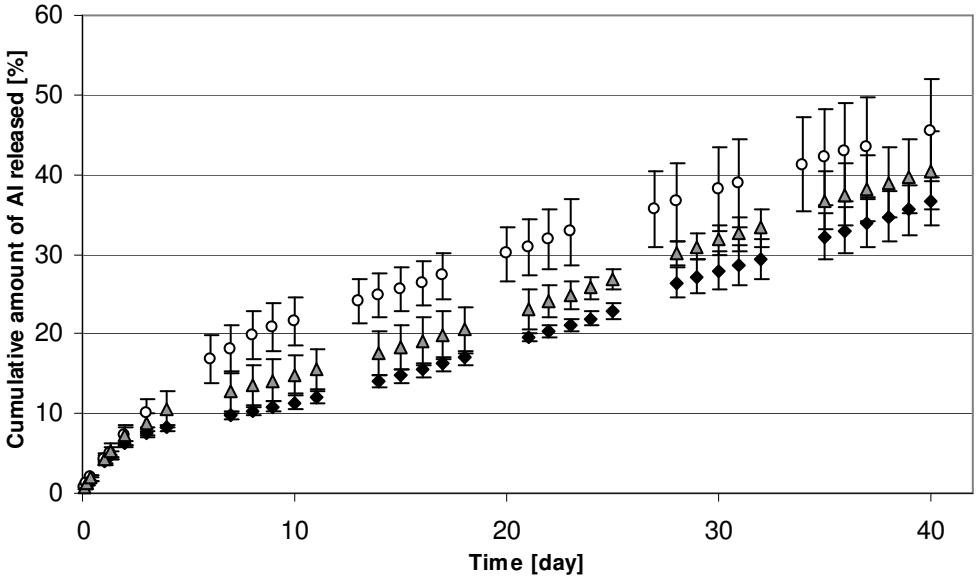


Figure 3.9: Influence of co-solvent anhydrous glycerol on release of NOA449851 [%(w/w)] from sustained release injectables formed *in-situ,* related to time. Formulation (○) had only triacetin as solvent. To the other formulations, 5.00 % (△) or 10.00 % (◆) of anhydrous glycerol was added.

3.2.4.3. Influence of glycerol formal as co-solvent

In order to examine the influence of a second hydrophilic co-solvent on the NOA449851 *in-vitro* release from sustained release injectables formed *in-situ*, two further release profiles were compared. The hydrophilic co-solvent investigated was glycerol formal in a concentration of 10.00 %. Both formulations were based on PLA polymer and triacetin. For composition details, see Tables 3.1 and 3.8.

It was found that 43 % of the drug was released after 40 days from formulation *"PLA-AI(1:1)"* and only 31 % from formulation *"Co-solvent (10 %) glycerol formal, PLA-AI(1:1)"*. Both release profiles showed a slight initial burst: after 7 days, formulation *"PLA-AI(1:1)"* released 18 % of the active ingredient and formulation *"Co-solvent (10 %) glycerol formal PLA-AI(1:1)"* 17 %. The main difference in the release behaviour was observed during the second linear phase. The release rate of the formulation *"PLA-AI(1:1)"* was 0.73 %/d, while drug release from formulation *"Co-solvent (10 %) glycerol formal, PLA-AI(1:1)"* was slower with a rate of 0.45 %/day. Both dissolution profiles are illustrated in Figure 3.10.

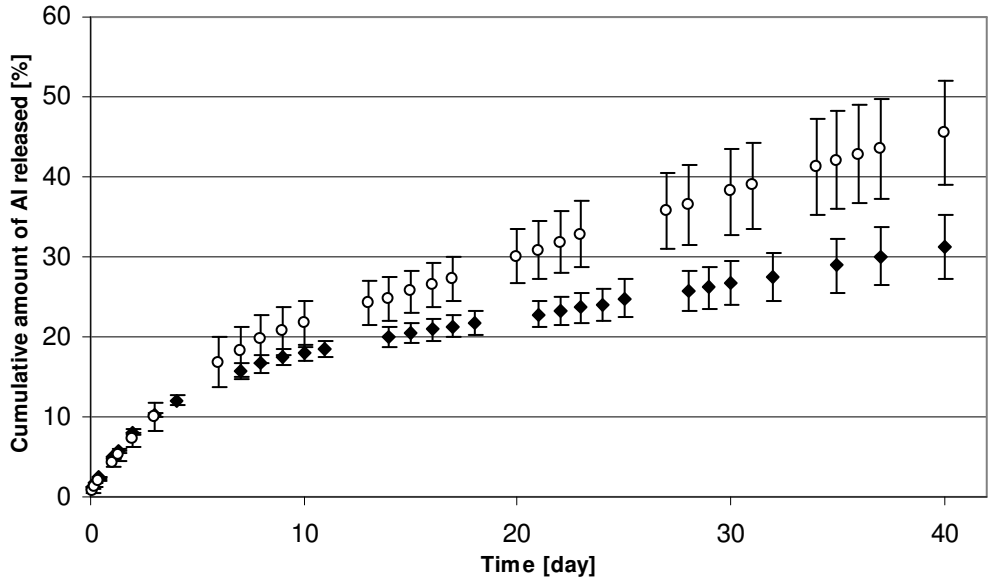


Figure 3.10: Influence of co-solvent glycerol formal on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ*, related to time. Formulation *"PLA-AI(1:1)"* (○) contained triacetin as unique solvent. In formulation *"Co-solvent (10 %) glycerol formal, PLA-AI(1:1)"* (◆), 10.00 % triacetin was substituted with glycerol formal.

3.2.4.4. Influence of NMP as co-solvent

The influence of NMP used as hydrophilic co-solvent at concentration of 10.00 % in the formulation on the drug release of NOA449851 from sustained release injectables formed *in-situ* was studied. For this purpose the release from this formulation was compared with the one of formulation *"PLA-AI(1:1)"*, which contains only triacetin as solvent. Both formulations were based on PLA polymer and triacetin. Formulation *"PLA-AI(1:1)"* consisted of PLA (17.00 %), NOA449851 (17.00 %) and triacetin (66.00 %). In formulation *"Co-solvent (10 %) NMP, PLA-AI(1:1)"*, 10.00 % triacetin was replaced by NMP. The amount of NOA449851 and PLA polymer in both formulations resulted in a polymer to drug ratio of 1:1. For composition details, see Tables 3.1 and 3.9.

Both release profiles are represented in Figure 3.11 where it can be seen that the burst effect was reduced with the use of NMP as co-solvent. In formulation *"PLA-AI(1:1)"*, which did not contain co-solvent, 18 % of the active ingredient was released during the first 7 days, while 12.4 % of drug were released from formulations *"Co-solvent (10 %) NMP, PLA-AI(1:1)"*. The release rate during the linear phase was similar for both formulations, i.e. about 0.7 %/d. Consequently, the "b/a" factor was lower for formulation *"Co-solvent (10 %) NMP, PLA-AI(1:1)"* than for formulation *"PLA-AI(1:1)"*, with values of 9.8 and 19.5, respectively .

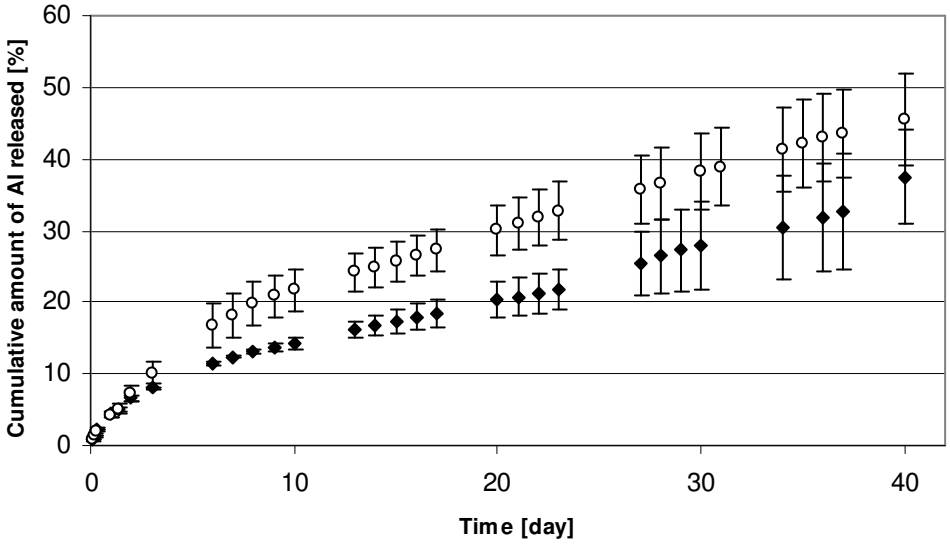


Figure 3.11: Influence of co-solvent NMP on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ*, related to time. Formulation *"PLA-AI(1:1)"* (O) contained triacetin as unique solvent. In formulation *"Co-solvent (10 %) NMP, PLA-AI(1:1)"* (◆), 10.00 % triacetin was substituted with NMP.

3.2.4.5. Influence of ethanol as co-solvent

The effect of 10 % absolute ethanol as co-solvent on the *in-vitro* release from sustained release injectables formed *in-situ* was studied by comparing the release profiles of the formulations *"PLA-AI(1:1)"* and *"Co-solvent (10 %) ethanol PLA-AI(1:1)"*. The amounts of PLA polymer and active ingredient were constant (17.00 %) in both formulations. There were only changes in the solvent which consisted of 66.00 % of triacetin in formulation *"PLA-AI(1:1)"* and of 56.00 % of triacetin and 10.00 % of ethanol in formulation *"Co-solvent (10 %) ethanol, PLA-AI(1:1)"*. For composition details, see Tables 3.1 and 3.9.

The release of active ingredient from both formulations was quite similar. The drug amount released during the 40 days of the experiment was 43 % for formulation *"PLA-AI(1:1)"* and 52.2 % for formulation *"Co-solvent (10 %) ethanol PLA-AI(1:1)"*. However, the slight initial burst effect observed for formulation *"PLA-AI(1:1)"* practically disappeared for formulation *"Co-solvent (10 %) ethanol PLA-AI(1:1)".* The drug release was linear from the second day after application and reached a release rate of 1.25 %/d. The drug release profile converged to the desired zero order kinetic with a "b/a" factor of 1.9. Formulation profiles of *"PLA-AI(1:1)"* and of *"Co-solvent (10 %) ethanol PLA-AI(1:1)"* are represented in Figure 3.12.

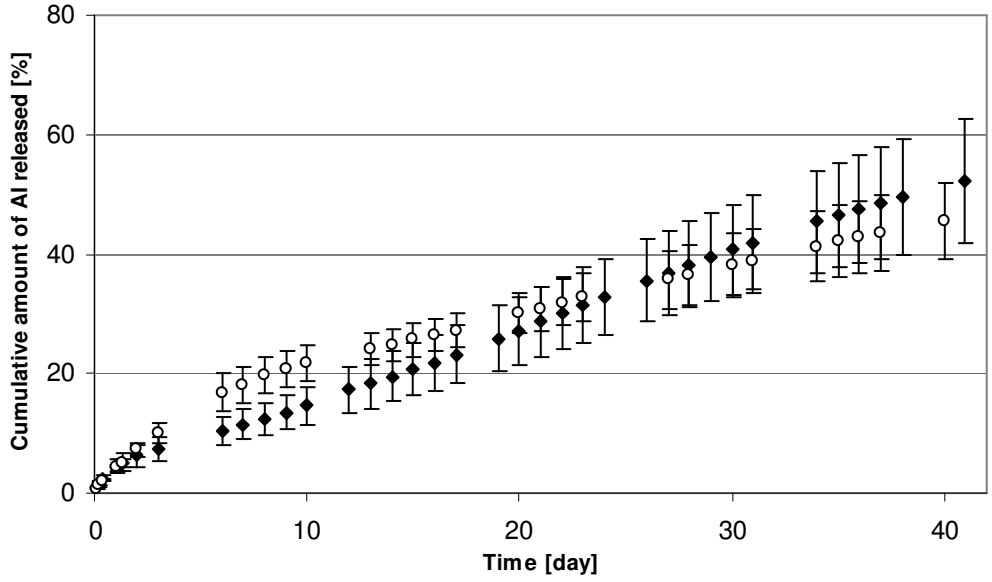


Figure 3.12: Influence of co-solvent ethanol on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ,* related to time. Formulation (O) contained triacetin as unique solvent, while in formulation (◆) 10.00 % of triacetin was replaced by ethanol.

Two additional formulations with 10.00 % ethanol as co-solvent and containing 12.75 % PLA polymer ("*Co-solvent (10 %) ethanol PLA-AI(3:4")* and 8.5 % PLA polymer ("*Co-solvent (10 %) ethanol PLA-AI(1:2")* were produced. The concentration of active ingredient was maintained at 17.00 %, leading to polymer to drug ratios of 3:4 and 1:2, respectively. Their release profiles were compared with the formulation *"Co-solvent (10 %) ethanol PLA-AI(1:1)"* which contained also 10.00 % ethanol as co-solvent and 17.00 % PLA polymer. The *in-vitro* release profiles of both new formulations were determined over a period of 120 days.

The three release profiles are represented in Figure 3.13. They showed high initial burst effects with a drug release after 7 days of 31 % for formulation "*Co-solvent (10 %) ethanol PLA-AI(3:4)"* and 50 % for formulation "*Co-solvent (10 %) ethanol PLA-AI(1:2)"*. The release rates of the second linear phase were of 0.3 %/d and 0.1 %/d, which resulted in high "b/a" factors of 129.7 and 505.3, respectively. The total drug released into the medium after 120 days was 70.6 % for "*Co-solvent (10 %) ethanol PLA-AI(3:4)"* and 64.9 % for "*Co-solvent (10 %) ethanol PLA-AI(1:2)"*.

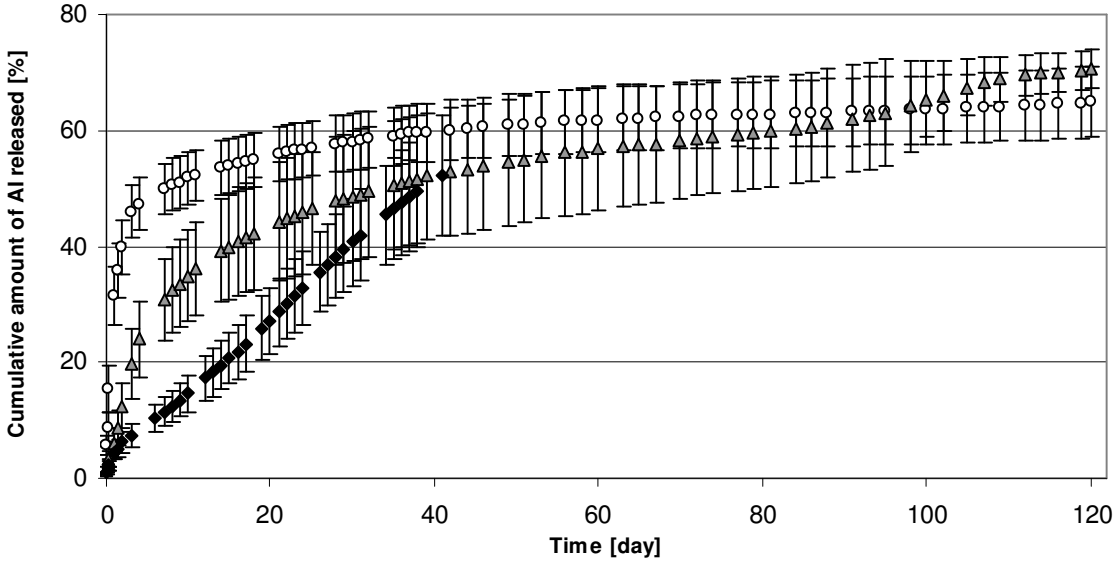


Figure 3.13: Influence of polymer load on release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ,* related to time. All three formulations contained 10.00 % ethanol as co-solvent. The amount of PLA polymer was 17.00 % (◆), 12.75 % (▲) and 8.50 % (O).

3.3. Ternary phase diagrams

3.3.1. PLA precipitation

Ternary phase diagrams were drawn to determine the necessary amount of demineralised water needed to induce PLA polymer precipitation. The exact composition of the mixtures based on the solvents triacetin or NMP at precipitation of the PLA polymers are represented in Figure 3.14. The compositions at the left of the precipitation curves are single phase systems while compositions at the right of the curves are two-phases systems with precipitated polymers and dissolved polymers. For NMP based polymer solutions, the higher the PLA polymer concentration of the initial solution was, the less water was needed to reach precipitation. The quantity of water needed for precipitation varied from 0.5 % to 11.9 %. For triacetin based polymer solutions, the amount of water needed for precipitation also reduced with increased polymer concentration of the initial solution. However, the amount of water needed to induce polymer precipitation remained in a range between 3.2 % and 4.3 %.

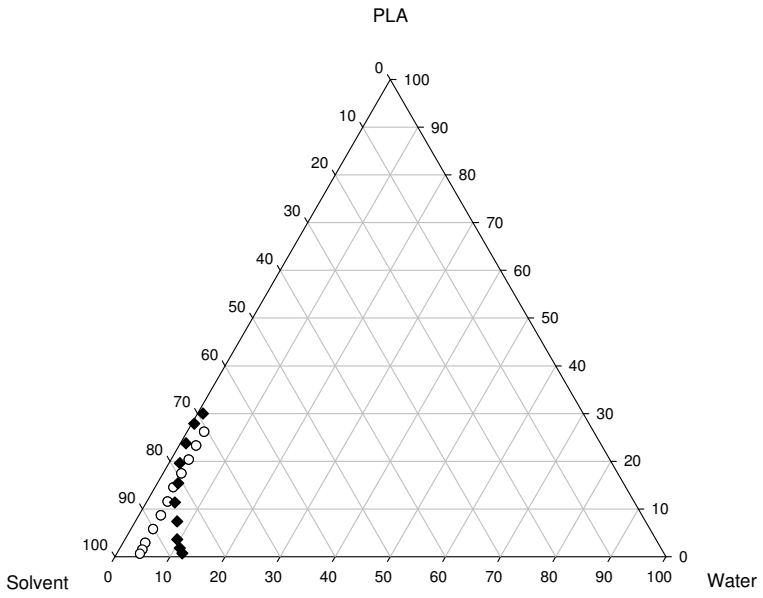


Figure 3.14: Ternary phase diagram of polymer precipitation as a function of PLA polymer, solvent and water concentration [% (w/w)]. The solvents used were triacetin (O) and NMP (◆).

Further ternary plots were constructed to investigate PLA polymer precipitation when water was added to polymer solutions based on various solvent mixtures. PLA polymers dissolved in triacetin mixed with 10 % of diverse co-solvents showed other precipitation properties as when dissolved in triacetin only. The co-solvents used were ethanol absolute, Miglyol 812 and glycerol formal. The compositions of all mixtures at precipitation of the PLA polymers are represented in Figure 3.15. When the lipophilic co-solvent Migylol 812 represented 10 % (w/w) of the solution, less water was needed for precipitation as without co-solvent. The water added to the Miglyol 812 containing polymer solution to observe PLA precipitation represented 0.7 % to 3.4 % of the mixture, while 3.2 % to 4.3 % of water were necessary for polymer precipitation when dissolved in the sole solvent triacetin. On the other hand, precipitation required more water when a hydrophilic co-solvent was added to the polymer solution. This was the case with the co-solvent ethanol absolute and with the co-solvent glycerol formal. For ethanol absolute containing solutions, the amount of water needed to reach PLA precipitation represented 6.1 % to 10.5 % of the mixture, while it represented 5.0 % to 7.2 % for glycerol formal containing polymer solutions. For all solvent mixtures, the quantity of water required for PLA precipitation slightly decreased as the PLA concentration was increased in the initial mixture.

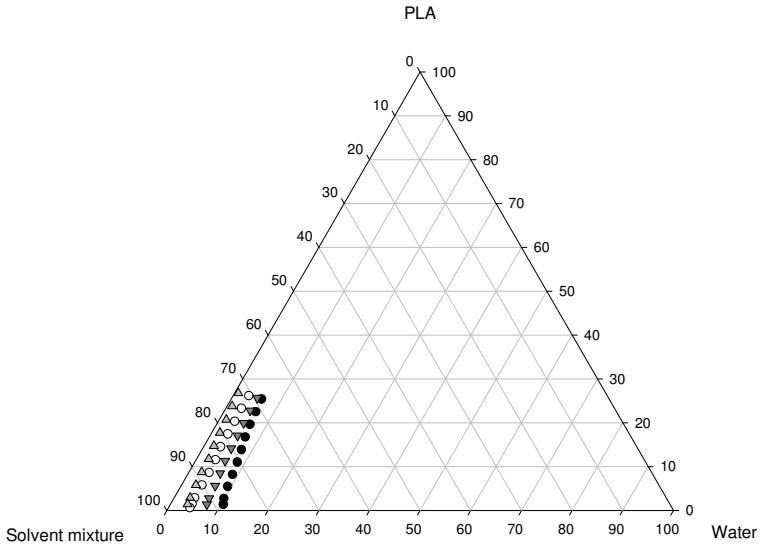


Figure 3.15: Ternary phase diagram of polymer precipitation as function of PLA polymer, solvent mixture and water concentration [% (w/w)]. The solvent mixtures were triacetin alone (○), triacetin/Miglyol 812 (△), triacetin/ethanol (●) and triacetin/glycerol formal (▽).

3.3.2. Active ingredient precipitation

The ternary phase diagrams for both systems active ingredient/triacetin/water and active ingredient/NMP/water are shown in Figure 3.16. The amount of water needed to observe active ingredient precipitation increased with increased concentration in the initial mixture. The higher the drug concentration of the initial solution was, the less water was needed to reach precipitation. The quantity of water needed for active ingredient precipitation in NMP based solutions varied from 11.5 % to 33.2 %. For triacetin based drug solutions, the amount of water needed to induce polymer precipitation was in a range between 3.4 % and 21.5 %.

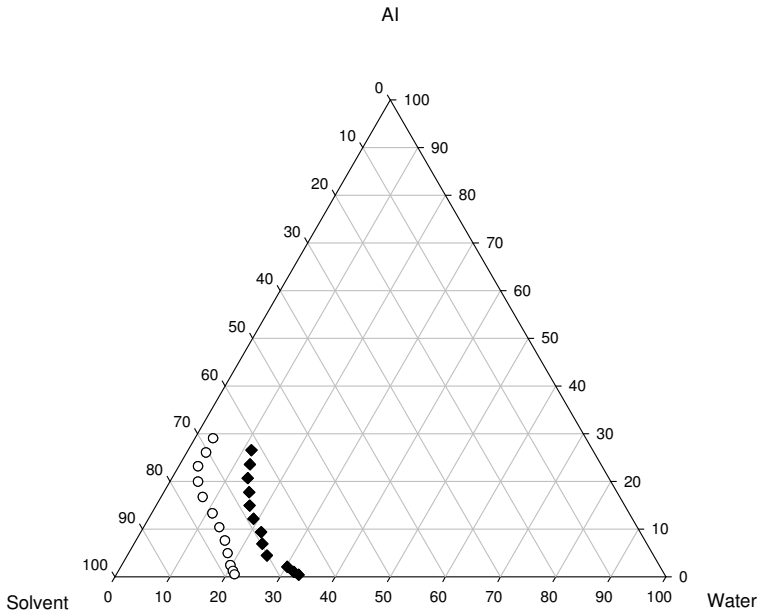


Figure 3.16: Ternary phase diagram of active ingredient precipitation as a function of NOA449851, solvent and water concentration [% (w/w)]. Solvents used were triacetin (O) and NMP (◆).

3.4. Raman spectroscopy

Raman spectroscopy was used to investigate conformational morphology of *in-vitro* solidified implants. The analysis focused mainly on possible polymorph conformations of the active ingredient in the solid implants as well as detection of possible interactions between the active ingredient and the polymer matrix. The investigated solid implants were gendered from the formulations "*PLA-AI(1:1)*" (curve a), "*Co-solvent (10 %) GF, PLA-AI(1:1)*" (curve b), "*Co-solvent (10 %) ethanol, PLA-AI(1:1)*" (curve c), "*Co-solvent (10 %) Miglyol, PLA-AI(1:1)*" (curve d).

Raman spectra of implants obtained after *in-vitro* solidification are illustrated in Figure 3.18 in a wavelength range from 300 cm^{-1} to 4000 cm^{-1}. The intensity of the Raman signal of each component was not identical for every samples, as the composition of each implants was different. Various solvent mixtures were used, and the active ingredient content of each implant was different due to the various release properties of each implant. However, the signal for the amorphous conformation of the active ingredient, as determined in Chapter 2, was consistent in each solid implant (dotted line). Furthermore, the Raman bands position of all component remained unchanged, indicating no detectable interaction. Therefore, no polymorphic conformation change or interaction between the active ingredient and the polymer matrix were found.

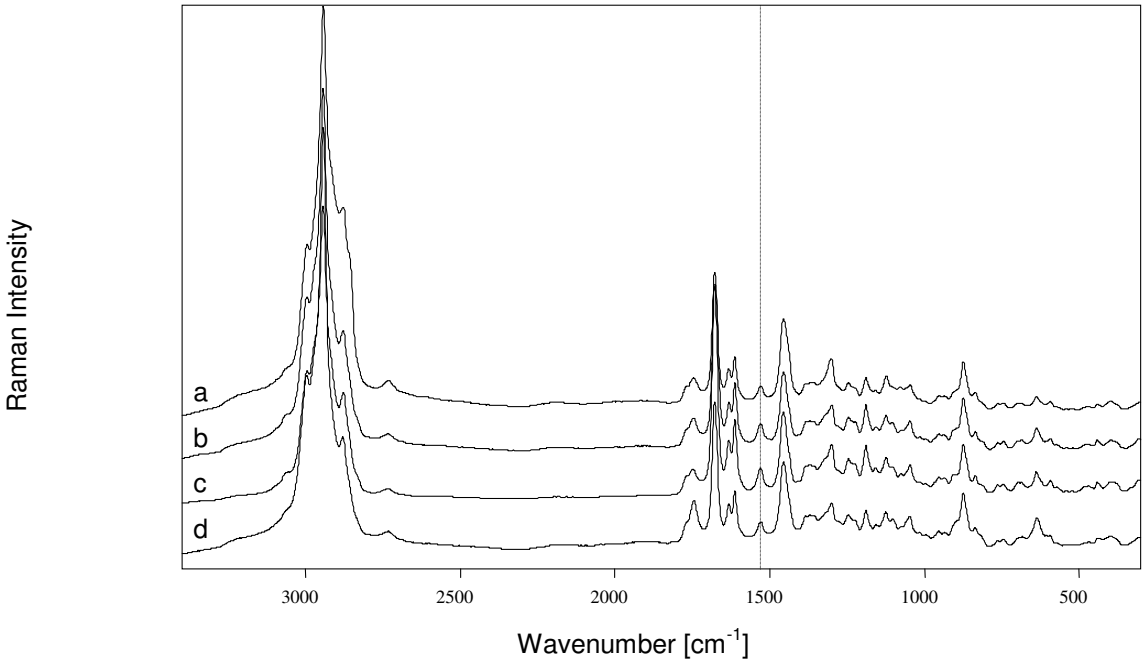


Figure 3.18: Raman spectra plotted in the region between 4000 cm^{-1} and 300 cm^{-1} of the *in-vitro* solidified implants from formulations "*PLA-AI(1:1)*" (a), "*Co-solvent (10 %) GF, PLA-AI(1:1)*" (b), "*Co-solvent (10 %) ethanol, PLA-AI(1:1)*" (c), "*Co-solvent (10 %) Miglyol, PLA-AI(1:1)*" (d).

4. Discussion

4.1. Dissolution test method

In order to investigate the *in-vitro* drug release from sustained release injectables formed *in-situ*, various dissolution tests were carried out. At the present time, no *in-vitro* method is described in the Pharmacopoeia for characterisation of such formulations. Therefore, a modified USP basket method was applied, using felt bags to prevent the liquid formulation from running out of the basket before solidification of the implant. This modified method has already been used previously for characterisation of release profiles from sustained release formulations formed *in-situ* and it showed sufficient discriminatory criteria to be further recommended [Matschke, 2002].

As the active ingredient NOA449851 is insoluble in water, it was important to determine an appropriate medium for the dissolution tests. *In-vitro*, the release rate depends on the active ingredient properties and on dissolution test conditions. These conditions can be modified to improve active ingredient solubility by adding alcohol or another organic solvent to the aqueous medium, or by increasing the agitation rate. Nevertheless, these methods lack physiological relevance. On the contrary, surfactants can be successfully added to the medium for dissolution tests without losing the physiological relevance, since the body has natural surfactants which help dissolving drugs with low water solubility [Noory et al., 2000; Shah et al., 1989].

The surfactant Solutol HS 15® (polyethylene glycol-660-12-hydroxy stearate) was selected to increase the solubility of the active ingredient in water. Therefore, the solubility of NOA449851 was measured in aqueous medium containing Solutol HS 15® at concentrations varying from 0 % to 10 % (w/w). The addition of Solutol HS 15® led to a slight but sufficient increase of solubility of the active ingredient in water. In pure water, its concentration was below the detection limit of the HPLC method. A concentration of 0.04 % of active ingredient is reached in an aqueous medium containing 3 % Solutol HS 15®. A concentration of 5 % Solutol HS 15® in water raised the maximal concentration of NOA449851 up to 0.08 %. The active ingredient solubility in an aqueous medium with 10 % Solutol HS 15® was 0.22 %.

The concentration of surfactant needed to be adjusted to maximise the sensitivity of the method without losing physiological relevance. One litre of an aqueous solution containing 5 % Solutol HS 15® was found to be an optimal dissolution test medium for NOA449851. The medium was replaced with a new solution to guarantee sink conditions as soon as the active

ingredient concentration reached 0.01 %. Medium replacement is often used to study implant release characterisation when the dissolution testing lasts for several weeks. When the entire volume of dissolution medium is replaced with fresh solution, it is important that the dosage form remains intact so that no undissolved drug particles are lost [Rohrs et al., 2001].

4.2. Drug release profiles

The *in-vitro* development studies were designed to ascertain the influence of critical parameters and variables on the final release characteristics of the formulation. The parameters included were diverse polymer types, the concentration of the components of the formulations as well as the use of co-solvents.

In the present experiments, the release profiles of NOA449851 from biodegradable PLA/PLGA polymer implants formed *in-situ* showed generally two distinct phases. The first phase, also called *initial burst*, is dependent on the solvent movements out of the implant formulations during the precipitation of the polymer. In addition, it is also related to the implant forming process. Directly after the injection into the basket of the dissolution apparatus, the solvents diffuse from the formulations into the aqueous dissolution medium, dragging the solved active ingredient with them. The burst is usually not desired because the drug which is released during this phase is not available for prolonged release. Even more important for potent substances or drugs with a narrow therapeutic window, this sudden release of a high drug dose may result in toxicity or other side effects.

After injection of the formulation into the aqueous medium and after the solvent had diffused out of the formulation into the outer aqueous medium, the active ingredient is entrapped in the precipitated polymer matrix. The drug release after this time point, which is also called the *linear phase,* is attributed to a mixed drug diffusion and polymer erosion process [Miyajima et al., 1997; Shah et al., 1992].

The transition from the initial burst phase to the mixed diffusion and biodegradation controlled phase was indicated in the drug release profiles by the decline of the release curves. From these considerations can be deduced that a simple first order process was not sufficient to describe the release behavior of drugs from bulk degrading polymers. Therefore, a factor “b/a” was introduced for comparison of the various drug release profiles. A high “b/a” factor describes a profile with a substantial initial burst effect and a low release rate during

the linear phase. For a profile of zero order kinetic without any initial burst, the “b/a” factor would converge to the ideal value of 1.

The mathematical results of all release profiles are listed in Table 3.11. The “b/a” factors can be divided in several groups, depending on the composition of the formulations. The “b/a” factor can be easily obtained from the graphical plots and allows rapid and objective comparisons of numerous release profiles. The formulation based on the solvent NMP reached a “b/a” factor of 67.0, while formulation based on glycerol formal reached a “b/a” factor of 30.1. For the formulations based on the solvent triacetin, and when the polymer to drug weight ratio was 1:1, all “b/a” factors were lower than 20. For all other polymer to drug ratios tested, the “b/a” factor was over 100, which is far away from the targeted drug release of zero order kinetic. There was no difference if the polymer or the active ingredient was in excess. Formulations with a polymer to drug weight ratio of 1:1 and containing co-solvents showed a variety of results. A “b/a” factor of 241.6 was obtained for the formulation containing the lipophilic co-solvent Miglyol 812. For the formulations with hydrophilic co-solvents, all “b/a” values were lower than 30, with an excellent result for the formulation containing absolute ethanol which reached the value of 1.9. Further formulations with ethanol as co-solvent were investigated, with polymer to drug weight ratios of 3:4 and 1:2. Substantial burst effects led to high “b/a” values of 129.7 and 505.3, respectively.

4.2.1. Influence of solvent type

In order to investigate the influence of the type of solvent on the drug release profile, three solvents were tested. Triacetin, NMP and glycerol formal were the excipients investigated as solvent in the sustained release formulations formed *in-situ*. It was found that the triacetin formulation *“PLA-AI(1:1)”* had a profile with a relative small initial burst and a great release rate during the linear phase. Formulations *“NMP; PLA-AI(1:1)”* and *“GF; PLA-AI(1:1)”* led to higher initial bursts, followed for the NMP based formulation by a linear phase with a lower release rate (Figures 3.2 and 3.3).

The negative partition coefficient of NMP (log P = -0.38) indicates that this solvent is highly water-miscible and therefore, after injection, diffused rapidly into the dissolution medium. This resulted in a high initial burst effect because the dissolved active ingredient is flushed out of the formulation simultaneously with the solvent. Apart from that, conditions leading to rapid phase separation, by decreasing the affinity between the solvent and the non solvent (here the dissolution medium) also promote the formation of a thick skin at the surface of the polymer-matrix [Graham et al., 1999]. It is then difficult for the active to diffuse through such

a dense barrier, and this may explain why the release rate during the second phase is reduced.

Because triacetin is a short chain triglyceride, it is only sparingly miscible with water (log P = +0.25). Its use in the formulation decreased the affinity between the water and the polymer solution which resulted in a decrease in the water influx rate during implant formation. Conditions leading to a delayed precipitation promote more uniform sponge- like sublayers in the body of the implant. This is the case when triacetin is used as solvent. The final morphology of the implant had a direct influence on the resulting transport characteristics [Graham et al., 1999].

The drug release profiles illustrated in Figure 3.2 are therefore in accordance with the predictions of the different authors [McHugh et al., 1999; Graham et al., 1999; Brodbeck et al., 1999 (1), (2); Yewey et al., 1997]. NMP as a completely water miscible and strong solvent for the PLA polymers leads to a rapid implant solidification and consequently to a higher burst effect than the solvent triacetin.

4.2.2. Different polymer types

In order to study the influence of polymers with different biodegradation properties on the *in-vitro* release of NOA449851 from sustained release injectables formed *in-situ*, formulations based on PLA, 85/15 PLGA and 75/25 PLGA polymers were investigated. As reported in the literature, the hydrolysis of the polymers, mostly in presence of a minor amount of either lipophilic or hydrophilic drug, occurs in a period of about three months for 75/25 PLGA polymers, of about five months for 85/15 PLGA polymers and between six and twelve months for PLA polymers [Wang et al., 1997; Middleton et al., 2000]. The increase in lactic acid content decreases the degradation rate by reducing water uptake and making the ester bond less accessible to water, due to the hindrance by the bulky methyl group [Göpferich, 1996].

The polymer to drug weight ratio was 1:1 in the experiment represented in Figure 3.4, which is extremely high compared to the cited references. Surprisingly, no significant differences between the three types of polymer were observed. Due to the erosion properties of the different types of biodegradable polymers, it was expected that the release of active ingredient during the second phase, the mixed diffusion and erosion process, occurred faster when the glycolic unit ratio in the polymers became higher. The release rate of the active

ingredient during the linear phase was similar for all three formulations, varying from 0.73 %/d for PLA, to 0.72 %/d for 85/15 PLGA and to 0.75 %/d for 75/25 PLGA.

In order to investigate whether the identical release rates were determined by the high drug content which influenced the break down of the polymers, the dissolution tests were repeated with a lower amount of drug in the formulations. The high concentration of drug substance at the surface of the implant may have minimised the biodegradation effects of the different types of polymer and reduced the water penetration rate. Again, even with a polymer to drug weight ratio of 5:1 instead of 1:1, all three curves were very similar and no significant differences were observed during the linear phase (Figure 3.5).

Obviously, the effects of the various biodegradation properties of PLA/PLGA polymers at the studied polymer to drug weight ratio of 5:1 and 1:1 on the release characteristics of NOA449851 were less important than expected. The experiments were stopped 40 days after injection into the dissolution apparatus. The erosion effects may have been more evident after a longer period.

An additional experiment was carried out to study the effect of the polymer type on the drug release. The amount of active ingredient was further reduced to obtain a polymer to drug weight ratio of 100:1. As represented in Figure 3.6, the drug release was similar for all three formulations during the first half of the experiment. An initial burst effect as well as a second linear phase were observed for all three formulations. The second phase ended for the formulation based on 75/25 PLGA at day 20, followed by a fast and exponential drug release. For the formulations based on PLA and 85/15 PLGA, the second phase continued until the end of the dissolution test experiment.

The profile of formulation *"75/25 PLGA(100:1*)" showed a triphasic drug release, also referred to as the S release pattern phases. This has already been described for formulations based on biodegradable polymers, particularly concerning the release from microspheres [Wang et al., 1996; Cleland, 1997]. The release of the active ingredient is then controlled by erosion of the matrix and can be modified by choosing the appropriate chemical composition of the polymer. In the particular experiments, the effect of the polymer type can only be seen when the amount of active ingredient is decreased. The lipophilic properties of the active ingredient may reduce the penetration of water into the polymer matrix, leading to reduced erosion due to hydrolytic break of the ester bonds of the polymer. By reducing the loading of lipophilic drug in the implant, water can better enter the matrix and erode the polymer.

The conclusion of these series of experiments is that the drug release from sustained release injections formed *in-situ* was probably mainly due to diffusion phenomena. Only the third phase of the *"75/25 PLGA(100:1*)" was effective matrix erosion controlled drug release. If erosion is slower than diffusion process, then the polymer controls the release by diffusion. Only if degradation is the fastest process, the drug release may be erosion controlled. Many systems made of degradable polymers are not truly degradation controlled drug delivery devices and their only advantage consists in the disappearance of the drug carrier through degradation, circumventing its removal [Göpferich, 1996].

4.2.3. Active ingredient load

The effect of active ingredient load was examined by comparing release profiles of formulations *"PLA-AI(1:1)"*, *"PLA-AI(5:1)"* and *"PLA-AI(100:1)"*, containing, respectively, 17.00 %, 3.40 % and 0.17 % active ingredient. As the PLA content was maintained at 17.00 %, the resulted polymer to drug weight ratios were 1:1, 5:1 and 100:1, respectively. Their *in-vitro* drug release profiles were compared with regard to the "b/a" factor. The "b/a" factor of formulation *"PLA-AI(1:1)"* was 19.5, while formulation *"PLA-AI(5:1)"* had a "b/a" factor of 296.2 and *"PLA-AI(100:1)"* of 329.3. The release patterns of the formulation with the polymer to drug weight ratio 1:1 was significantly closer to the targeted zero order release kinetic than the two other profiles.

It is generally assumed that drug is released through the matrix by diffusion with a concentration gradient as driving force [McHugh et al., 1999; Graham et al., 1999; Dunn et al., 1996; Lambert et al., 1995]. If the release is mainly due to the Fickian's diffusion, then the driving force is generated by the concentration gradient. As the dissolution medium was changed to keep sink conditions, the formulation containing 17.00 % of drug had a bigger concentration gradient than the implant containing only 3.40 % of active ingredient. The diffusion phenomena during the linear phase was then much more important for formulation with the higher driving force and therefore the higher drug load.

Another reason for these important differences in drug release profiles was that the amount of the solvent triacetin in formulation *"PLA-AI(5:1)"* and *"PLA-AI(100:1)"* was higher than in formulation *"PLA-AI(1:1)"* to compensate for the reduced active ingredient content. A higher amount of solvent led to a more pronounced initial burst because the fluid movement out of the implant was faster. Therefore the amount of active ingredient flushed out of the matrix together with the solvent was increased.

Eliaz et al., using 75/25 PLGA polymers dissolved in glycofurol as sustained release formulation, also observed a release rate which was highly dependent on drug loading. They suggested that, for low loading levels, the protein release was predominantly controlled by matrix degradation. For high protein loadings, the formation of large and interconnected pores throughout the matrix were responsible for the faster release [Eliaz et al., 2000 (1), (2); Eliaz et al., 1997]. The hypothesis of formation of pores within the polymer matrix leading to a faster release was already mentioned by Shah [Shah et al., 1993] and Chandrashekar [Chandrashekar et al., 1996]. It is also possible in the present study that channels were formed in the polymer matrix, leading to a higher drug release for formulation containing higher active ingredient concentrations.

The effect of protein loading was also investigated by Yewey et al., by varying bovine serum albumin (BSA) concentrations from 0.01 % to 20 %. In contrast to the results obtained above, as the protein load was increased, a smaller percentage of the total protein in the formulation was released. The authors suggested an increased protein-protein interaction within the polymer matrix [Yewey et al., 1997].

4.2.4. Polymer load

In an attempt to modify the initial burst effect, release kinetics of drug from PLA formulations with different polymer loads were evaluated and followed over a period of 40 days. The release profiles of the formulations *"PLA-AI(3:4)"* and *"PLA-AI(1:2)"* containing, respectively, 12.75 % and 8.50 % of PLA polymer both showed very large initial burst effects which represented a drug release during the first 6 days of 60.13 % and 72.89 %, respectively. As can be seen in Figure 3.7, the *in-vitro* initial burst was almost eliminated by increasing the polymer concentration to 17.00 % as in formulation *"PLA-AI(1:1)"*.

At the initial phase, after injection of the polymer solution into an aqueous medium, the drug and the solvent diffuse out of the formulation into the surrounding medium. The higher the polymer concentration and therefore the viscosity are, the slower is the diffusion, which resulted in a lower burst. The influence of the polymer load on the *in-vitro* drug release is already widely described in the literature and appears to be a robust parameter. This effect has been described independent of the solvent used: NMP [Graham et al., 1999; Lambert et al., 1995], triacetin [Singh et al., 1997 (1); Chandrashekar et al., 1996; Shah et al., 1993] or glycofurol [Eliaz et al. 2000 (1); Eliaz et al. 1997]. It appears to be also independent if the polymer type used was PLA [Yewey et al., 1997], 75/25 PLGA [Eliaz et al. 2000 (1), (2)] or 50/50 PLGA [Singh et al., 1997]. The influence of the polymer load on the initial drug release

was described for hydrophilic active ingredients [Graham et al., 1999] as well as for lipophilic active ingredients [Singh et al., 1997 (1), (2)].

4.2.5. Use of co-solvents

As a further objective of the *in-vitro* experiments, the influence of co-solvents on the release behaviour was investigated. For these experiments various formulations were prepared, substituting a fraction of the main solvent triacetin with excipients of various properties. Miglyol 812 was used as lipophilic co-solvent, anhydrous glycerol, glycerol formal, NMP and ethanol were used as hydrophilic co-solvents.

Miglyol 812, a mixture of medium chain triglicerides, was added to the formulation as lipophilic co-solvent, replacing 10.00 % of the main solvent triacetin. As investigated in Chapter 2, Section 3.1, only 5.6 % of active ingredient could be dissolved in Miglyol 812. Miglyol 812 can only solubilze the PLA/PLGA polymers up to 1 % [Matschke, 2002].

As hydrophilic co-solvent, anhydrous glycerol was a candidate which was, like Miglyol 812, neither a good solvent for the active ingredient (Chapter 2) nor for the PLA polymers. Glycerol of anhydrous quality was used to prevent hydrolysis of the PLA polymers during storage. Two formulations were prepared, containing 5.00 % respectively 10.00 % of anhydrous glycerol. The poor miscibility of the co-solvent with triacetin resulted in the formation of emulsions, which were very viscous and therefore difficult to inject. The stability of the emulsions was also very critical, as it was mainly due to the high viscosity of the polymer phase. The addition of an emulsifier to stabilise the emulsion would have made a direct comparison impossible since two parameters would have been changed and the addition of tenside to polymer systems is known to increase the drug release rate [Graham et al., 1999; Yewey et al., 1997; Rohrs, 2001].

As further hydrophilic co-solvents, glycerol formal and NMP were tested. They showed good solubility properties for the active ingredient (Chapter 2) as well as for the PLA polymers [Matschke, 2002]. The formulations were clear solution of high viscosity.

Ethanol was the fourth hydrophilic co-solvent investigated. Apart from the very high solubilizing power for NOA449851, it reduced the viscosity of the formulation, which was very advantageous for the injection procedure.

Solvents like PEG 300 and propylenglycol, which are generally well tolerated excipients for parenteral application, were not tested as co-solvent for the sustained release injectables formed *in-situ*.

The effects of addition of co-solvent on the release profile were statistically relevant. Changes could be observed during the initial burst effect as well as during the linear phase. The addition of a lipophilic co-solvent like Miglyol 812 increased the initial burst (Figure 3.8) while the addition of hydrophilic co-solvents (anhydrous glycerol, NMP and ethanol) tended to minimise it (Figures 3.9, 3.11 and 3.12).

The effects were dependent also on the amount of co-solvent used, since the initial burst effect of formulations containing 10.00 % anhydrous glycerol was smaller than the burst of formulations containing 5.00 % of this co-solvent (Figure 3.8).

A slight increase of the release rate in the second phase was observed for the co-solvent glycerol anhydrous (0.84 %/d) while the release rate of the active ingredient was significantly accelerated with ethanol as hydrophilic co-solvent (1.25 %/d) in comparison with the formulation containing only triacetin as solvent (0.73 %/d). In contrary, the release rate during the linear phase was reduced for the formulation containing the lipophilic co-solvent Miglyol 812 (0.15 %/d) and for the hydrophilic co-solvent glycerol formal (0.45 %/d).

The lipophilic respectively the hydrophilic characteristics of the co-solvents had an obvious effect on the drug release from the implant. It can be assumed that the addition of 10.00 % of Miglyol 812 gave to the formulation a more lipophilic character and that the affinity of the active ingredient to the polymer system changed. On the other hand, the mixture of solvent and co-solvent had a different affinity to the dissolution medium and therefore different rate of fluid-convection and of the hardening process of the implant. As already seen in the drug release with NMP or glycerol formal as sole solvent (Figures 3.2 and 3.3), this parameter had a very strong influence on the hardening process of the implant, on the coagulation rate of the polymer and on the internal structure of the implant.

4.3. Phase diagrams

The interactions between polymer, solvent and non-solvent were evaluated through the construction of ternary phase diagrams. Analysis of ternary phase diagrams indicated that the quantity of water needed to initiate PLA precipitation increased with polymer concentration of the initial mixture. However, as illustrated in Figure 3.14, the influence of polymer concentration was much more important for NMP based solutions than for triacetin based solutions.

For Brodbeck et al., the nature of the solvent as well as the solvent/non-solvent miscibility are essential parameters for explaining the difference in the implant forming process. NMP is a strong solvent for PLA polymers and is completely miscible with water. These both factors accelerate the hardening process of the matrix, in contrast to solutions based on triacetin which is a solvent with lesser power and having a lower water miscibility [Brodbeck et al., 1999 (1)]. As a result of the limited water miscibility of triacetin, the system shows, in Figure 3.14, a significantly lower influence of the polymer concentration than for the NMP system.

The interactions between polymer, solvent and non-solvent were further studied with a fraction of the main solvent triacetin substituted with co-solvents. The amount of water needed to add for PLA polymer precipitation was found to be also slightly dependent on the solvent mixture. Figure 3.15 illustrated that for Miglyol 812 containing mixtures, less water was needed to initiate polymer precipitation than for mixture containing only triacetin as solvent. The lipophilic co-solvent Miglyol 812 is not soluble in water and is also not a good solvent for the PLA polymers. However, for mixtures containing the hydrophilic co-solvents ethanol absolute and glycerol formal, more water was needed to initiate polymer precipitation than for the mixtures containing only triacetin as solvent. Co-solvent glycerol formal is a strong solvent for PLA polymers, while co-solvent ethanol absolute is weak solvent for the polymeric excipients, but completely water miscible.

Interactions between active ingredient, solvent and non-solvent was also investigated for the two solvents triacetin and NMP. As for the PLA polymer system, the non-solvent was demineralised water. After injection of the formulation into the aqueous milieu not only the polymer but also the solved active ingredient precipitate to form the solid implant. As investigated in Chapter 2, the active ingredient was found not to be soluble in water. As illustrated in Figure 3.16, much more water was needed for NMP based mixtures than for triacetin based mixtures to observe drug precipitation.

4.4. Raman spectroscopy

Many types of polymer based drug delivery systems are in current use, with the polymer acting either as carrier or as rate-controlling modulator of drug release. The physicochemical properties of both the polymer and the active ingredient are critical parameters to the performance of these delivery system by determining the nature of the release process.

IR spectroscopy was not an adequate method for analysing the *in-vitro* solidified implants, as one of the limitation of use of this technique is the presence of water in the samples. Water is a very strong absorber and its total remove from the implants after solidification was technical not feasible. Therefore, Raman spectroscopy was applied for characterisation of the solid state of the implants. This method does not require specific preparation and also does not affect the morphological properties of the components in the implant. Raman spectroscopy is a technique which can be employed to monitor changes in the physicochemical properties of such systems by non-invasive analysis.

Strength and specificity of interactions between a drug and a polymer plays an important role in the dissolution rate of the drug. It is important to determine the molecular state of the drug within the polymer matrix, and how the drug interacts with the polymer matrix. Raman spectroscopy has already been successfully used for investigations of interactions between drug and polymer matrix [Taylor et al., 1997; Watts et al., 1991; Broman et al., 2001; Breitenbach et al., 1999; Tudor et al., 1990].

The difference spectra of the active ingredient loaded implants did not reveal any discernable modification as compared with their corresponding Raman patterns, as illustrated in Figure 3.18.

In the solid state of the implant, the active ingredient has always the same conformation, independently of the solvent or co-solvent used. It was concluded that no polymorphic form was present in detectable amounts in these samples. Based on Raman and X-ray diffraction patterns as described in Chapter 2, the active ingredient is in the amorphous conformation in all investigated implants. The hypothesis that the active ingredient dissolved in different solvent mixtures could have taken, after precipitation, different polymorphic conformation with various solubility properties was therefore not corroborated.

Also the PLA polymer had identical parameters for all investigated implants and did not show any influence due to the solvent composition. No interaction between polymer matrix and active ingredient was observed.

5. Conclusions

A dissolution test method was used to compare dissolution profiles of various sustained release injectables formed *in-situ*. The changes in the composition of the formulations concerned type of solvent, type of polymer, amount of polymer and of active ingredient as well as use of co-solvents. The comparison of the dissolution profiles showed little influence of the polymer type on the drug release during the dissolution test period of 40 days. Only when the content of the lipophilic active ingredient was reduced to reach a polymer to drug ratio of 100:1, drug release was dependent on the hydrolysis rate properties of the various polymer type used. The biodegradable polymers are known to have different hydrolysis kinetics properties and it was concluded that the drug release was mainly regulated, for formulations with polymer to drug ratios of 1:1 or 5:1 by a diffusion-controlled process rather than by an erosion-controlled process. The amount of polymer admixed to the formulation highly influenced the release profiles. The high viscosity of the formulations containing a high amount of polymer reduced the convection of liquids and solvents, with a probable influence on the rate of the hardening process of the implant. The initial burst effect was mainly influenced by this factor. A change in solvents or even the substitution of a fraction of the solvent with a co-solvent led to significant differences in drug release profiles. Physicochemical properties of the solvent and co-solvents like log partition coefficient, miscibility with the aqueous outer medium and viscosity are determining factors for the diverse movements of the fluids and for the implant forming process. The speed of hardening conferred to the implant the definitive matrix structure and release characteristics. The focus on bio-relevancy should be increased, with major interest in *in-vivo-in-vitro correlation* and therefore, *in-vivo* tests were needed.

Chapter 4

Injectability properties and pharmacokinetic data of sustained release injectables formed *in-situ* tested *in-vivo*

1. Objectives and introduction

One of the major tasks during development of sustained release injectables formed *in-situ* is to determine the active ingredient release patterns from the implants. Ideally, *in-vitro* drug dissolutions reflect properties of the *in-vivo* dissolution process. However, since this correlation is mostly unclear, it was the objective of this chapter to test the formulations in the target animal to assess the effective active ingredient release profiles. In addition, the pharmacokinetic properties and local tolerability of the injectables were investigated in detail.

Therefore, six formulations investigated *in-vitro* for drug release were selected to be applied to experimental animals. After subcutaneous administration in Beagle dogs, the blood concentrations of the active ingredient NOA449851 were measured during several months. The pharmacokinetic data were used for further characterisation of the subcutaneous implants. To test local tolerability, the related placebos were also injected to the same experimental animal and possible reactions were recorded with visual and palpatory examination.

As the formulations were highly viscous and therefore difficult to inject with standard needles, the rheological properties of the formulations were measured using a cone-plate rheometer for shear rates in a range from 1 s^{-1} to 100 s^{-1}. Additionally, a texture analyser apparatus was used for determining the force to be applied on the syringe as well as the total work required for the injection of the formulations through three different needle sizes within predetermined time frames.

2. Materials and methods

2.1. Materials

The drug substance NOA449851 was manufactured by CarboGen Laboratories, Aarau, CH. The PLA polymers (inherent viscosity: 0.68 dl/g in trichloromethane at 30°C) were purchased from Birmingham Polymers Inc., Birmingham, AL, USA. Triacetin, glycerol formal and anhydrous glycerol were obtained from Fluka AG, Buchs, CH. The solvents ethanol, acetonitrile, methanol and water for chromatography were purchased from Merck Inc., Darmstadt, D. All solvents were at least of reagent analytical grade.

2.2. Methods

2.2.1. Preparation of formulations and placebos

The sustained release injectables formed in-situ were prepared as already described in Chapter 3, Section 2.2.1.1. To assure sterility of the formulations, they were manufactured under aseptic conditions in a laminar flow bench (VS 120 AFX, Skan AG, Allschwil, CH). All solvents were filtered through sterile filters with 0.22 µm pore size, and all beakers and spatula were sterilized in the autoclave (Melag Autoklav, Typ 23, Berlin, D) before manufacturing.

All formulations contained 17.00 % (w/w) of the active ingredient NOA449851 beside PLA polymers and triacetin. The formulations differed in concentration of the PLA polymers (17.00 % (w/w) or 12.75 % (w/w)) as well as in the composition of the solvent mixture: a fraction of the main solvent triacetin was substituted with hydrophilic co-solvents such as ethanol, glycerol formal or anhydrous glycerol. In the placebos, the active ingredient was replaced by the equivalent amount of the solvent triacetin. The exact compositions of the formulations tested *in-vivo* are listed in Table 4.1.

Table 4.1: Composition [% (w/w)] of the sustained release injectables formed *in-situ* for *in-vivo* experiments in dogs.

Formulation	Component	Content in formulation [% (w/w)]	Content in placebo [% (w/w)]
A	NOA449851 PLA triacetin	17.00 17.00 66.00	- 17.00 83.00
B	NOA449851 PLA triacetin	17.00 12.75 70.25	- 12.75 87.25
C	NOA449851 PLA ethanol triacetin	17.00 17.00 10.00 56.00	- 17.00 10.00 73.00
D	NOA449851 PLA ethanol triacetin	17.00 12.75 10.00 60.25	- 12.75 10.00 77.25
E	NOA449851 PLA glycerol formal triacetin	17.00 17.00 10.00 56.00	- 17.00 10.00 73.00
F	NOA449851 PLA anhydrous glycerol triacetin	17.00 17.00 10.00 56.00	- 17.00 10.00 73.00

2.2.2. Viscosity of formulations and placebos

The high viscosity of the sustained release injectables formed *in-situ* is a challenging parameter to be adapted for applicability of the formulation as well as for acceptance of the patient. Therefore, the viscosity of the six formulations and placebos administered to experimental animals was determined using a cone-plate rheometer (StressTech Rheometer, Reologica, Lund, Sweden).

Measurements were carried out at shear rates in a range from 1 s^{-1} to 100 s^{-1} and at a constant temperature of 20°C. The viscosity of formulations and of placebo solutions was recorded for the shear rates 1 s^{-1}, 5.5 s^{-1}, 30 s^{-1} and 100 s^{-1}.

2.2.3. Syringeability of formulations and placebos

The force necessary to apply on the plunger of the syringe for injection of the formulation through a needle with a specific size was determined using a TA-XT2 texture analyser apparatus in compression mode (Stable Micro System, Surrey, UK). The formulations as well as the placebos were loaded into plastic syringes (Braun Inc., Melsungen, D) with a 16 G, a 18 G or a 19 G needle attached (Becton and Dickenson, Basel, CH). The force was applied to the syringe plunger by using the arm of the texture analyser. The piston moved during 5 seconds at a rate of 3 mm/s into the syringe. The pressure for delivery of the formulation was measured five times for every sample. The force applied versus injection time was graphically plotted and the area under the force-time curve was integrated to obtain the total work.

2.2.4. Experimental animals

A total of twelve healthy Beagle dogs of various age, breed, body weight and sex were selected. They were supplied by the CRA dog facilities of Novartis Animal Health Inc.. Dogs were identified by an individual collar number as well as a subcutaneous tag ("RID" RF Identification Technology by Data Mars® SA, CH-6814 Cadempino-Lugano, CH). The study was reviewed by an ethic committee according to the Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985) and the Swiss regulation for animal experiments (Schweizerisches Tierschutzgesetz).

2.2.5. Dose and method of administration

Each formulation was tested in two dogs, each dog receiving one injection with the active ingredient and the corresponding placebo. The NOA449851-containing injectables formed *in-situ* were administered subcutaneously behind the shoulder and over the ribs of the left side, while the related placebo solution was injected on the right side of the same animal. The supervising veterinarian administered all injections. A 18 G or 16 G needle was used for injection to the experimental animals, depending on the viscosity of the formulation.

The total injection volume per formulation was 2 ml, leading to a total amount of 400 mg NOA449851 per dog, corresponding to a dose of 40 - 44 mg/kg of active ingredient, depending on the body weight of the selected dog.

2.2.6. Clinical observation

At the beginning of the study all dogs were examined by a veterinarian. Only healthy dogs were included in the trial. The dogs were observed weekly with respect to their general health, behavior and appetite by the maintenance staff. During the whole duration of the experiment and especially on the day of injection, the dogs were monitored by the veterinarian, with special attention to possible general side effects and to local reactions at the injection site. Possible general side effects were described by quality and severity of respective reactions. Localized reactions at the injection site were measured by visual and palpatory examination. All qualitative characteristics, such as signs of inflammation, hyperthermia, painfulness, size and persistence of local changes, were recorded.

2.2.7. Collection of blood specimens

Blood samples were collected from the *vena jugularis* of each dog into sterile tubes (S-Monovette®, Medi-prax GmbH, München, D) of 2.7 ml volume containing EDTA as anticoagulant. Blood was collected at the following nominal time points: at pre-test and at 2 h, 8 h, 24 h, 48 h after administration of test items and continued weekly until 462 days for dogs receiving the formulations A and C, until 468 days for formulations B, 189 days for formulation D and 426 days for formulations E and F. The blood samples were kept frozen until HPLC-MS analysis.

2.2.8. Analytical method for S-1238

S-1238 is the major homologue and represents approximately 80 % of the active ingredient NOA449851. Besides S-1238, the active ingredient also contains approximately 20 % of the component S-1262. Only S-1238 was quantitatively analysed in the blood samples, by comparison with an internal standard solution of NOA409797.

2.2.8.1. Extraction method

After mixing 0.5 ml of blood sample with 50 µl internal standard solution (2500 ng/ml NOA409797 in acetonitrile), 0.5 ml water and 1.0 ml acetonitrile, the samples were centrifuged, the supernatant solution was extracted and washed, using a Bond Elut cartridge (Varian Inc., California, USA). Then, 2.0 ml acetonitrile were used for the elution before evaporation to dryness. The residues were dissolved in 125 µl of a mixture acetonitrile/water (75:25) and quantified by HPLC-MS.

2.2.8.2. HPLC-MS method

A LC-MS 1100 apparatus (Agilent, Basel, CH) was used for the quantitative analysis of the extracted samples. The column (Nucleosil 100-3, C18, 70/2, Macherey-Nagel, Düren, D) was maintained at a temperature of 40°C. The mobile phase consisted of a constant mixture of water, methanol and acetonitrile. The flow rate was kept at 0.5 ml/min during the whole analyze run. The injection volume of the samples was 10 µl. The samples were detected at a wavelength of 247 nm.

The positive ion mode of API-ES electrospray was used for the measurements. The parameters controlling the electrospray chamber were determined as follow: the fragmentor voltage was set at 200 V, the capillary voltage at 4500 V, the nebulizer pressure at 40 psi. The drying gas nitrogen was set to a rate of 10 l/min and at a temperature of 350°C.

The detected blood concentrations of S-1238, the major homologue of NOA449851, were graphically plotted over time for each dog separately.

3. Results

In order to determine the release characteristics of the active ingredient NOA449851 from sustained release injectables formed *in-situ*, *in-vivo* trials in Beagle dogs were initiated. Six formulations and their respective placebos were administered subcutaneously in twelve healthy dogs, and tolerability and blood profiles were followed during several months.

3.1. Viscosity of formulations and placebos

The viscosity of all formulations and placebos applied to the experimental animals was determined using a cone-plate rheometer. The viscosity of the different formulations varied in a large range from 0.4 Pa*s (placebo D at shear rate 1 s^{-1}) to 118 Pa*s (formulation F at shear rate 1 s^{-1}). The viscosity of the placebos was significantly lower than the one of formulations with active ingredient. This can be explained by the substitution of the active ingredient with the equivalent amount of the solvent triacetin in the placebos.

The amount of polymer greatly influenced the viscosity of the formulations. For example, the viscosity of formulation A with 17.00 % of PLA polymer was 49.0 Pa*s at shear rate 1 s^{-1},

while formulation B with only 12.75 % of PLA polymer had a viscosity of 12.3 Pa*s at the same shear rate. Similarly, the viscosity of formulation C with 17.00 % of PLA polymer dissolved in a solvent mixture consisting of triacetin and ethanol was 5.5 Pa*s at shear rate 1 s^{-1} while its related formulation D with only 12.75 % of PLA polymer had a reduced viscosity of 2.2 Pa*s at the same measurement conditions. The substitution of the solvent triacetin with hydrophilic co-solvents also modified the viscosity. Ethanol and glycerol formal tended to reduce the viscosity of the formulations, while anhydrous glycerol increased it dramatically. Formulations C and E had a viscosity of 5.5 Pa*s and 30.3 Pa*s at shear rate 1 s^{-1}, as formulation F had a viscosity of 118 Pa*s at the same measurements conditions.

All placebo PLA polymers solutions showed Newtonian rheological behavior, since the viscosity seemed independent of the shear rate. Also the active ingredient loaded formulations B, C and D showed the ideal Newtonian behavior, whereas formulations A, E and F decreased in viscosity at increased shear rates. This type of fluid comportment is referred to as shear thinning or pseudoplastic flow behavior.

The measured viscosity at the four pre-selected shear rate 1 s^{-1}, 5.5 s^{-1}, 30 s^{-1} and 100 s^{-1} are listed in Table 4.2.

Table 4.2: Viscosity of the *in-vivo* tested formulations and their respective placebos at four different shear rates.

Formulation	**Viscosity [Pa*s] at shear rate:**			
	1 s^{-1}	**5.5 s^{-1}**	**30 s^{-1}**	**100 s^{-1}**
formulation A	49.0	49.5	46.1	17.6
placebo A	7.2	7.3	7.4	7.2
formulation B	12.3	12.9	12.9	12.0
placebo B	2.3	2.3	2.3	2.3
formulation C	5.5	5.7	5.8	5.6
placebo C	1.4	1.4	1.5	1.5
formulation D	2.2	2.3	2.3	2.2
placebo D	0.4	0.5	0.5	0.5
formulation E	30.3	31.7	30.9	19.4
placebo E	5.3	5.5	5.5	5.4
formulation F	118	113	53.4	12.4
placebo F	12.4	12.6	12.2	11.1

3.2. Injectability of formulations and placebos

Formulations and placebos solutions were filled into syringes with a 16 G, a 18 G or a 19 G needle attached. The parameters for injections with the texture analyser apparatus were defined as follows: the arm pushed the plunger into the syringe at a rate of 3 mm/s during 5 seconds.

The texture analyser allowed to determine different parameters such as the maximal force during injection [N] as well as the total work [N*s]. The maximal force measured with the texture analyser increased with the decrease of diameter of the needle. It varied in a range from 2.0 N to 103.8 N for 16 G needles, from 4.9 N to 172.5 N for 18 G needles and from 6.5 N to 212.3 N for 19 G needles. The maximal force average (n = 5 ± S.D.) applied on the plunger of the syringe during injection procedure for each needle and formulation is depicted in Figure 4.1.

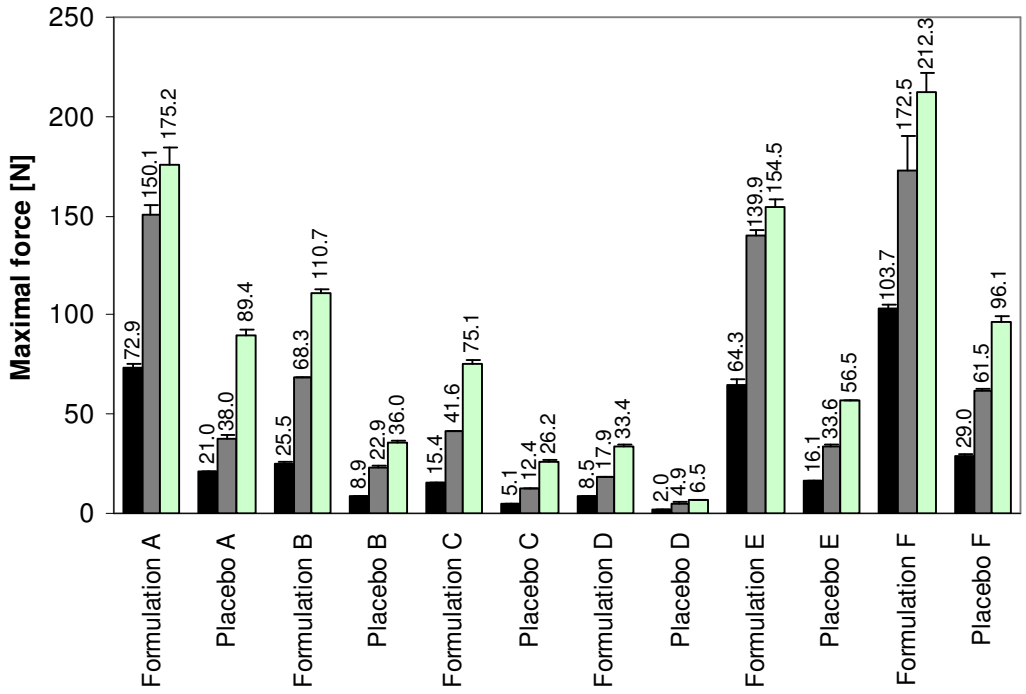


Figure 4.1: Maximal force average [N] measured with the texture analyser apparatus during injection of the formulations and placebos investigated *in-vivo* through 16 G (■), 18 G (■) and 19 G (□) needles (n = 5 ± S.D.).

The total work to be applied on the syringe plunger for delivery of the formulation was also determined by integrating the area under the force-time curve. As the force was not constant during injection procedure, the total work was not directly proportional to the maximal force. All the results are depicted in Figure 4.2.

The total work required from the texture analyser apparatus for pushing the plunge of the syringe during 5 seconds at a rate of 3 mm/s into the syringe varied in a range from 9.9 N*s to 421.3 N*s for 16 G needles, from 24.5 N*s to 613.5 N*s for 18 G needles and from 31.8 N*s to 764.2 N*s for 19 G needles.

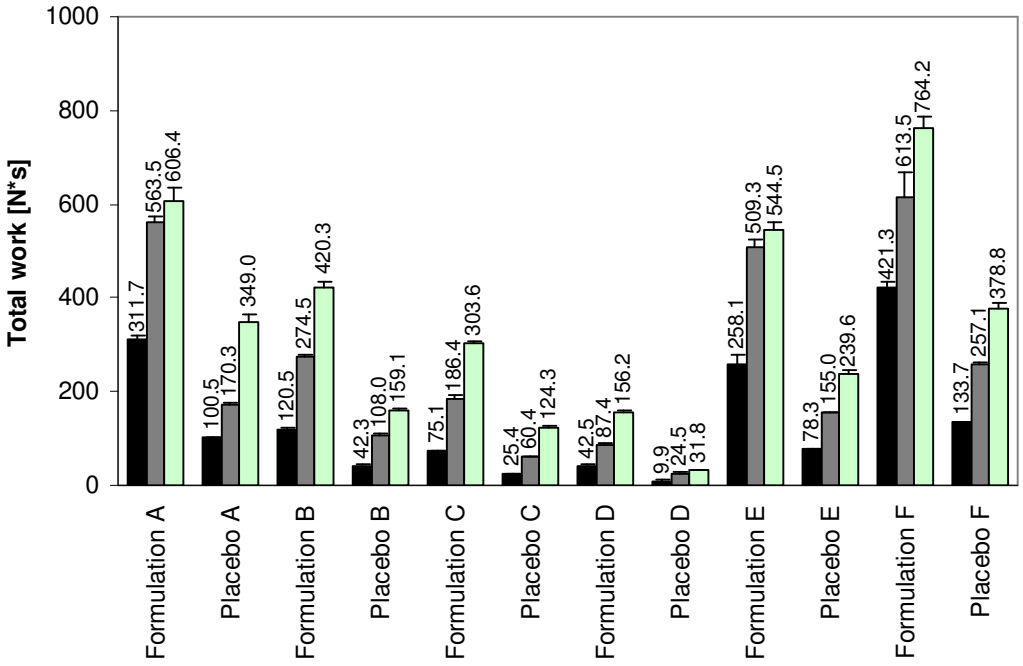


Figure 4.2: Total work required in average [N*s] for pushing the plunge during 5 seconds at a rate of 3 mm/s into the syringe, to which a 16 G (■), 18 G (▩) and 19 G (□) needle was attached (n = 5, ± S.D.).

The force applied to the plunger of the syringe did not remain constant but tended to increase during injection procedure. The less viscous the formulation, the sooner the arm of the texture analyser apparatus reached the maximal force to apply to the plunger. For the remaining distance to push, the force was maintained constant. An example of graphic of the injection force over the time for a relatively low viscous formulation is shown in Figure 4.3. The injected solution was the placebo solution D through a 19 G needle. The maximum force was reached within the first second of injection procedure.

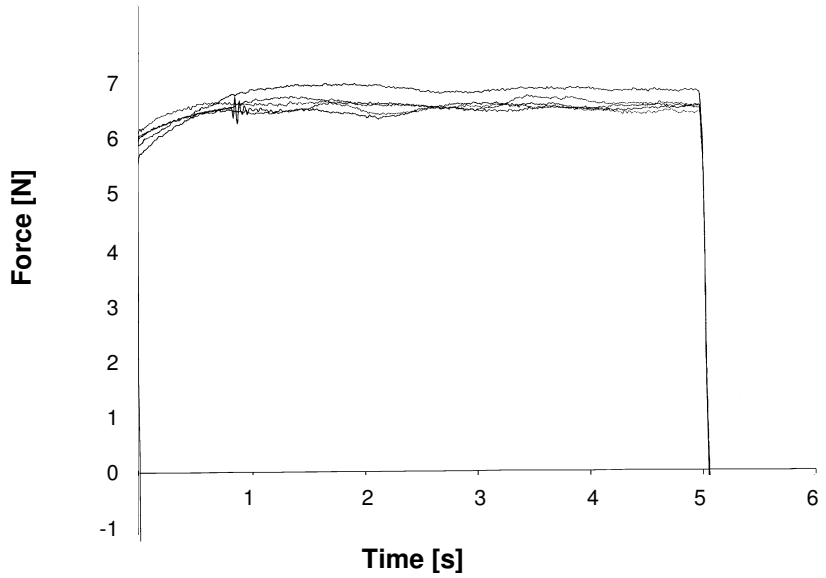


Figure 4.3: Force [N] to be applied on the syringe piston against time measured with the texture analyser for the placebo solution D through a 19 G needle as an example of a relatively low viscous formulation.

In case of highly viscous formulations, the maximal force is reached after a longer period of time. Figure 4.4 illustrates the plot "Force versus Time" of the injection of formulation A through a 18 G needle as an example of a highly viscous solution. The maximum force is reached with a delay of about 3 seconds.

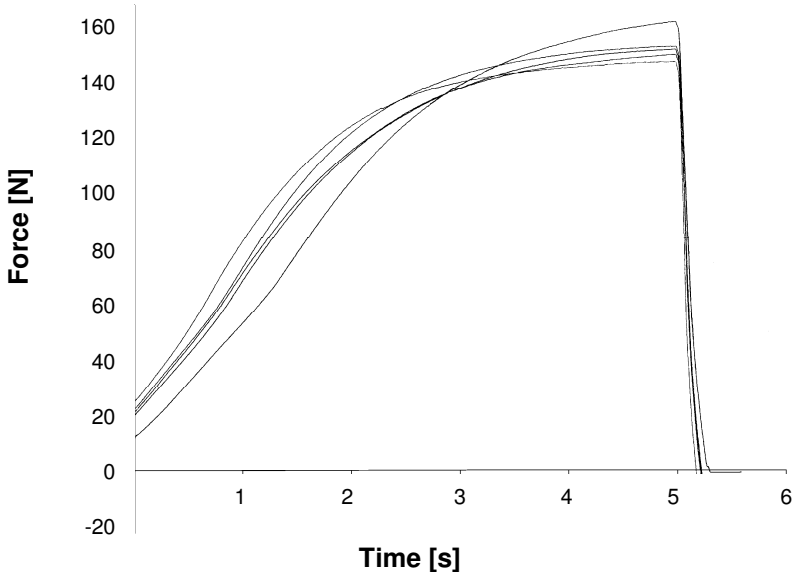


Figure 4.4: Force [N] to apply on the syringe piston against time measured with the texture analyser for the formulation A through a 18 G needle as an example of a relative highly viscous formulation.

For extremely highly viscous solutions such as formulations A, E or F, the force applied to the plunger when a needle with a reduced internal diameter (19 G) was attached did not result in the injection of the formulation through the needle, but rather in deformation of the plastic syringe due to the increase of the internal pressure.

In practice, a comfortable injection should not need more than 5 N for its application. No formulation was injectable with less than 8.5 N, by using the selected needles diameters. Only placebo solutions C and D were close to this value by using the largest investigated needle diameter (16 G).

3.3. Local and systemic tolerability

Each dog received 2 ml of formulation on the left shoulder and 2 ml of the respective placebo on the right side. Minor localized reactions at the injection site were observed. It consisted in slight and painless oedema which spontaneously shrinked within 1-2 days after the subcutaneous application. No systemic side effects were noticed in any dog.

3.4. Blood levels profiles

Six formulations investigated in Chapter 3 for *in-vitro* dissolution were selected for *in-vivo* trials to Beagle dogs. After subcutaneous injection of the formulations, the blood concentrations were determined over a period of several months. There were two major criteria to assess the results. First, the magnitude of the initial burst and second, the duration and consistency of the drug release above the therapeutic concentration estimated at 10 ng/ml.

Blood concentrations of S-1238 after application of formulation A to dogs # 2014 and # 2026 versus time are represented in Figure 4.5. Blood levels were measured during a period of 462 days. They reached a maximal blood concentration of 89.2 ng/ml and of 87.6 ng/ml, respectively during the first two days. A second burst was observed for dog # 2026 at day 52, leading to a blood concentration of 69 ng/ml. Blood levels almost one year (350 days) after application were 3.5 ng/ml and 1.8 ng/ml, respectively. However, as illustrated in the chart, there is a big difference in the blood concentration profiles of both dogs which have received the formulation A during the first 200 days of treatment.

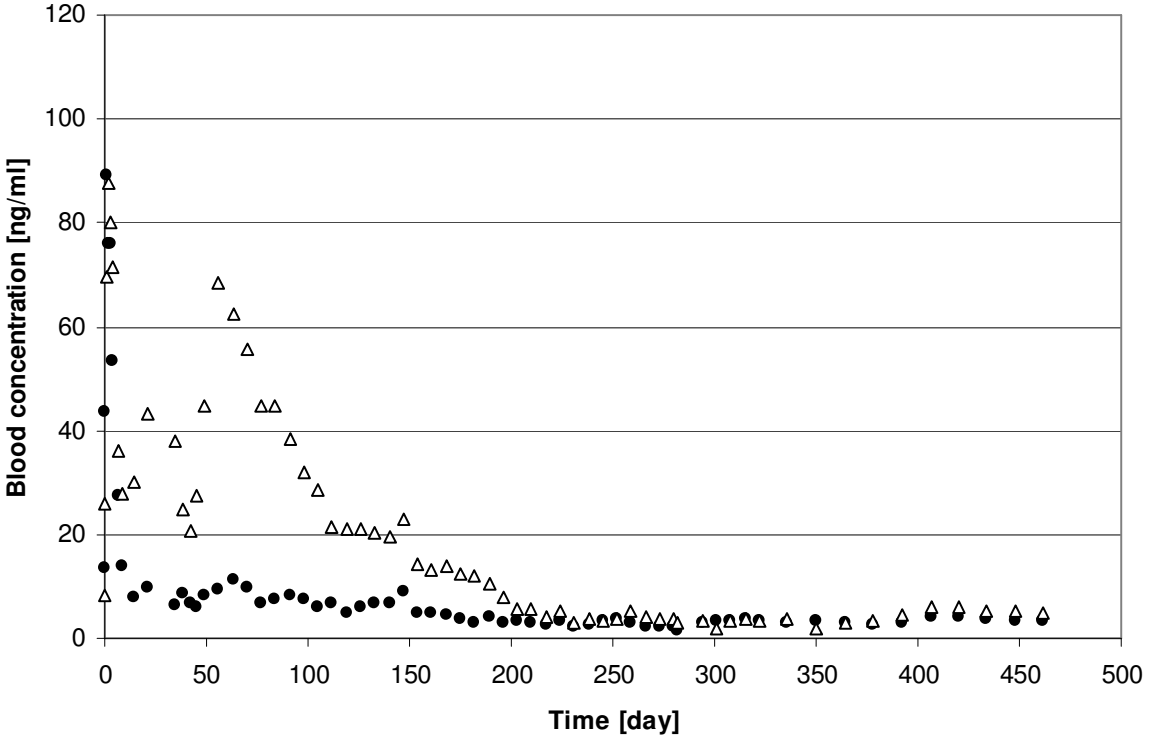


Figure 4.5: Pharmacokinetics of S-1238 [ng/ml], the major homologue of NOA449851, after subcutaneous injection of formulation A to dogs # 2014 (●) and # 2026 (Δ), measured over a period of 462 days.

Formulation B, which contained a reduced amount of PLA polymers in comparison to formulation A, was applied to dogs # 2030 and # 2032. Maximal blood concentrations were reached after 2 and 13 days, with concentrations of 92.7 ng/ml and 117.2 ng/ml, respectively. After almost one year (370 days), blood concentrations were found to be 9.1 ng/ml and 9.7 ng/ml, respectively. S-1238 blood profiles of both dogs which received formulation B are represented in Figure 4.6.

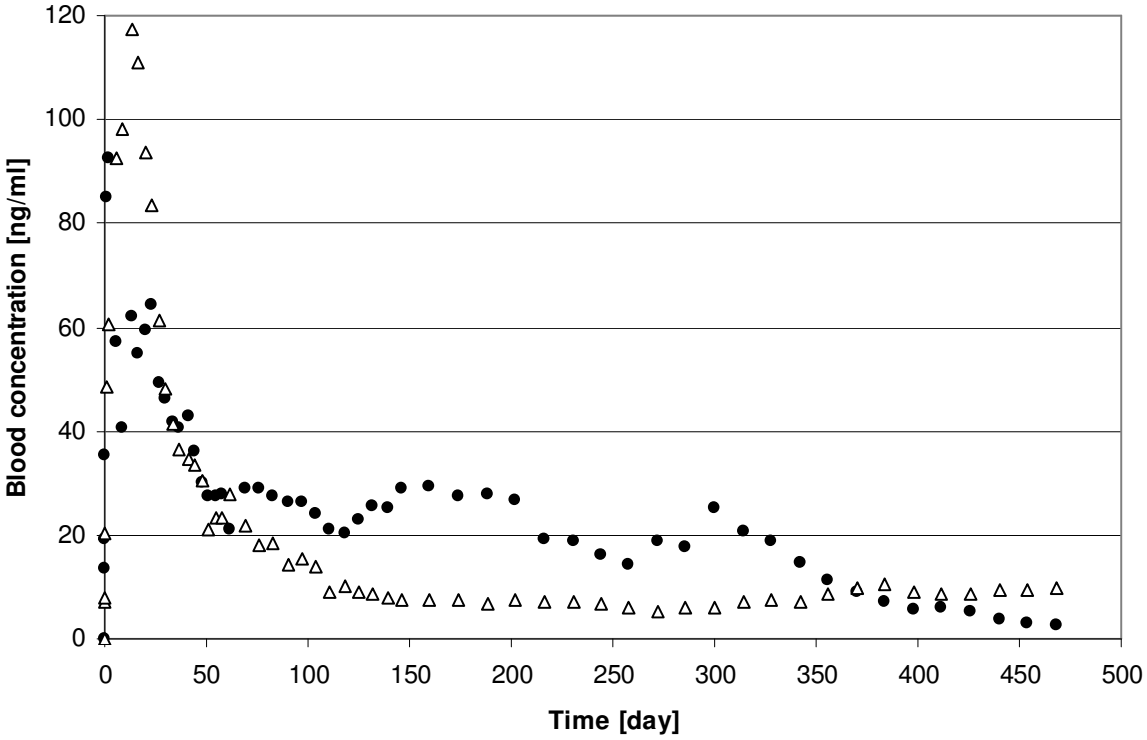


Figure 4.6: Pharmacokinetics of S-1238 [ng/ml], the major homologue of NOA449851, after subcutaneous injection of formulation B to dogs # 2030 (●) and # 2032 (Δ), measured over a period of 468 days.

Dogs # 2063 and # 2028 each received subcutaneously 2 ml of the formulation C, containing ethanol as co-solvent. The maximal blood levels S-1238 were measured 3 and 4 days after administration at blood concentrations of 44.2 ng/ml and 47.5 ng/ml, respectively. For dog # 2063, an additional and unexplained burst was observed after 158 days, reaching a blood concentration of 38 ng/ml. After almost one year (364 days), blood concentration was found to be 9.5 ng/ml for dog # 2063 and 1.5 ng/ml for dog # 2028. The blood level profiles of the experimental animals to which formulation C was applied are represented in Figure 4.7.

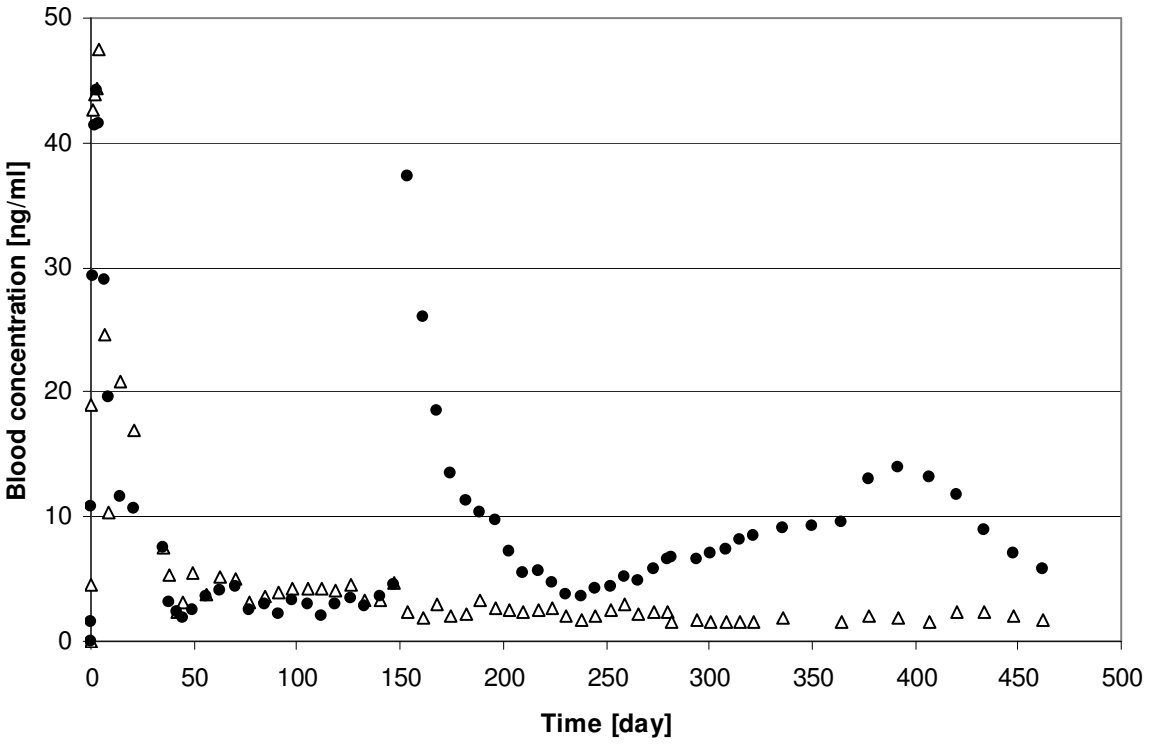


Figure 4.7: Pharmacokinetics of S-1238 [ng/ml], the major homologue of NOA449851, after subcutaneous injection of formulation C to dogs # 2063 (●) and # 2028 (Δ), measured over a period of 462 days.

In Figure 4.8, the blood profiles of both experiment animals (dogs # 957 and # 973) after administration of 2 ml of the formulation D are illustrated. The initial burst was observed at day 1 and 2, respectively, and blood levels reached concentrations up to 61.2 ng/ml and 48.9 ng/ml. After 49 and 91 days, respectively, blood levels decreased to concentrations lower than 10 ng/ml.

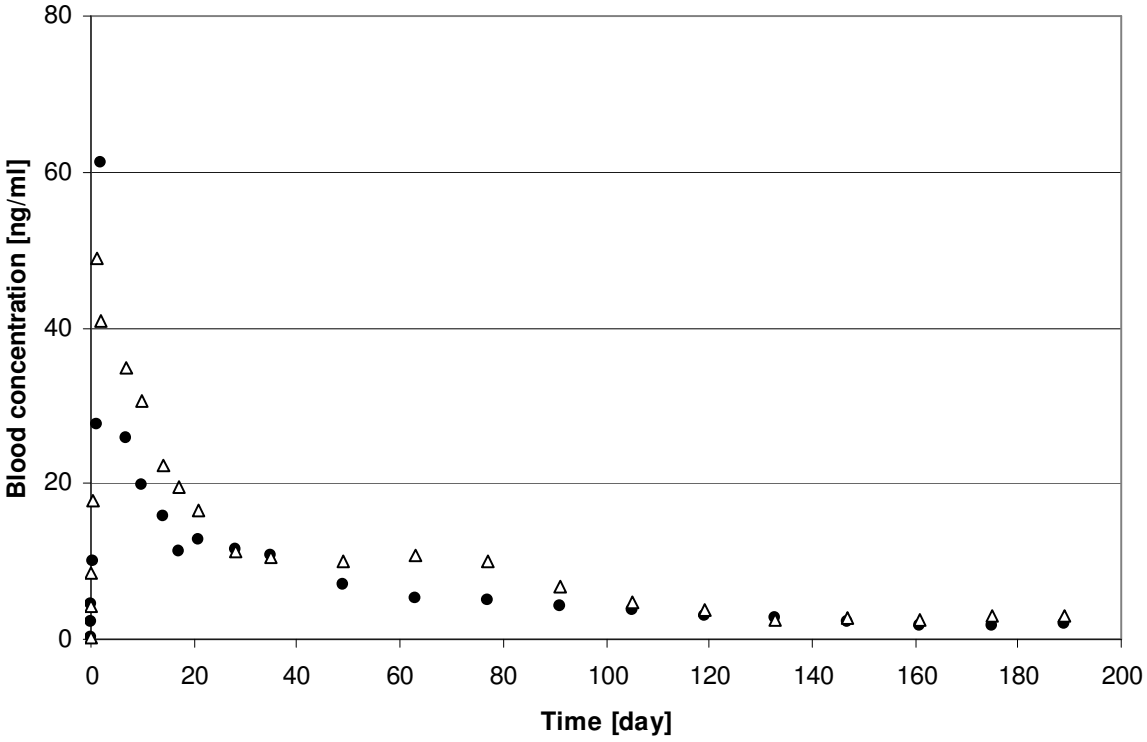


Figure 4.8: Pharmacokinetics of S-1238 [ng/ml], the major homologue of NOA449851, after subcutaneous injection of formulation D to dogs # 957 (●) and # 973 (Δ), measured over a period of 189 days.

Formulation E, which contained glycerol formal as co-solvent was applied to dogs # 2009 and # 2010. Maximum blood levels were reached at day 2 and 6, with blood concentrations of 35.6 ng/ml and 37.4 ng/ml, respectively. Blood levels almost one year (370 days) after application were 2.8 ng/ml and 3.3 ng/ml. Figure 4.9 illustrates the pharmacokinetics after injection of formulation E .

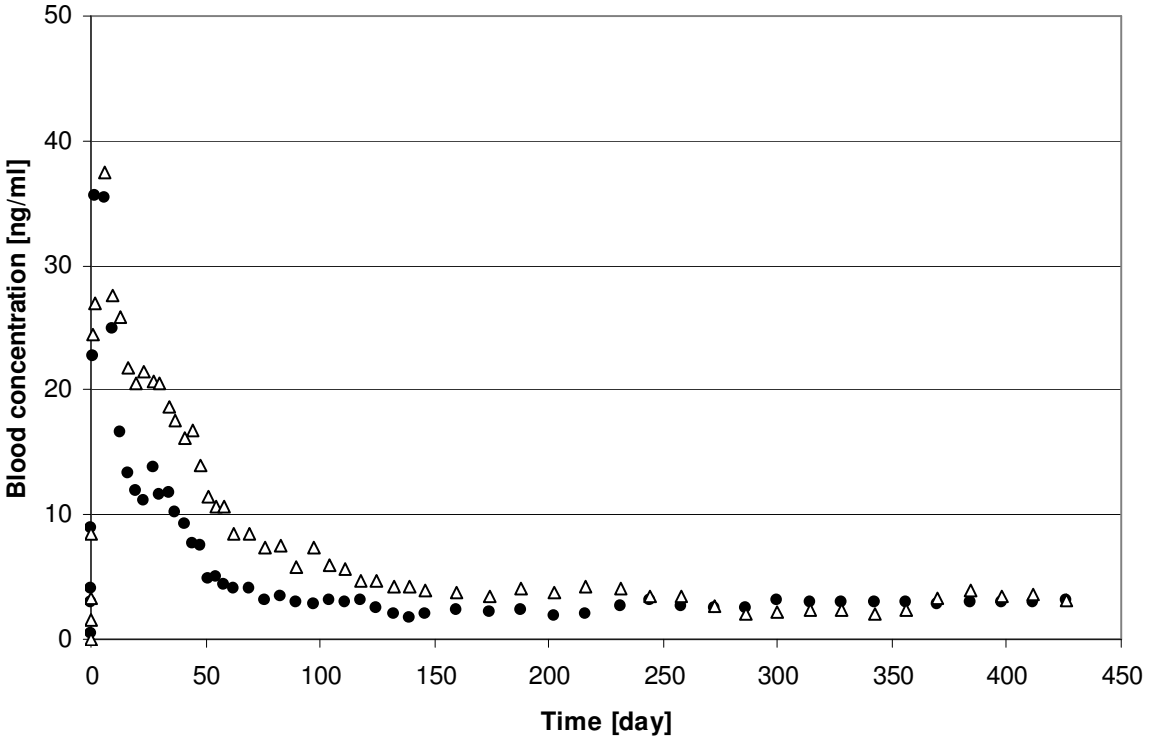


Figure 4.9: Pharmacokinetics of S-1238 [ng/ml], the major homologue of NOA449851, after subcutaneous injection of formulation E to dogs # 2009 (●) and # 2010 (Δ), measured over a period of 426 days.

Formulation F, containing anhydrous glycerol as co-solvent, was applied to dogs # 2005 and # 2007. Maximal blood concentration were obtained in both cases two days after subcutaneous administration and reached concentrations of 35.0 ng/ml and 40.6 ng/ml. Blood levels almost one year after application (370 days) reached concentrations of 2.0 ng/ml and 2.6 ng/ml, respectively. They passed the critical blood concentration of 10 ng/ml 23 days, respectively 30 days after injection. Blood levels of both experimental animals are illustrated in Figure 4.10.

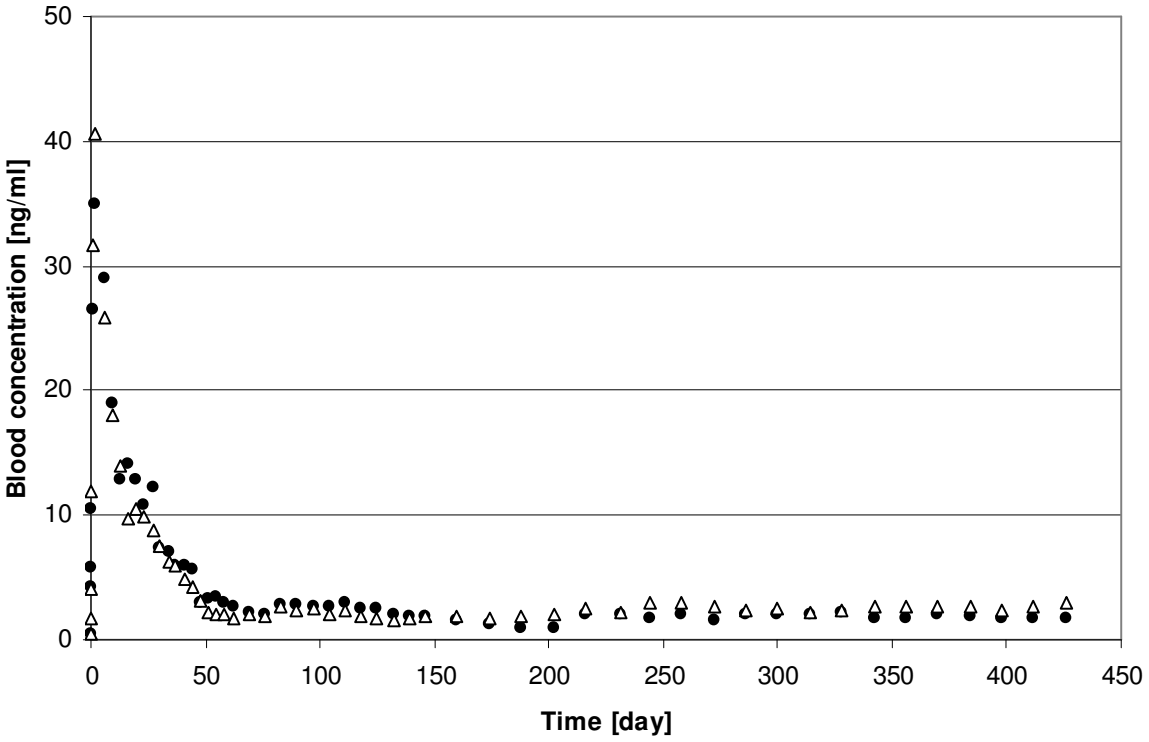


Figure 4.10: Pharmacokinetics of S-1238 [ng/ml], the major homologue of NOA449851, after subcutaneous injection of formulation F to dogs # 2005 (●) and # 2007 (Δ), measured over a period of 426 days.

3.5. Assessment of pharmacokinetic parameters

Blood levels were collected at selected time points and release characteristics of the formulations were determined based on these analytical results. Pharmacokinetic parameters such as time at maximal blood concentration (T_{max}), maximal blood concentration (C_{max}) and area under the concentration-time curve $AUC_{(0\text{-}x\ d)}$ are summarized in Table 4.3. Each dog received 400 mg of NOA449851, representing an amount of 320 mg of its major homologue S-1238.

The time at maximal blood concentration varied between 1 and 13 days after the subcutaneous application of the different formulations, but was most of the time between 2 and 4 days. The maximal blood concentration (C_{max}) was dependent on the composition of the formulation. It was higher for formulations with a concentration of polymers of 12.75 % (w/w) than for the related formulation containing 17.00 % (w/w) of polymers. The maximal blood concentrations for experimental animals to which formulation A (containing 17.00 % (w/w) PLA) was applied were 89.2 ng/ml (dog # 2014) and 87.6 ng/ml (dog # 2026). The dogs to which the related formulation B (containing 12.75 % (w/w) PLA) was injected had higher maximal blood concentrations of 92.7 ng/ml (dog # 2030) and 117.2 ng/ml (dog # 2032).

A similar comparison can be made for formulations C and D, containing both a solvent mixture consisting of triacetin and ethanol. The maximal blood concentrations after application of formulation C (containing 17.00 % (w/w) PLA) were 44.2 ng/ml (dog # 2063) and 47.5 ng/ml (dog # 2028), while dogs to which formulation D (containing 12.75 % (w/w) PLA) was injected had maximal blood concentrations of 61.2 ng/ml (dog # 957) and 48.9 ng/ml (dog # 973).

The substitution of a fraction of the solvent triacetin with various co-solvents resulted in the decrease of the maximal blood concentrations. Formulation A, containing only triacetin as solvent, lead to maximal blood concentrations of 89.2 ng/ml (dog # 2014) and 87.6 ng/ml (dog # 2026). With the same amount of polymer (17.00 % w/w) and of active ingredient (17.00 % w/w) and the substitution of 10.00 % of the solvent triacetin with various co-solvents, the maximum blood concentration was reduced to 44.2 ng/ml (dog # 2063) and 47.5 ng/ml (dog # 2028) for the formulation containing the co-solvent ethanol (formulation D), 35.6 ng/ml (dog # 2009) and 37.4 ng/ml (dog # 2010) for the formulation containing the

co-solvent glycerol formal (formulation E), and 35.0 ng/ml (dog # 2005) and 40.6 ng/ml (dog # 2007) for the formulation with the co-solvent anhydrous glycerol (formulation F).

Table 4.3: Overview of pharmacokinetic parameters of the *in-vivo* trials: time at maximal blood concentration (T_{max}), maximal blood concentration (C_{max}) and $AUC_{(0\text{-}x\ d)}$.

Formulation	Dog #	Weight of dog (beginning) [kg]	Weight of dog (end) [kg]	T_{max} [d]	C_{max} [ng/ml]	$AUC_{(0\text{-}x\ d)}$ [(ng/ml)d]
A	2014	9.4	11.0	1	89.2	$AUC_{(0\text{-}462\ d)}$: 2576
	2026	10.4	12.0	2	87.6	$AUC_{(0\text{-}462\ d)}$: 7210
B	2030	8.6	8.6	2	92.7	$AUC_{(0\text{-}468\ d)}$: 10034
	2032	8.9	8.7	13	117.2	$AUC_{(0\text{-}468\ d)}$: 7240
C	2063	11.5	12.0	3	44.2	$AUC_{(0\text{-}462\ d)}$: 3981
	2028	10.0	11.0	4	47.5	$AUC_{(0\text{-}462\ d)}$: 1807
D	957	10.0	10.6	2	61.2	$AUC_{(0\text{-}189\ d)}$: 1271
	973	11.0	10.5	1	48.9	$AUC_{(0\text{-}189\ d)}$: 1664
E	2009	9.4	10.2	2	35.6	$AUC_{(0\text{-}426\ d)}$: 1817
	2010	9.8	10.0	6	37.4	$AUC_{(0\text{-}426\ d)}$: 2597
F	2005	12.0	13.4	2	35.0	$AUC_{(0\text{-}426\ d)}$: 1367
	2007	9.1	10.6	2	40.6	$AUC_{(0\text{-}426\ d)}$: 1458

The pharmacokinetic parameters can be used to further calculate the release characteristics of the implants. The overall mean concentration (ng/ml) is calculated with the $AUC_{(0\text{-}x\ d)}$ divided by the time period of the experiment ($AUC_{(0\text{-}x\ d)}/t$). Further, the fraction of drug release from the implants was evaluated using the $AUC_{(0\text{-}x\ d)}$, multiplied with the clearance and the weight of the experimental animal. In Table 4.4 the release is represented in weight [mg] and in percentage [%] of the total active ingredient integrated in the formulation. The clearance of the active compound for dogs was previously determined to be 3 L/kg*d (unpublished internal data).

The fraction of drug released after application of formulation A during the duration of the animal trials (462 days) represented 25 % (85 mg) and 76 % (259 mg) of the total active ingredient integrated in the implant for the dogs # 2014 and # 2026. A fraction of 89 % (301 mg) and 64 % (217 mg) was found to be released from the implants for the dogs # 2030 and # 2032, 468 days after application of the formulation B. The fraction of active ingredient released till day 462 from the implant emerged form the formulation C was 42 % (143 mg)

and 18 % (60 mg) for the dogs # 2063 and # 2028, respectively. After 189 days following the injection of formulation D, the fraction of active ingredient released was 11 % (38 mg) for dog # 957 and 15 % (50 mg) for dog # 973. The fraction of drug released 426 days after application of formulation E was 16 % (55 mg) and 23 % (78 mg) for dogs # 2009 and # 2010, respectively. After 426 days of treatment with formulation F, 12 % (41 mg) of the active ingredient were released in dog # 2005 and 13 % (44 mg) in dog # 2007.

Table 4.4: Overall mean concentration C [ng/ml] as well as amount of drug released ([mg] and [%]) during the *in-vivo* experiment.

Formulation	Dog #	$AUC_{(0\text{-}x\ d)}$ [(ng/ml)d]	$AUC_{(0\text{-}x\ d)}$ /t [ng/ml]	$AUC_{(0\text{-}x\ d)}$ x Cl x weight [mg]	$AUC_{(0\text{-}x\ d)}$ x Cl x weight [%]
A	2014	$AUC_{(0\text{-}462\ d)}$: 2576	5.6	85	25
	2026	$AUC_{(0\text{-}462\ d)}$: 7210	15.6	259	76
B	2030	$AUC_{(0\text{-}468\ d)}$:10034	21.4	301	89
	2032	$AUC_{(0\text{-}468\ d)}$: 7240	15.5	217	64
C	2063	$AUC_{(0\text{-}462\ d)}$: 3981	8.6	143	42
	2028	$AUC_{(0\text{-}462\ d)}$: 1807	3.9	60	18
D	957	$AUC_{(0\text{-}189\ d)}$: 1271	6.7	38	11
	973	$AUC_{(0\text{-}189\ d)}$: 1664	8.8	50	15
E	2009	$AUC_{(0\text{-}426\ d)}$: 1817	4.3	55	16
	2010	$AUC_{(0\text{-}426\ d)}$: 2597	6.1	78	23
F	2005	$AUC_{(0\text{-}426\ d)}$: 1367	3.2	41	12
	2007	$AUC_{(0\text{-}426\ d)}$: 1458	3.4	44	13

4. Discussion

4.1. Viscosity and injectability of formulations and placebos

4.1.1. Rheology

The PLA polymer solutions gave wide ranges of viscosity and appeared to be either Newtonian or non-Newtonian. All formulations with a viscosity lower than 20 Pa*s showed Newtonian flow behavior. This was valid for all placebo polymer solutions as well as active ingredient loaded formulations B, C and D. However, active ingredient loaded formulations A, E and F showed pseudoplastic flow behavior, with a significant decrease of viscosity at higher shear rates. In the practice, it is not possible to take advantage of the pseudoplasic flow behavior of formulations A, E and F, because, at the moment, it exists no application device capable of generating high shear rates to lower the viscosity at injection time.

The data in Table 4.2 show that the viscosity of the formulations was reduced by decreasing the amount of PLA polymers from 17.00 % (w/w) to 12.75 % (w/w) (formulations B versus A and formulation D versus C). As the PLA polymers form the matrix of the implant and leads to the sustained release effect of the active ingredient, reduction of the polymer concentration is restricted.

Another way for lowering the viscosity of the formulations was to reduce the active ingredient concentration. As illustrated in the experiment, the placebo solutions were less viscous than the related formulations with active ingredient. For evident reasons, the drug content in the formulation should be sufficient to provide long release of an adequate amount of active ingredient.

A further alternative to reduce viscosity and therefore improve syringeability is to use a co-solvent which is less viscous than the main solvent triacetin. As successful examples, both formulations containing ethanol as co-solvent (formulations C and D) were significantly less viscous and easier to inject than the related formulations without co-solvent (formulation A and B).

4.1.2. Injection procedure to dogs

The largest needle with respect to length and diameter, that can be used for a given indication is determined by a number of parameters including the fluidic requirements of the formulation, the application way (i.m., s.c. or i.v.), the frequency of injection, the seriousness of the disease, the target population and the availability of alternative therapies. The acceptability of the injection system is important for human patient as well as for companion animal patients.

The high viscosity of the formulations is inconvenient for the injection procedure. To prevent a long injection time and to reduce the necessary force to be applied on the syringe plunge, 18 G needles had to be used. For formulations containing glycerol formal or anhydrous glycerol, the even larger 16 G needles were necessary, resulting in a pain reaction during subcutaneous insertion of the needles into the skin. However, the injection of the formulations themselves did not seem to be painful to the dogs.

A force of maximum 5 N is needed for a comfortable injection. Combined with the fact that in practice 19 G needles as maximum size for subcutaneous injections to dogs is acceptable, it can be concluded that the high viscosity of the sustained release injectables formed *in-situ* is a serious issue for further development of this technology. No formulation could fulfill both requirements. Only the placebo solutions C and D were close to these desired specifications. Development of an applicator suitable to inject subcutaneously highly viscous formulations would overcome these issues. At present time, there is no such applicator to be found on the market. A novel injection system which has the potential to enhance the delivery of complex formulations such as viscous and particulate material is described by Maynard et al. [Maynard et al., 2003].

4.2. Tolerability

All formulations, placebos as well as formulations with active ingredient, were well tolerated by the experimental animals. The excipients used in the formulations like triacetin, PLA, and anhydrous glycerol are known to be generally well tolerated after subcutaneous injection [Bertholom et al., 2000; Bartsch et al., 1976; Bailey et al., 1991]. Glycerol formal and ethanol are more critical substances [Bertholom et al., 2000], but at concentrations of 10.00 % as in formulations C, D and E, no side effects were observed.

Clinical tolerability is a parameter which is regularly checked in the experiments cited in the literature. Authors using the solvents NMP and DMSO mentioned inflammation or tissue irritation [Kranz et al., 2001; Royals et al., 1999; Yewey et al., 1997]. Encapsulation with connective tissues of implant is cited also several times, however without causing any problems for acceptability [Eliaz et al., 2002; Royals et al., 1999]. The encapsulation of the subcutaneous implant can lead to modifications in the active ingredient release rate and extent. The capsule builds an additional barrier for water to enter the implant and therefore for biodegradation of the polymers, as well as for the active to be released from the implant into the subcutaneous milieu and therefore the systemic circulation.

Sustained release injectables implants formed *in-situ* with the solvent triacetin were also tested *in-vivo* for tolerability: very slight irritations were observed after i.m. or s.c. application in rats of 0.2 ml of a 5 % 50/50 PLGA solution in triacetin [Shah et al., 1993].

In the present experiments, all injections resulted in a mild swelling surrounding the injection site due to the injected volume. No skin changes were noted on top of these swellings and there were no clinical signs of irritation or pain. Formulations with active ingredient as well as the related placebos were also very well tolerated by the experimental animals.

The placebo implants disintegrated quite rapidly and were no more noticeable at the injection site 30 days after application. The implants containing active ingredient were more voluminous than the placebos and were resorbed more slowly. Interestingly, the opposite was observed by Eliaz for 75/25 PLGA implants containing plasmid DNA as active ingredient [Eliaz et al., 2002]. In this *in-vivo* experiment, the 75/25 PLGA polymers of the implants containing the plasmid DNA disintegrated more rapidly than the polymers of the related placebo implants. Even if these observations seem contradictory, there is evidence that the erosion of the polymers is influenced by the substances contained in the implant [Vert et al., 1998]. The bioerosion of the polymers is due to the hydrolysis of ester bonds and is therefore dependent on the amount of water penetrating the implant matrix. The physicochemical characteristics of the active ingredient may influence water permeation. A lipophilic substance such as NOA449851 is an hindrance for the water to enter the polymer matrix, whereby a more hydrophilic substance like a plasmid DNA may enhance water diffusion and therefore increase the rate of the polymer erosion. This may be the explanation for these seemingly contradictory observations.

4.3. Blood level profiles

The present *in-vivo* trials are carried out in an early clinical stage and should be considered as an explorative pilot study. To obtain more robust results, it would be necessary to carry out confirmatory trials in accordance with the current guideline for Good Clinical Practice. The appropriate minimal number of animals per group should be carefully estimated to produce robust data, including factors such as variance of responses and high inter-animal variations. However, the collected data give good indications with regard to tendency of drug release from the sustained release injectables formed *in-situ* and allow a first assessment of the properties of the different formulations.

Because of a very high risk of local and systemic toxicity, it was not considered ethical to inject a triacetin solution containing the same amount of active ingredient but without the PLA polymers. Without PLA polymers, no sustained release effect would be generated and the amount of active ingredient would probably be released in a short period of time, leading to acute high blood concentrations and possibly systemic side effects. Therefore, no control tests were carried out to measure the effective impact of PLA polymers on the sustained drug release.

Based on the *in-vitro* results obtained from formulations with different polymer types (PLA, 85/15 PLGA and 75/25 PLGA), only formulations containing PLA polymers were tested. The *in-vitro* dissolution tests showed that formulations with high drug loads had very similar drug release profiles, independently of polymer type (Chapter 3, Figures 3.3 and 3.4). This was attributed to the drug release mechanism which seemed to be mainly drug diffusion controlled and not polymer erosion controlled. The goal of these experiments was to reach *in-vivo* a sustained release of the active ingredient at therapeutic levels over the longest possible time period. Therefore, the polymer type selected for the *in-vivo* trials was the one with the lowest degradation rate: the PLA polymer [Middleton et al., 2000].

The *in-vivo* trials were stopped after the active ingredient blood concentrations decreased and remained below the therapeutic level of 10 ng/ml. The fractions of drug released, estimated from the total AUC, multiplied with the clearance and the weight of the experimental animal were surprisingly low (Table 4.4). It represented 25 % (85 mg) and 76 % (259 mg) of the total injected active ingredient for the dogs # 2014 and # 2026, 462 days after application of the formulation A, and 89 % (301 mg) and 64 % (217 mg) for the dogs # 2030 and # 2032, 468 days after application of the formulation B. The fraction of drug

released after application of formulation C was 42 % (143 mg) and 18 % (60 mg) for the dogs # 2063 and # 2028, respectively. After 189 days treatment with formulation D, the fraction of active ingredient released was only 11 % (38 mg) for dog # 957 and 15 % (50 mg) for dog # 973. The fraction of drug released after application of formulation E was 16 % (55 mg) and 23 % (78 mg) for the dogs # 2009 and # 2010, respectively. The values were even lower 426 days after the injection of formulation F, with only 12 % (41 mg) of the active ingredient released in dog # 2005 and 13 % (44 mg) in dog # 2007.

The low fraction of active ingredient released during the *in-vivo* experiments may indicate a too high concentration of the PLA polymer in the formulations: the formulations B with a reduced amount of polymer had over 60 % of the drug released for both experimental animals 468 days after application. It could also be an indication that the implant matrix should not consist of PLA polymers but of faster degrading polymers such as 85/15 PLGA or 75/25 PLGA. The small fraction of active ingredient released *in-vivo* after long period of time may also be explained by the possible encapsulation of the solid subcutaneous implant. As already mentioned, this may build an additional barrier which inhibits water permeation into the implant matrix and therefore prevents hydrolysis of the polymeric ester bounds and concomitantly complicates drug diffusion into the subcutaneous milieu.

In some cases, very high inter-animal variations in the blood drug concentration were noticed for dogs which had received the same formulation. The pharmacokinetic data represented in Table 4.3, and particularly the AUC values, illustrate these differences. This was the case for the experimental animals to which formulations A and C were applied, as illustrated in Figures 4.5 and 4.7. The injection of these formulations led in one of the experimental animal per group to an unexplained spontaneous burst at 60, respectively 150 days after application. This effect of spontaneous late burst has also been observed in published literature [Ravivarapu et al., 2000 (3)]. For explorative trials, two dogs per group allow some correlation with *in-vitro* findings. However, the number of animals per group must be higher in future confirmatory experiments.

The amount of active ingredient injected was 400 mg for all dogs. As they have body weight ranging from 8.6 kg to 13.4 kg, all dogs should have similar AUC values. In Table 4.3, the values of the AUC showed evident differences. These differences may be the consequence of encapsulation of the subcutaneous implant. It has been reported in previous papers that connective tissue was found around the implant [Jain et al., 1999; Royals et al., 1999; Eliaz et al., 2002]. This can build a barrier for the active ingredient and inhibit its diffusion into the body. As an other explanation, the active ingredient may have degraded within the implant

[Burgess et al., 2002]. According to stability investigations of the active ingredient in the liquid formulations A, B and C (Chapter 6, Figure 6.4), the refund of the drug NOA449851 was between 92.89 % and 95.46 % after six months storage at 40°C. Therefore, the degradation of the active ingredient within the polymer matrix seems not to explain alone the low fraction of drug released.

Attention was paid to test *in-vivo* formulations which were already investigated *in-vitro* with dissolution tests. The trends of the release profiles were therefore already known. This is further elaborated as part of a correlation between the *in-vivo* and the *in-vitro* data in Chapter 5.

4.3.1. Influence of polymer concentration in the formulation

A reduction of the PLA polymer concentration in the formulation lowered the viscosity and therefore improved its injectability. For this reason, such formulations were tested, despite in-vitro release profiles showing a dramatic increase in the initial burst effect (Chapter 3, Figures 3.6 and 3.11).

A comparison can be made between formulation A, containing 17.00 % (w/w) PLA polymers and formulation B, containing only 12.75 % (w/w) PLA polymers. According to the *in-vitro* dissolution tests, an important intensification of the initial burst effect was expected for the formulation with a reduced amount of polymer. Surprisingly, the differences of blood concentrations during the first days of the *in-vivo* trial between the animals receiving the formulation A and formulation B were not so evident. The maximal blood concentrations for dogs which received formulation A with 17.00 % (w/w) PLA polymers were 89.2 ng/ml (dog # 2014) and 87.6 ng/ml (dog # 2026); dogs which received formulation B with 12.75 % (w/w) PLA polymers reached maximal blood concentrations of 92.7 ng/ml (dog # 2030) and 117.2 ng/ml (dog # 2032). The formulations containing less polymer led to a slightly increased initial burst effect during the *in-vivo* testing.

Similarly, a comparison can be made between formulations C and D. The PLA polymer concentration was 17.00 % (w/w) for formulation C and 12.75 % (w/w) for formulation D. In this case, the solvent of the formulations was a mixture of triacetin and ethanol. The maximal blood concentrations for dogs which received formulation C with 17.00 % (w/w) PLA polymers were 44.2 ng/ml (dog # 2063) and 47.5 ng/ml (dog # 2028); dogs which received the formulation D with 12.75 % (w/w) PLA polymers reached maximal blood concentrations of 61.2 ng/ml (dog # 957) and 48.9 ng/ml (dog # 973). Here again, reduction of PLA polymer

content in the formulations increased slightly the initial burst, but not in the same proportions as in the *in-vitro* dissolution tests carried out with the same formulations (Chapter 3, Figure 3.11).

The effects of polymer concentrations on the *in-vivo* drug release and particularly on the magnitude of the initial burst have already been widely discussed in various papers. In an experiment using the lipophilic drug naltrexone base in rats, Shively et al. [Shively et al., 1995] described the *in-vivo* initial release being highly dependent on the polymer concentration. The percent of active ingredient released from a formulation containing 57 % 75/25 PLGA polymers was significantly lower than that observed for a formulation containing 35 % of the same polymer. No explanation about the prolonged release was possible as the blood concentrations were only monitored for four hours following injection [Shively et al., 1995].

Eliaz et al. described in diverse papers the effect of different polymer concentrations on the release of water soluble TNF receptors p55-R from sustained release injectables formed *in-situ*. The formulation containing 10 % of the 75/25 PLGA polymer had a higher initial burst effect than the formulation containing 20 % of the polymer. However, following the initial burst, the sp55-R serum concentration at 6 hours after injection was similar for both polymer concentrations [Eliaz et al., 2000 (1); Eliaz et al., 1997].

In the experiments described by Ravivarapu et al., in which formulations containing the water soluble active ingredient leuprolide acetate were investigated, the efficacy on testosterone level in rats was tested. The polymer concentrations ranged from 40 % to 50 % (w/w). No significant effect of polymer concentration on formulation efficacy could be noted, except for one day (day 9) when the testosterone level was statistically higher for animals having received the formulation with the highest polymer concentration. However, it was concluded that the whole range of polymer concentrations tested was suitable for producing efficacious formulations for a period of 100 days [Ravivarapu et al., 2000 (1)].

4.3.2. Influence of hydrophilic co-solvents

The initial burst effects are often mentioned by authors who have performed *in-vivo* experiments with sustained release injectables formed *in-situ* [Ravivarapu et al., 2000 (2); Yewey et al., 1997; Chandrashekar et al., 1996; Dunn et al., 1996; Randomsky et al., 1993]. The two main reasons to prevent an initial burst is that the released active ingredient is no longer available for the prolonged release and that the early concentrations can reach toxic

blood levels and generate systemic side effects. Therefore, a major objective of this formulation development was to determine and test parameters capable to reduce the magnitude of the initial burst effect.

The use of various hydrophilic co-solvents resulted in the decrease of the initial burst effect. Maximum blood concentrations of the formulations containing only triacetin as solvent (formulation A) were 89.2 ng/ml (dog # 2014) and 87.6 ng/ml (dog # 2026). With the same amount of polymer and active ingredient, but substitution of 10 % triacetin with hydrophilic co-solvents, the maximum blood concentration was decreased as follows: to 44.2 ng/ml (dog # 2063) and 47.5 ng/ml (dog # 2028) for the formulation containing the co-solvent ethanol (formulation D), to 35.6 ng/ml (dog # 2009) and 37.4 ng/ml (dog # 2010) for the one containing glycerol formal (formulation E), and to 35.0 ng/ml (dog # 2005) and 40.6 ng/ml (dog # 2007) for the formulation with the co-solvent anhydrous glycerol (formulation F).

This effect was also observed for formulations B and D which both contained a reduced amount of PLA polymers (12.75 % w/w). Formulation B, with triacetin as unique solvent, had an initial drug concentration of 92.7 ng/ml (dog # 2030) and 117.2 ng/ml (dog # 2032) while formulation D, with ethanol as co-solvent, had a reduced burst with a maximal blood concentration of 61.2 ng/ml (dog # 957) and 48.9 ng/ml (dog # 973).

The reduction of magnitude of initial burst by the substitution of the main solvent triacetin with hydrophilic co-solvents was already observed during the *in-vitro* dissolution tests (Chapter 3, Figures 3.8, 3.9 and 3.10). The hydrophilic co-solvent may affect the convections of water into the implants and out of the polymer matrix, altering the implant forming process. Attempts to make a mathematical correlation between *in-vitro* and *in-vivo* data of drug release from sustained release injectables formed *in-situ* can be found in Chapter 5.

The ideal blood profile with a sustained release injectable formed *in-situ* is a zero order kinetic during several months. Formulations containing glycerol formal (formulation E) and anhydrous glycerol (formulation F) clearly showed a continuous decrease in the blood level concentration over time. These formulations are therefore no good candidates for such application. In addition, due to the high viscosity, both formulations were very difficult to inject with a normal syringe.

5. Conclusion

A total of twelve healthy Beagle dogs were used for the *in-vivo* studies, with two dogs assigned to each formulation. Six different formulations were tested, each dog receiving both an active ingredient loaded formulation and the related placebo injection.

The rheological properties as well as the syringeability of each formulation tested *in-vivo* were evaluated using a cone-plate viscosimeter and a texture analyser apparatus. All PLA placebo solutions showed Newtonian flow behaviour whereas some highly viscous active ingredient loaded formulations had pseudoplastic rheological properties. Injection through normal needles of these formulations with high viscosity required excessive pressure on the syringe plunger. The high viscosity of the sustained release injectables formed *in-situ* is a serious issue for further development of this technology. However, the viscosity of the formulation was reduced when the concentration of PLA polymers was lowered as well as when the main solvent triacetin was substituted to a fraction by co-solvents such as ethanol.

All PLA placebo solutions and all formulations applied subcutaneously were very well tolerated by the experimental animals with only a slight and painless swelling at the injection site for a few days. The blood profiles showed a release of the active ingredient over a prolonged period for all formulations. The magnitude of the initial burst was slightly increased by the reduction of the amount of PLA polymers in the formulation. However, a significant decrease of the initial burst was observed for all formulations containing hydrophilic co-solvents such as ethanol, glycerol formal or anhydrous glycerol. The fractions of active ingredient released were in general very low and may have indicated a too high concentration of PLA polymer in the formulations. As alternative to increase the fraction of drug released over the whole duration of the treatment, faster degrading polymer types such as 85/15 PLGA or 75/25 PLGA could be used instead of the selected PLA polymers.

The present *in-vivo* trials were carried out in an early clinical stage and should be considered as an explorative pilot study. In some cases, very high inter-animal variations in the blood drug concentration were measured for dogs which received identical formulations. It would be necessary to carry out confirmatory trials in accordance with the current guideline for Good Clinical Practice and carefully estimated the appropriate number of animals per group to produce more robust data.

Chapter 5

In-vitro in-vivo correlation (IVIVC) for sustained release injectables formed *in-situ* the active ingredient NOA449851

1. Introduction and objectives

Scientifically based methods to predict the *in-vivo* drug release performance of pharmaceutical dosage forms are beneficial in order to minimize animal experiments, reduce drug development duration and allow a quality control of the release properties of the dosage forms. Various physicochemical, biopharmaceutical and physiological factors need to be considered to achieve a successful correlation between *in-vitro* data and *in-vivo* parameters. Guidance for IVIVC models are proposed for oral [Guidance for industry, 1997; EMEA, 1999, USP 28, 2005 <1088>], parenteral [Burgess et al., 2004; USP 28, 2005 <1088>] and transdermal dosage forms [EMEA, 1999, USP 28, 2005 <1088>]. IVIVC models are divided into three categories known as levels A, B and C. Level A correlations apply to models than can predict the entire *in-vivo* time course from *in-vitro* data. They represent point-to-point correlations between *in-vivo* absorption profiles and *in-vitro* release profiles. Level B correlations are based on statistical moment analysis and compare mean *in-vitro* dissolution time of the formulation with mean residence time in the body. Level C correlations establish a single point relationship between an *in-vitro* dissolution parameter and a pharmacokinetic parameter [Guidance for industry IVIVC; EMEA, 1999; Burgess et al., 2004].

The active ingredient release from sustained release injectables formed *in-situ* is a complex process determined mainly by properties of the formulation as well as by the physicochemical characteristics of the active ingredient. After an initial burst phase related to solvent diffusion into the surrounding milieu and solidification of the subcutaneous implant,

the drug is released at slower rates, mainly by diffusion through the polymeric matrix coupled with erosion of the biodegradable polymers.

The objective of this chapter is to find a correlation between *in-vitro* release properties of active ingredient NOA449851 from sustained release injectables formed *in-situ* obtained with dissolution tests (Chapter 3) and *in-vivo* drug release data obtained after subcutaneous application of the same formulations to experimental animals (Chapter 4).

2. Materials and methods

2.1. Materials

In-vitro release profiles and *in-vivo* pharmacokinetic data were obtained from experiments described in Chapter 3 and 4, respectively. More information on the materials used for manufacturing of the formulations and for analytics can be found in Section 2.1 of the related chapters.

2.2. Methods

2.2.1. Selection of formulations

IVIVC was performed for the six sustained release injectables formed *in-situ* tested *in-vivo* (Chapter 4) as their *in-vitro* drug release characteristics were previously evaluated with dissolution tests (Chapter 3). Compositions of the six evaluated formulations are summarized in Table 5.1. All formulations contained 17.00 % (w/w) of the active ingredient NOA449851 beside PLA polymers and triacetin. The formulations differed in concentration of the PLA polymers (17.00 % (w/w) or 12.75 % (w/w)) as well as in composition of the solvent mixture: a fraction of the main solvent triacetin was substituted with co-solvents such as ethanol, glycerol formal or anhydrous glycerol. Furthermore, the different used nomenclatures for formulations in Chapters 3 and 4 are mentioned in Table 5.1, as well as the Figures in which the related drug release profile is represented. In this chapter, the same nomenclature for the formulations will be used as in Chapter 4 (formulations A to F). Preparation method of formulations can be found in Chapter 3, Section 2.2.1.1.

Table 5.1: Compositions [% (w/w)] of sustained release injectables formed *in-situ* used for determination of IVIVC. It is also indicated which Figure shows the corresponding *in-vitro* drug release profile and the *in-vivo* profile.

Nomenclature of formulation in Chapters 4 and 5	Nomenclature of formulation in Chapter 3	Components	Content of components [% (w/w)]	*In-vitro* release profile	*In-vivo* release profile
A	PLA-AI(1:1)	NOA449851 PLA triacetin	17.00 17.00 66.00	Chapter 3, Figure 3.6	Chapter 4, Figure 4.5
B	PLA-AI(3:4)	NOA449851 PLA triacetin	17.00 12.75 70.25	Chapter 3, Figure 3.6	Chapter 4, Figure 4.6
C	Co-solvent (10 %) ethanol, PLA-AI(1:1)	NOA449851 PLA ethanol absolute triacetin	17.00 17.00 10.00 56.00	Chapter 3, Figure 3.10	Chapter 4, Figure 4.7
D	Co-solvent (10 %) ethanol, PLA-AI(3:4)	NOA449851 PLA ethanol absolute triacetin	17.00 12.75 10.00 60.25	Chapter 3, Figure 3.11	Chapter 4, Figure 4.8
E	Co-solvent (10 %) glycerol formal PLA-AI(1:1)	NOA449851 PLA glycerol formal triacetin	17.00 17.00 10.00 56.00	Chapter 3, Figure 3.9	Chapter 4, Figure 4.9
F	Co-solvent (10 %) glycerol, PLA-AI(1:1)	NOA449851 PLA anhydrous glycerol triacetin	17.00 17.00 10.00 56.00	Chapter 3, Figure 3.8	Chapter 4, Figure 4.10

2.2.2. Correlation approaches

IVIVC is a mathematical relationship between *in-vitro* and *in-vivo* data. Plasma concentration data cannot be directly correlated with *in-vitro* dissolution data and need first to be translated into suitable dimensions to be able to assess whether there is a correlation. Therefore, the fraction of drug released *in-vivo* was evaluated using the corresponding AUC values. The fraction of drug released *in-vivo* for a given time period was calculated with a model-independent evaluation, based on mean $AUC_{[0,t]}$ for the determined period of time, related to the predicted $AUC_{[o,\infty]}$ to infinite time, as represented in equation 5.1,

Equation 5.1:
$$Fraction\_released\_\%(t) = \frac{AUC_{[0,t]}}{AUC_{[o,\infty]}} \cdot 100\%$$

where $AUC_{[o,t]}$ is the area under the curve from injection time to time point t and $AUC_{[o,\infty]}$, the area under the curve from injection time to infinite time, as extrapolated from the model-independent evaluation.

2.2.2.1. Development of level C correlation model

Level C correlation establishes a single point relationship between a dissolution parameter obtained with an *in-vitro* experiment and a pharmacokinetic parameter. Here, the pharmacokinetic parameters are the maximal blood concentration C_{max} and the evaluated percentages of drug released at three predetermined time points. Level C IVIVC does not reflect the complete shape of the plasma level and is generally not useful for supporting major variations in composition or manufacturing process of the product. However, it can provide useful information during formulation development and can be used in setting release specifications.

Level C IVIVC modeling was first attempted using C_{max} values as pharmacokinetic parameter, form which it is claimed to be related with the cumulative percentage of drug released *in-vitro* during dissolution test until the time point T_{max}. The correlation points for the six formulations were represented in a scatter plot, with the C_{max} value reported on the Y-axis and the percentage of drug released *in-vitro* at T_{max} reported on the X-axis.

Further level C IVIVC models were attempted for three predetermined time points. The selected time points were set at 2, 7 and 14 days, to investigate the initial burst effect of sustained release injectables formed *in-situ.* Regression lines and related correlation coefficients were calculated from the various scatter plots.

2.2.2.2. Development of level A correlation model

Level A IVIVC represents a point-to-point relationship between *in-vitro* cumulative dissolution curves of the product and *in-vivo* dissolution profiles generated by pretreatment of the plasma level data.

For a given sustained release injectable formed *in-situ* formulation, the average cumulative percentages of drug released *in-vitro* during dissolution test were reported as a function of the mean evaluated percentage of active ingredient released *in-vivo* at the same time point. Correlation was possible only for the first 40 days of the experiments, as the *in-vitro* dissolution tests carried out in Chapter 3 were stopped after this period of time. The correlation profiles were reported graphically on a chart where the percentage of drug released *in-vitro* was represented on the X-axis and the corresponding evaluated *in-vivo* drug released on the Y-axis.

3. Results

3.1. Level C correlations

3.1.1. Level C correlations using C_{max}

Level C IVIVC was performed using the average C_{max} values of *in-vivo* drug release profiles, as function of the average percentage of drug released *in-vitro* at the time point T_{max}.

For each formulation, maximal drug concentration in the *in-vivo* release profile was reported on the Y-axis, while percentage of drug released at this time point (T_{max}) during *in-vitro* dissolution test was represented on the X-axis. Correlation points were reported as scatter plot in chart of Figure 5.1.

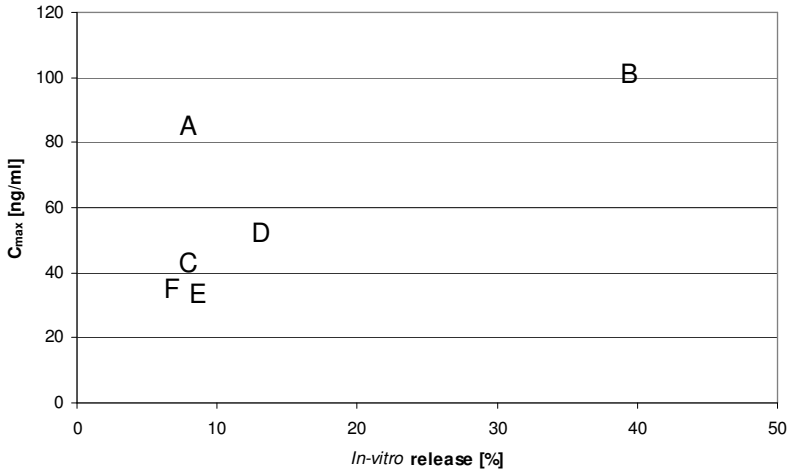


Figure 5.1: Level C IVIVC for formulations A to F, using C_{max}-values. Labels A, B, C, D, E and F indicate the related formulations.

No significant correlation could be found for level C IVIVC using C_{max} values as pharmacokinetic parameter. Specifically correlation point of formulation A differs highly from the other formulation correlations data.

However, excluding formulation A, a clear trend could be observed, in which the *in-vitro* drug release performances from the different formulations were positively and linearly correlated to the obtained *in-vivo* C_{max} values. The higher the value of C_{max}, the higher the *in-vitro* drug release in the dissolution test at this time point.

3.1.2. Level C correlation for time point t = 2 days

Level C IVIVC of NOA449851 for time point t = 2 days is shown in a scatter plot in Figure 5.2, for the four formulations containing 17.00 % of PLA polymer (formulations A, C, E and F). The percentage of drug released *in-vitro* two days after injection is presented on the X-axis and the evaluated percentage of drug released *in-vivo* two days after subcutaneous application is reported on the Y-axis. Based on relationships of the various formulations for this time point, a linear regression level C IVIVC model was constructed. The equation of the regression line and the correlation coefficient are indicated in Figure 5.2.

The slope of the linear regression obtained from these data is significantly different from 0 (p-value = 0.0362). The good correlation coefficient indicates a strong predictability. However, the negative slope of the regression line is surprising, as it suggest an inverse proportional correlation of the drug release for the investigated range.

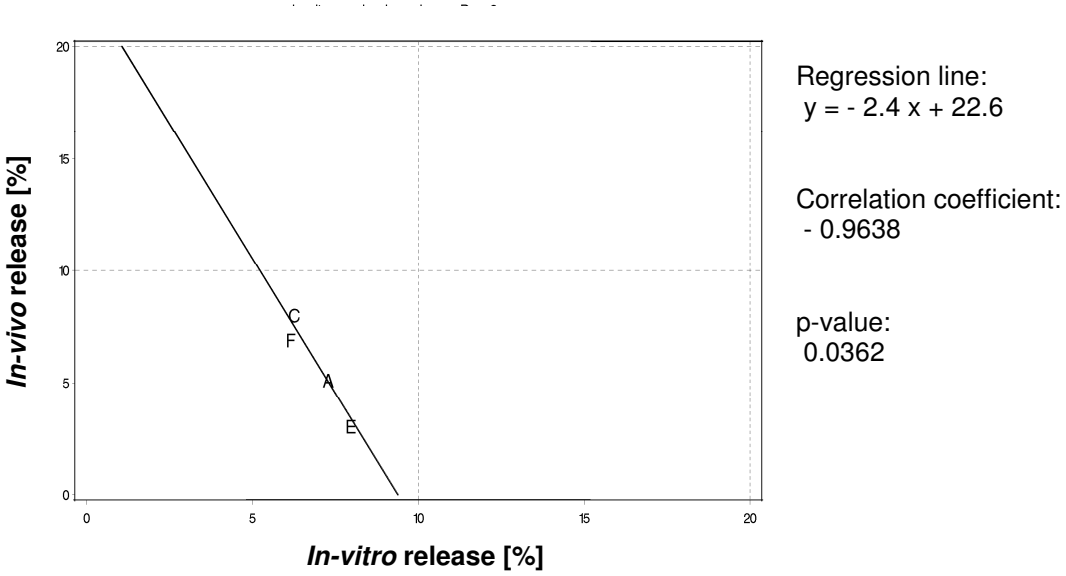


Figure 5.2: Percentage of active ingredient released *in-vivo* from formulations A, C, E and F versus *in-vitro* release, two days after injection. Regression line, related equation and correlation coefficient are indicated. The labels A, C, E and F indicate the related formulations.

By using all formulations (formulations A to F) for level C IVIVC at time point t = 2 days, no significant correlation could be found. The results for the correlation of the formulations containing a reduced amount of polymer (formulations B and D with 12.75 % of PLA polymers) differed significantly from the results of formulations containing 17.00 % of PLA

polymers (formulations A, C, E and F). The slope of the regression line obtained from this data set was not significantly different from 0 (p-value = 0.1589). The correlation coefficient of -0.65 indicated a bad predictability. However, similarly to level C correlation at time t = 2 days for formulations A, C, E and F, the slope of the regression line is negative.

Correlation for formulations B and D differed highly compared to the other formulations investigated. After two days, none of the formulations released more than 10 % *in-vivo*, and all formulations containing 17.00 % PLA polymers released between 6 % and 8 % *in-vitro*. Formulations B and D however, had both extensive *in-vitro* release, representing 39 % and 13 % of the total amount of active ingredient integrated in the formulation, respectively.

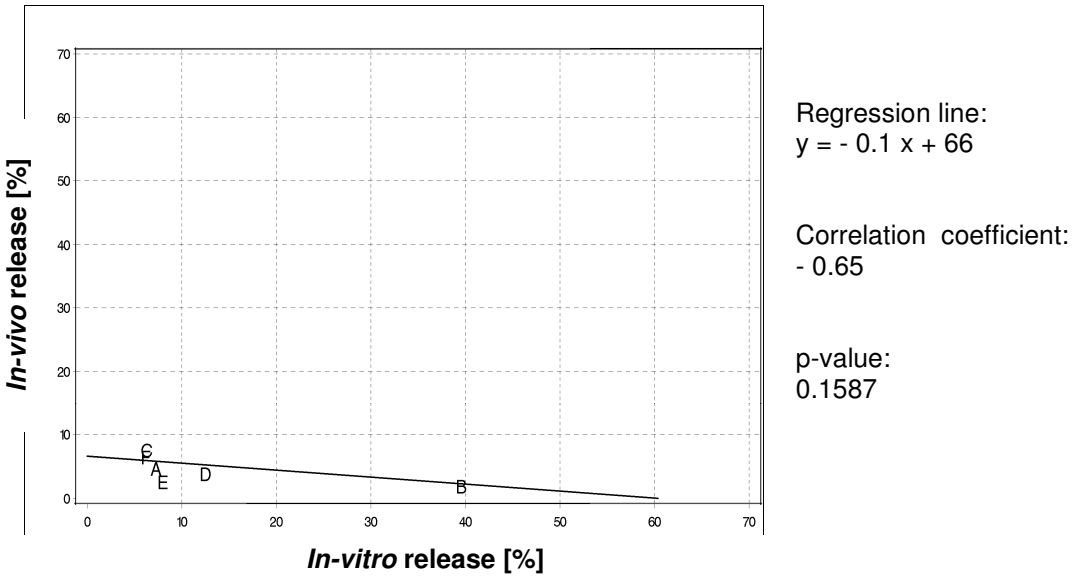


Figure 5.3: Percentage of active ingredient released *in-vivo* from formulations A to F versus *in-vitro* release, two days after injection. Regression line, related equation and correlation coefficient are indicated. The labels A, B, C, D, E and F indicate the related formulations.

3.1.3. Level C correlation for time point t = 7 days

A level C IVIVC is presented as scatter plot in Figure 5.4 for the time point t = 7 days. For each formulation containing 17.00 % of PLA polymer, percentage of drug released *in-vitro* after seven days of the dissolution test is presented on the X-axis, while the percentage of drug released *in-vivo* seven days after subcutaneous application is reported on the Y-axis. According to the obtained relationship of drug released from formulations A, C, E and F for this time point, a linear regression was calculated. The equation of the regression line and its correlation coefficient are also indicated in Figure 5.4.

The slope of the linear regression obtained from this data set was not significantly different from 0 (p-value = 0.2047). This can be due to the small number of data used for this correlation calculation. The inverse proportional correlation observed in Figure 5.2 between *in-vitro* and *in-vivo* performance was again observed after seven days of treatment with a negative slope of the regression line. However, no significant correlation could be determined between the various formulations after seven days of treatment compared to two days of treatment.

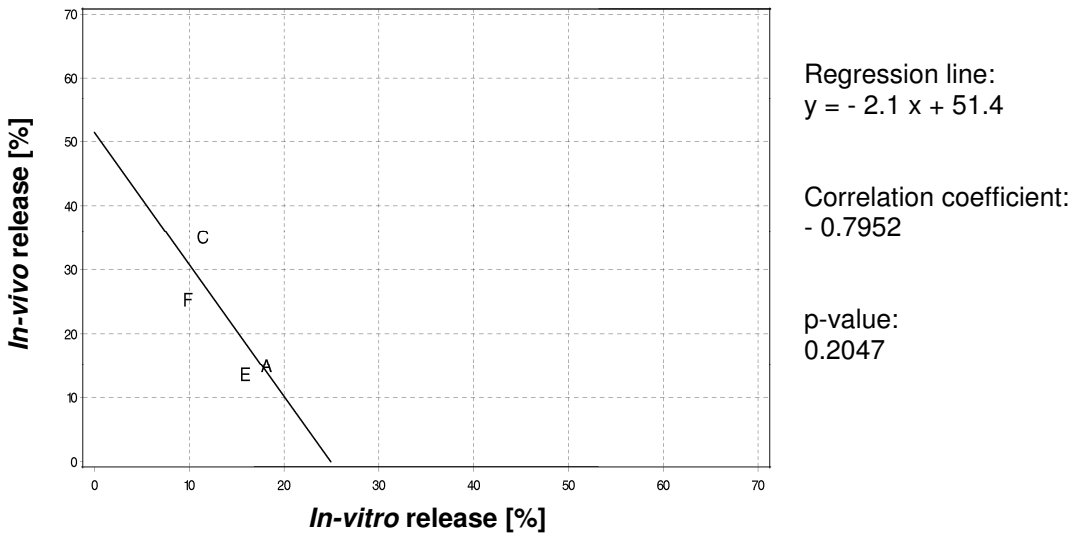


Figure 5.4: Percentage of active ingredient released *in-vivo* from formulations A, C, E and F versus *in-vitro* release, seven days after injection. Regression line, related equation and correlation coefficient are indicated. The labels A, C, E and F indicate the related formulations.

Level C correlation at time point t = 7 days, including all formulations (formulations A to F) did not show any significant correlation (chart not shown). The slope of the linear regression was not significantly different from 0 (p-value = 0.1551). Equation of regression line was - 0.3 x + 27.9 and correlation coefficient - 0.66.

3.1.4. Level C correlation for time point t = 14 days

Level C IVIVC for formulations A, C, D and F at time point t = 14 days is represented in a scatter plot in Figure 5.5. Linear regression level C IVIVC model was calculated from the relationships between *in-vitro* and *in-vivo* performances of the investigated formulations for this time point. The equation of the regression line and the correlation coefficient are indicated in Figure 5.5.

Similarly to IVIVC level C model at time points t = 7 day, the slope of the linear regression obtained from the data set for t = 14 days was not significantly different from 0 (p-value = 0.2860). Correlation coefficient of the linear regression with the value - 0.7141 indicates an insufficient relationship between investigated formulations with regard to percentage of drug released during *in-vitro* dissolution test and *in-vivo* trials. Similarly to level C correlation at time point t = 2 and 7 days, slope of regression line was negative. From these considerations, no significant level C correlation could be assessed between the various formulations after two weeks treatment.

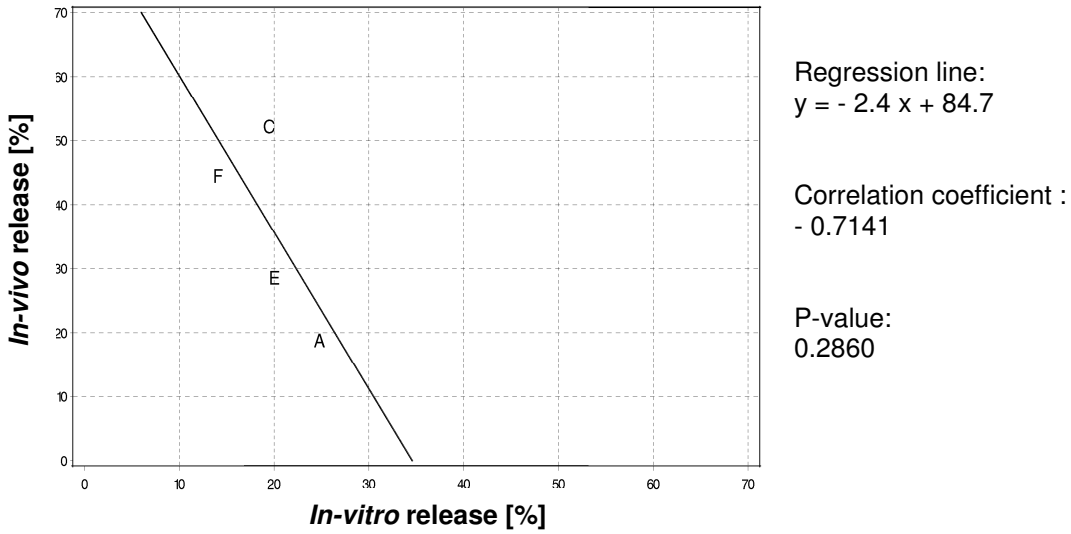


Figure 5.5: Percentage of active ingredient released *in-vivo* from formulations A, C, E and F versus *in-vitro* release, two weeks after injection. Regression line, related equation and correlation coefficient are indicated. The labels A, C, E and F indicate the related formulations.

Level C correlation at time point t = 14 days for all formulations (formulations A to F) did not show any significant correlation (chart not shown). The slope of the linear regression was not significantly different from 0 (p-value = 0.2566). Equation of regression line was - 0.4 x + 44.8 and correlation coefficient - 0.55.

3.2 Level A evaluation model

Level A correlation profiles represent percentage of drug released *in-vitro* as a function of percentage of *in-vivo* drug released at the same time point. Predictability of the performed dissolution tests can therefore be evaluated for each time point. The correlation profiles of each formulation is presented in Figure 5.6 as a solid line for a test period of 40 days. None of the correlation profiles is linear, however, some trends could be observed, as described below.

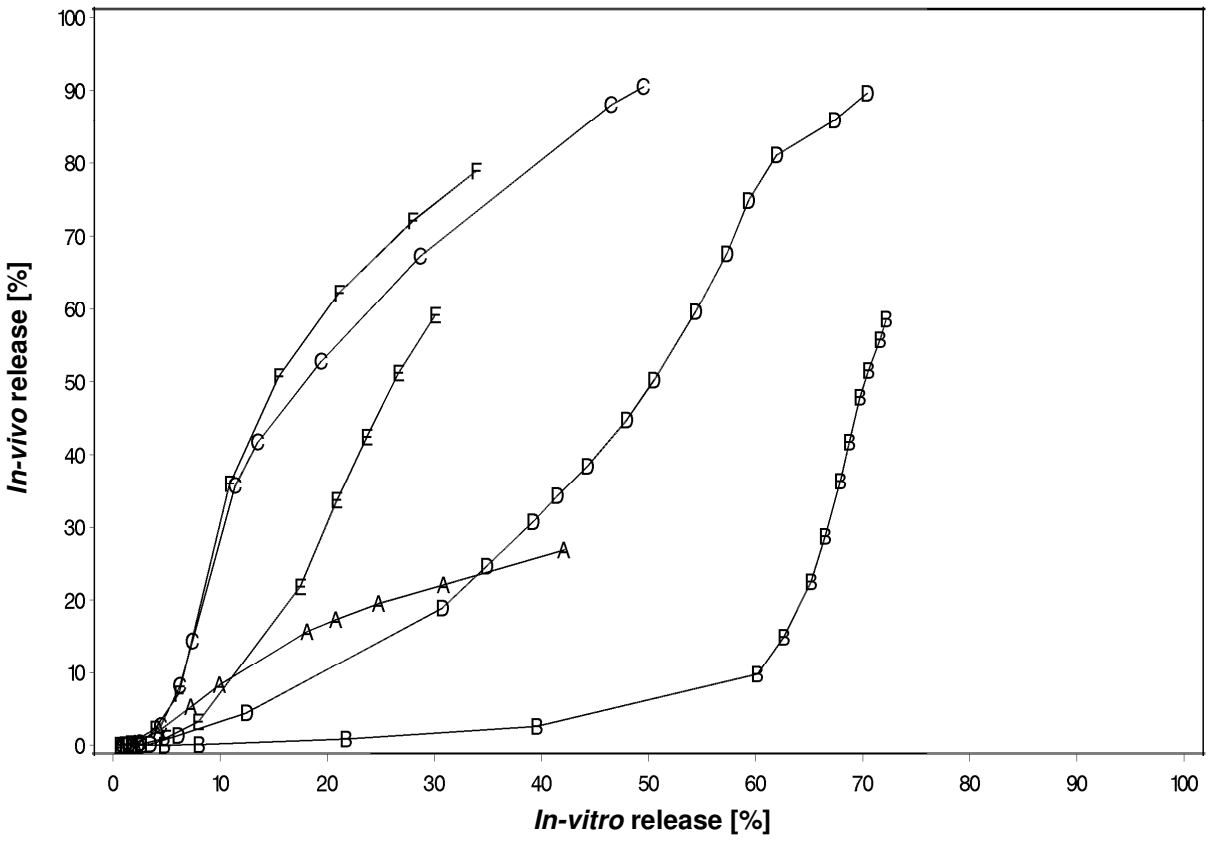


Figure 5.6: Correlation profiles level A for formulations A to F, for a drug release period of 40 days. The cumulative drug released *in-vivo* is reported for the cumulative drug release *in-vitro* for each time point.

Formulation A is based on the sole solvent triacetin and has a weight polymer to drug ratio of 1:1. The *in-vitro* as well as the *in-vivo* drug release profiles are characterized by two

distinguished phases (Figure 3.6, Chapter 3 and Figure 4.5, Chapter 4). First, there is a phase during which the drug is rapidly released, the *initial burst*, followed by a second *linear phase* with lower and more constant drug release rates. Level A IVIVC for formulation A is presented by an almost linear curve. According to the obtained correlation profile, it seems that during the first 40 days of the experiment, the *in-vitro* drug release could be found presented in average twice the *in-vivo* drug release. The two phases of the drug release profiles from the sustained release injectables formed *in-situ* were found to occur concomitantly in both drug release tests performed with formulation A, with an overall greater magnitude for *in-vitro* dissolution test.

Similarly to formulation A, formulation B contained only triacetin as solvent. However, polymer concentration in formulation B was reduced from 17.00 % (w/w) to 12.75 % (w/w), resulting in a weight polymer to drug ratio of 3:4. As reported before, initial burst from formulation B was increased for both *in-vitro* and *in-vivo* drug release experiments in comparison to formulation A, which contained higher polymer concentration (Figure 3.6, Chapter 3 and Figure 4.6, Chapter 4). Level A correlation profile of formulation B exhibits two distinguished phases: first, a phase with a much faster drug release *in-vitro* than *in-vivo* followed by a reversed tendency, where the *in-vivo* release occurred at higher rates than *in-vitro*. During the first phase of the release, already 60 % of the active ingredient was released *in-vitro*, while only 10 % of the drug was released *in-vivo*. During the second phase, the drug release was clearly faster for the *in-vivo* drug performance than for the *in-vitro* drug release. Level A correlation profile for formulation B indicates a poor predictability of the *in-vitro* dissolution test for *in-vivo* drug release performances. The increased initial burst resulting from the reduction of the polymer amount was obviously more pronounced in the *in-vitro* dissolution tests than in the *in-vivo* experiments.

The overall percentage of drug released during the 40 days of the experiment was greater for formulation B than for formulation A, for both *in-vitro* and *in-vivo* experiments. This was reported to the reduced amount of polymer and the consequently increased initial burst release.

Similarly to formulation A, formulation C had a weight polymer to drug ratio of 1:1, but contained, in addition to the main solvent triacetin, 10.00 % of co-solvent ethanol. Drug release from formulation C, for *in-vitro* and *in-vivo* experiments, showed a reduced initial burst compared to formulation A (Figure 3.10, Chapter 3 and Figure 4.7, Chapter 4). Correlation curve of formulation C exhibits a sigmoid profile. At the very beginning, *in-vitro* drug release occurs at a faster rate than *in-vivo* drug release. Then, during the second phase

the *in-vivo* drug release was greater than the concomitant *in-vitro* performance. Finally, and until the end of the 40 days of the experiment, the slope of the correlation curve is reduced and suggest similar releasing rates for both, *in-vitro* and *in-vivo* conditions.

Like formulation B, formulation D had a weight polymer to drug ratio of 3:4, and similarly to formulation C, it contained, in addition to the main solvent triacetin, 10.00 % of co-solvent ethanol. Drug release performance from formulation D showed, for *in-vitro* as well as *in-vivo* experiments, a reduced initial burst compared to formulation B due to the addition of the co-solvent ethanol, and an increased initial release compared to formulation C due to the reduced amount of PLA polymers (Figure 3.10, Chapter 3 and Figure 4.7, Chapter 4).

Correlation profile of formulation D suggested a good predictability of the drug release performance. The fraction of drug released during the first two weeks of the experiments was slightly higher for *in-vitro* experiment than for *in-vivo* experiment. For the second half of the test, the inverse tendency was observed, with an increased *in-vivo* release compared to the *in-vitro* performance. This profile is similar, but in a less accentuate way, to the correlation profile of formulation B, which also contained a polymer to drug ratio of 3:4.

Like formulations C, formulations E and F had weight polymer to drug ratios of 1:1 and 10.00 % co-solvent above the main solvent triacetin. Glycerol formal is used as co-solvent for formulation E and anhydrous glycerol for formulation F. *In-vitro* as well as *in-vivo* drug release tests for formulation E (Figure 3.9, Chapter 3 and Figure 4.9, Chapter 4) and for formulation F (Figure 3.8, Chapter 3 and Figure 4.10, Chapter 4) showed reduced initial burst release, compared to formulation A, which contained no co-solvent but only triacetin as polymer solvent. Correlation profiles of formulations E and F were similar to correlation profile of formulation C. At the very beginning, level A correlation curves showed a faster drug release *in-vitro* than *in-vivo*. Then, during the second phase, the inverse tendency is observed, during which, the *in-vivo* drug release is faster than the *in-vitro* performance. For formulation F, a further phase was observed, during which the slope of the correlation profile indicates similar release rate for both experimental conditions for the last days of the investigated period.

4. Discussion

IVIVC methods are increasingly used in development of extended release dosage forms and are now FDA regulated [Guidance for industry, 1997; EMEA, 1999; Burgess et al., 2002; Burgess et al., 2004]. Such methods aim to establish quantitative and reproducible relationships between some easily measurable *in-vitro* characteristics of a dosage form, such as the *in-vitro* dissolution rate and *in-vivo* biological parameters of interest that are considerably more time, labor and costs expensive. However, the ultimate goal is to develop a predictive model connecting the time course of relevant *in-vivo* pharmacokinetic parameters to the *in-vitro* release profile. Predictability is a desirable attribute in the assurance of drug product quality, since extrapolation is often the practical solution to achieve a scientific decision, regarding drug product performance. Correlation between *in-vitro* testing and *in-vivo* performances are encouraged, guidelines were published and since 1995, a chapter about *in-vitro* and *in-vivo* evaluation of the dosage forms is included in the USP edition. [USP 28, 2005 <1088>; Guidance for industry, 1997; EMEA, 1999].

Dissolution tests were carried out in Chapter 3 for evaluation of the *in-vitro* drug release over a period of 40 days from sustained released injectables formed *in-situ* in regards to initial burst and overall drug release rate, as function of drug content, polymer content, polymer type and solvent type. Based on *in-vitro* drug release profiles, injectability properties of the formulations, therapeutic and toxic parameters of the active ingredient as well as tolerability parameters of the excipients, six sustained release formulations were selected for *in-vivo* testing in dogs (Chapter 4). There were similarities between *in-vitro* and *in-vivo* drug release properties, however, correlations were not really observed. For example, it was evident that the reduction of the amount of polymer, increased the initial burst in both *in-vivo* and *in-vitro* experiments. However, it was not explicit if the magnitude of this effect was comparable in both conditions. Therefore, an attempt was made to correlate *in-vitro* and *in-vivo* data sets, to evaluate the predictive quality as well as the relevancy of the *in-vitro* dissolution model used.

4.1. Level C evaluation model

Level C correlation relates a dissolution parameter to a pharmacokinetic parameter. It represents a single point correlation and does not reflect the complete shape of the plasma concentration time curve. Due to its restrictive prediction, it is usually used as a guide for the formulation development or for routine production quality control [Guidance for industry, 1997, EMEA, 1999].

Level C IVIVC represented in Figure 5.1 correlates maximal blood concentration with the cumulative percentage of drug released during dissolution test at this time point. No significant correlation could be calculate. However, with the exception of the correlation point of formulation A, a clear trend could be observed, in which the *in-vivo* maximal blood concentration was positively correlated to the magnitude of drug released *in-vitro* at the same time point. For this correlation model, *in-vivo* data (C_{max}) were used as measured and therefore all parameters of the *in-vivo* test conditions were included.

Level C IVIVC was then calculated for the four formulations which contained 17.00 % (w/w) PLA polymers, excluding formulations B and D which contained reduced amount of PLA (12.75 % w/w). Formulations A, C, E and F differed only in their solvent mixture (for exact composition of formulations, see Table 5.1). As investigated in Chapters 3 and 4, solvent mixture is an important parameter influencing both *in-vitro* as well as *in-vivo* drug release properties of the formulation.

For time point t = 2 days, a significant level C IVIVC could be found by evaluating the relationship between the cumulative percentage of drug released *in-vitro* and the calculated percentage of drug released *in-vivo* using equation 5.1. The slope of regression line was significantly different from 0 ($p = 0.0362$) and the correlation coefficient was - 0.9638.

Surprisingly, the slope of the regression line of level C IVIVC at time t = 2 days was negative in a significant manner, with regards to p-value and the correlation coefficient. In a first approach, it can be interpreted that the more active ingredient is released during *in-vitro* dissolution test, the less is released during *in-vivo* experiments. However, this would contradict the level C correlation with C_{max} values, which was positively correlated as well as the data set measured in Chapters 3 and 4. *In-vivo* values used for correlation, more specifically percentage of drug released, were calculated as function of total AUC. As already mentioned in Chapter 4, total AUC differs highly for each particular formulation, despite the

same amount of drug injected to each dog. Therefore, at the same *in-vivo* concentration values, percentage of drug released is higher for a formulation with poor bioavailability (low total AUC) than for a formulation with high bioavailability (high total AUC). Level C IVIVC at day 2 indicates therefore that formulations with poor bioavailability released, in a significant manner, a relative higher amount of active ingredient during the first two days.

By using data set of the six investigated formulations (formulations A to F), it was impossible to establish any confident correlation for *in-vitro* and *in-vivo* drug release at time point t = 2 days. Level C IVIVC is generally not useful for supporting major variations in composition. Polymer concentration is a factor which influences too radically drug release at the beginning of the treatment to allow a level C IVIVC. As example, relationship between *in-vitro* and *in-vivo* drug release for formulation B at time point t = 2 days could by far not be related to the release performances of the other formulations (Figure 5.3).

It was impossible to establish any confident correlation for the *in-vitro* and *in-vivo* drug release for time points t = 7 days and t = 14 days. However, by using the set of formulations containing 17.00 % PLA polymer, predictability was higher than by using all six formulations (p-value and correlation coefficient in Figures 5.2 to 5.4). The lack of significant correlation for the formulations containing 17.00 % of PLA polymer was attributed to the small amount of samples used, as there was a similar clear trend in the scatter plot represented in Figures 5.4 and 5.5.

For the three time points to which level C IVIVC correlation was evaluated by using equation 5.1 (the fraction of drug released at a given time point related to the cumulative percentage of drug released at the same time point), slopes of regression lines were found to be all negative. Furthermore, to correlate the formulation data set with the same amount of polymer (formulation A, C, E and F containing 17.00 % of PLA polymer), the slope of the related regression lines for the three different time points were found to be very similar, between -2.4 and -2.1. The negative value of the slope of the regression lines in level C IVIVC can be explained by the high variability of total AUC values for the different formulations during *in-vivo* trials. Furthermore, kinetic of the drug release profile was not strictly considered during transformation of *in-vivo* drug concentration time profiles values. By using the AUC values the kinetic parameter of the drug release was restrained.

IVIVC must be validated by estimating the magnitude of error in prediction for *in-vivo* release. It is generally recognized that the IVIVC for special dosage forms are difficult to obtain, because of complexity of drug release mechanism. Therefore, expectations for predictability of IVIVC of special dosage forms should not be set as high as for traditional solid oral dosage forms with immediate release and loaded with water soluble compounds [Siewert et al., 2003]. However, specifications for sustained release dosage forms are set as for oral extended release formulations: the percent prediction error must be less than or equal to 10 % for the mean absolute prediction error, and must be less than or equal to 15 % for all formulations [Guidance for industry, 1997]. However, excellent level C correlation was found for slow release biodegradable implant formulations containing the water soluble active ingredient buserelin with correlation coefficients varying from 0.977 and 0.999 [Schliecker et al., 2004].

4.2. Level A correlation model

For level A IVIVC, the calculated fraction of drug absorbed *in-vivo*, as calculated with equation 5.1, was plotted versus the cumulative percentage released *in-vitro* for each time point. The *in-vitro* dissolution curve is therefore compared to the drug input rate curve which may be obtained through various methods for mass balance model techniques such as Wagner-Nelson procedure, in case the absorption curve adjusts to a model of one compartment, Loo-Riegelman method in case the adjustment is significant for a model of two compartments, or by model-independent evaluation using pharmacokinetic parameters. The straightest way of demonstrating a correlation is to plot the fraction absorbed *in-vivo* versus the fraction released *in-vitro*.
None of the correlation profile illustrated in Figure 5.6 was linear. However, some correspondences could be found between the various releasing rate patterns. The correlation profiles of formulation A showed constant faster release for *in-vitro* as for *in-vivo* conditions.

Formulations B and D, which both contained only 12.75 % of PLA polymer, resulting in a polymer to drug ratio of 3:4, exhibited in the first period of the experiments a faster *in-vitro* release, followed by a second period where *in-vivo* release rate was higher. Correlation profile of formulation B was clearly divided into these two phases, as the changes in correlation profile of formulation D were smoother.

Correlation profiles of formulations C, E and F (containing all 17.00 % of PLA polymer and 10.00 % of co-solvent) were characterized by a short initial phase where the *in-vitro* release was greater than the *in-vivo* release, followed by a longer second phase with inverse

tendency. For formulations C and F, the slope of the correlation profiles indicates for the end of the experiment, similar release rates for both conditions tested.

The principle of system analysis primarily deals with linear schemes showing dose proportionality and time invariance. Although this requirements are never completely met in biological systems, in most applications it provides a correct approach. In case of level A IVIVC, drug release profiles are directly super-imposable or can be made super-imposable by applying a time scaling factor. The time scaling factor takes into account that *in-vitro* release do not always follow the same time scale than *in-vivo* absorption [Schliecker, 2004].

If the system is essentially non-linear, traditional linear treatment would not provide suitable results with respect to blood concentration or physiological effects. However, principles of systems analysis can be extent to non-linear cases. According to the high complexity of static and dynamic non-linearity in biological systems, no general mathematical solutions can be used but individual solutions must be found. An example of non-linear IVIVC model development has been described for multi-particulate bead extended release capsule formulations containing the highly water soluble active ingredient diltiazem [Sirisuth et al., 2002]. Although IVIVC did not fully met the criteria for a valid correlation as specified in the IVIVC guidance (2.4 % over the acceptable limits), the complexity and the most relevant parameters which must be taken into account are clearly presented.

The advantage of a validated level A IVIVC is that, *in-vitro* dissolution profile can serve as a substitute for *in-vivo* performance with high predictability. Thus, modifications of manufacturing site or method, change of raw materials supplier, small changes in formulation or in product strength, may be assessed without the need for additional studies on living organisms.

4.3. Limitations of *in-vitro* dissolution test models

An essential feature to obtain a good IVIVC is to have an *in-vitro* dissolution test method which shows sufficient relevance concerning drug release at *in-vivo* physiological conditions. The challenge remains to develop and identify suitable and reliable methods which can predicatively characterize drug release from various formulations. Furthermore, the experimental test conditions should be discriminating enough to detect the influence of composition or manufacturing modifications that affect biopharmaceutical product performance [Siewert et al., 2003, Dash et al., 1999].

The current USP test apparatus for *in-vitro* release testing are primarily designed for oral and transdermal products and may not be appropriate for sustained release parenteral formulations. The probably most relevant dissolution test apparatus for sustained or controlled release parenteral drug delivery system is the USP apparatus 4. It allows flexibility in volume, sample cell, flow rate and can be altered for specific product applications [Burgess et al., 2004].

IVIVC can help in objectively evaluating *in-vitro* dissolution test methods for given products. Bioequivalence experiments performed with three marketed oral formulations containing 250 mg of the very slightly water soluble active ingredient primidone revealed that the *in-vitro* dissolution test method which gave significant different drug release profiles was too discriminating in comparison to the *in-vivo* pharmacokinetic data which were all statistically equivalent [Meyer et al., 1998].

Physiological conditions at the site of administration should be considered when selecting the *in-vitro* dissolution test parameters [Siewert et al., 2003]. Composition of the medium should include buffer capacity of the fluids at site of administration. In the body, natural surfactants increase the dissolution and subsequent the absorption of drugs with limited aqueous solubility. Therefore, the use of the surfactant Solutol HS 15® in the dissolution medium of the *in-vitro* tests performed in Chapter 3 is physiologically relevant. Sink conditions refer to the solubilizing capacity of the dissolution medium [Rohrs, 2001].

The solidification of a subcutaneous implant in the case of sustained release injectables formed *in-situ*, is dependent on the rate of fluid flow at injection site. A certain quantity of water is required for precipitation of the polymers and for formation of the solid biodegradable matrix [Shively et al., 1995]. As determined in Chapter 3, quantity of water needed for implant formation tend to decrease as the polymer concentration is increased. This has important implications with regard to the design of appropriate *in-vitro* dissolution test methods, particularly in which concerns dissolution medium. The subcutaneous and intramuscular environment has been shown to have limited amounts of water [Shively et al., 1995]. Since fluid flow in adipose tissue may range from 7 to 53 ml/100 g per min., *in-vitro* implant formation in an aqueous milieu may be erratic. A delay in the *in-vivo* solidification of the implant may result in a higher initial release as the drug dissolved in the polymer solvent is more likely to diffuse out of the polymer and into the surrounding tissue [Shively et al.,1995]. Furthermore, movement of muscles and tissues may alter *in-vivo* drug release conditions.

The Biopharmaceutical Classification System (BCS) can provide a basis for predicting the likelihood of achieving a successful IVIVC, essentially for oral drug delivery systems. Based on drug solubility and permeability, the BCS divided active ingredients into four classes. The BCS suggest that for substances of high solubility and high permeability (class I), as well as for substances with high solubility and low permeability (class III), the bioavailability is not limited by dissolution. In the case of active ingredient of low solubility and high permeability (class II), drug dissolution may be the rate limiting step for drug absorption and a IVIVC may be expected. Concerning drug of low solubility and low permeability (class IV), likelihood of a successful IVIVC is not assumed [Guidance for industry, 1997]. *In-vitro* dissolution test should be used as a surrogate for the *in-vivo* performance of a drug only if the rate limiting step is the release of the drug from the formulation [Siewert et al., 2003]. *In-vitro* dissolution release test for special dosage forms should take into account relevant bioavailability data obtained by clinical research. The complexity of *in-vivo* release mechanisms from sustained release dosage forms increases the difficulty to design physiologically based *in-vitro* tests.

The *in-vitro* dissolution test provides a simplified model in comparison to the multi-compartments physiological models. Usually, the *in-vivo* parameter used for correlation is the detected blood level of the experimental animals. However, the *in-vivo* drug release from the implant is not necessary directly correlated to the measured blood level. After the injection of the liquid formulation in the subcutaneous milieu and the solidification of the polymeric matrix, the active ingredient has to pass various physiological barriers before it can be detected in the blood. Biopharmaceutical parameters influencing the active ingredient blood concentration include clearance, distribution volume, permeation rate from the subcutaneous milieu to the blood stream, eventual formation of a capsule around the polymeric matrix, possible degradation of the drug by enzymes, metabolism of the drug, protein bindings and even spontaneous drug degradation at 37°C. All these parameters, with exception of the spontaneous chemical degradation, are neglected during the *in-vitro* dissolution test and may, on their own, or in combination, dramatically influence the plasma drug profile. Therefore, it must be emphasized that the measured blood concentration data resulting from numerous parameters and may not necessarily primarily reflect the drug release from implants.

An essential feature to obtain a good IVIVC is to have an *in-vitro* dissolution test method which shows sufficient relevance concerning drug release at *in-vivo* physiological conditions. The challenge remains to develop and identify suitable and reliable methods which can predicatively characterize drug release from various formulations. Furthermore, the experimental test conditions should be discriminating enough to detect the influence of

composition or manufacturing modifications that affect biopharmaceutical product performance [Dash et al., 1999; Siewert et al., 2003].

The current USP method apparatus for *in-vitro* release testing are primarily designed for oral and transdermal products and may not be appropriate for sustained release parenteral formulations. The probably most relevant dissolution test apparatus for sustained or controlled release parenteral drug delivery system is the USP apparatus 4. It allows flexibility in volume, sample cell, flow rate and can be altered for specific product applications [Burgess et al., 2004].

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Physiological conditions at the site of administration should be considered when selecting the *in-vitro* dissolution test parameters [Siewert et al., 2003; Dash et al., 1999]. Composition of the medium should include buffer capacity of the fluids at site of administration. In the body, natural surfactants increase the dissolution and subsequent absorption of drugs with limited aqueous solubility. Therefore, the use of the surfactant Solutol HS 15® in the dissolution medium of the *in-vitro* tests performed in Chapter 3 is physiologically relevant. Sink conditions refer to the solubilizing capacity of the dissolution medium [Rohrs, 2001].

The solidification of a subcutaneous implant in the case of sustained release injectables formed *in-situ*, is dependent on the rate of fluid flow at injection site. A certain quantity of water is required for precipitation of the polymers and for formation of the solid biodegradable matrix [Shively et al., 1995]. As determined in Chapter 3, quantity of water needed for implant formation tend to decrease as the polymer concentration is increased. This has important implications with regard to the design of appropriate *in-vitro* dissolution test methods, particularly in which concerns dissolution medium. The subcutaneous and intramuscular environment has been shown to have limited amounts of water [Shively et al., 1995]. Since fluid flow in adipose tissue may range from 7 to 53 ml/100 g per min., *in-vitro* implant formation in an aqueous milieu may be erratic. A delay in the *in-vivo* solidification of the implant may result in a higher initial release as the drug dissolved in the polymer solvent is

more likely to diffuse out of the polymer and into the surrounding tissue [Shively et al.,1995]. Furthermore, movement of muscles and tissues may alter *in-vivo* drug release conditions.

In-vitro dissolution release test for special dosage forms should take into account relevant bioavailability data obtained by clinical research. The complexity of *in-vivo* release mechanisms from sustained release dosage forms increases the difficulty to design physiologically based *in-vitro* tests. *In-vitro* dissolution test should be used as a surrogate for the *in-vivo* performance of a drug only if the rate limiting step is the release of the drug from the formulation [Siewert et al., 2003].

The *in-vitro* dissolution test provides a simplified model in comparison to the multi-compartments physiological models. Usually, the *in-vivo* parameter used for correlation is the detected blood level of the experimental animals. However, the *in-vivo* drug release form the implant is not necessary directly correlated to the measured blood level. After the injection of the liquid formulation in the subcutaneous milieu and the solidification of the polymeric matrix, the active ingredient has to pass various physiological barriers before detection in blood. Above the effective drug release from the implant matrix, further physiological or biopharmaceutical parameters influence active ingredient blood concentration. It includes clearance, volume of distribution, permeation rate from the subcutaneous milieu to the blood stream, eventual formation of a capsule around the polymeric matrix, eventual degradation of the drug by enzymes, metabolism of the drug, protein bindings and even spontaneous drug degradation at 37°C. All these parameters, with exception of the spontaneous chemical degradation, are neglected during the *in-vitro* dissolution test and may, on their own, or in combination, dramatically influence the plasma drug profile. Therefore it must be emphasized that measured blood concentration are *in-vivo* data resulting from numerous parameters and may not necessarily primarily reflect the drug release from implants.

Therefore, it was suggested to establish IVIVC for various dissolution test parameters and choose the variables in order to reach optimal bio-relevant conditions. The *in-vitro* tests allow a first selection of formulations parameters, and helps to understand drug release mechanisms. In addition they, are important for batch control quality as well as similarity and reproducibility of the drug release for given conditions. Adjustments of the *in-vitro* data to fit better physiological conditions are justified for validation of level A IVIVC, as an established correlation may reduce the number of bioequivalence studies during product development, be helpful in setting specifications and be used to facilitate certain regulatory decisions such a scale up or post-approval variations [EMEA, 1999]

4.3. Evaluation of pharmacokinetic parameters

Different approaches and methods have been designed to set IVIVC for biodegradable parenteral dosage forms. However, in the literature, only few examples can be found where an *in-vitro* drug method predicts the *in-vivo* release profiles for parenteral biodegradable depot systems [Negrín et al., 2001]. This points out the challenge in establishing IVIVC for these formulation types due to the various parameters affecting *in-vivo* and *in-vitro* drug release [Schliecker et al., 2004; Negrín et al., 2001; Dash et al., 1999; Woo et al., 2001]. Expectations with respect to quality and level of IVIVC of special dosage form should not be set as high as for solid oral dosage forms, because of their higher level of complexity [Siewert et al., 2003].

The evaluation of *in-vivo* drug release was based on the observed total $AUC_{[0,\infty]}$ using a independent profile model. According to the pharmacokinetics reported in Chapter 4, Table 4.4, fraction of drug released during the whole duration of *in-vivo* experiments were surprisingly low and dissimilar. They represented 25 % and 76 % of the total active ingredient integrated in the implant of formulation A, 89 % and 64 % of formulation B, 42 % and 18 % of formulation C, 11 % and 15 % of formulation D, 16 % and 23 % of formulation E and 12 % and 13 % of formulation F. As the injected active ingredient amount was identical for all tested formulations (400 mg) and as the body weight of the experimental animals varied between 8.6 kg and 12.0 kg, the overall AUC for the different release profiles were expected to be more similar. Hypotheses such as incomplete release from the implant due to a too dense polymeric matrix, encapsulation of the implant with connective tissues, degradation of the active ingredient within the implant or incomplete absorption of the active ingredient from the subcutaneous milieu have been proposed (Chapter 4). Such elements cannot be simulated during *in-vitro* drug release testing. As already mentioned in Chapter 4, *in-vivo* data obtained with two dogs per groups are not robust enough for further analysis like IVIVC. Inter-animal variation after injection of such formulation was in some cases too important, that the prediction based on such data are unreliable.

5. Conclusion

The purpose of this study was to investigate the possibility to develop different levels of correlation between *in-vitro* dissolution parameters and *in-vivo* pharmacokinetic parameters for NOA449851 sustained release injectables formed *in-situ*.

Therefore attempt for level C IVIVC was performed, using as pharmacokinetic parameter C_{max} or an evaluated value of the fraction of drug released at given time points, in relation to cumulative *in-vitro* drug release at the same time point. The correlation level C for C_{max} with the cumulative *in-vitro* drug release was positively correlated, however not in significant manner. A significant correlation, but negative, was found for formulations containing 17.00 % of PLA polymers two days after injection. The relationship was negatively correlated, probably because of important differences in the total AUC values obtained *in-vivo* for the various formulations. Level A correlation profiles showed no linearity correlation for any formulation profile. However, some similarities for *in-vitro* and *in-vivo* drug release relationship could be observed. In general, it should be concluded that for this type of dosage form and drug no satisfactory IVIVC could be observed.

A simple kinetic model is unlikely to explain the overall *in-vivo* release behavior since the drug release is influenced by factors such as diffusion, dissolution and erosion. The *in-vitro* drug release studies permit a better comprehension of the different drug release mechanism from the particular delivery device, and help selecting parameters of the formulation to approach an ideal drug release. However, the model used for *in-vitro* drug release testing neglect some crucial aspects of the physiological conditions and cannot replace biological systems. Furthermore, the technique for mass balance model should be carefully evaluated to prevent alteration of data as function of relevant biopharmaceutical parameter, as in the present case.

In view of the use of IVIVC as a surrogate for *in-vivo* performance, it should be verified that the predictability of the *in-vivo* performance of a product based on its *in-vitro* dissolution profile is valid for the *in-vitro* dissolution rates covered by the specifications. This evaluation should focus on the estimation of the predictive performance or, conversely prediction error.

Chapter 6

Stability Studies of three Formulations containing the active ingredient NOA449851, PLA Polymers and Triacetin

1. Introduction and objectives

As part of development of new drug delivery systems, one of the most decisive issues is long term stability of formulations during storage. In sustained release injectables formed *in-situ*, various parameters should be investigated. Firstly, stability of the active ingredient NOA449851 and possible formation of by-products should be determined. Secondly, stability of the used PLA polymers, the excipient which confers to formulations modified release properties, should be evaluated.

Three formulations investigated for their *in-vitro* and *in-vivo* release properties (see Chapter 3 and 4) were selected for stability studies. These formulations, as well as their related placebos, were stored during six months at four different temperatures. An HPLC-method was utilized to determine drug content as well as appearance and quantification of four of known by-products. GPC was used to analyze molecular weight of the PLA polymers. Additionally, a NIR screening method was used as rapid, non destructive and innovative analytical method to follow various stability parameters of formulations and related placebos. To provide functional control, *in-vitro* release properties was investigated for a formulation stored for six months at 40°C, and compared with *in-vitro* release properties of the same formulation evaluated directly after manufacturing.

2. Materials and methods

2.1 Materials

The active ingredient NOA449851 was obtained from CarboGen Laboratories, Aarau, CH. PLA polymers (inherent viscosity: 0.68 dl/g in trichloromethane at 30°C) were provided by Birmingham Polymers Inc., Birmingham, AL, USA. Triacetin and THF were obtained from Fluka AG, Buchs, CH. Ethanol absolute, acetonitrile, phosphoric acid 85 % and water for chromatography were purchased from Merck Inc., Darmstadt, D. All solvents were of reagent analytical grade.

2.2. Preparation of formulations

Formulations were prepared as already described in Chapter 3, Section 2.2.1.1. All formulations contained PLA as biodegradable polymers, NOA449851 as active ingredient and triacetin as main solvent. Formulation A contained 17.00 % (w/w) of active ingredient NOA449851 as well as 17.00 % (w/w) of PLA polymers, resulting in a weight polymer to drug ratio of 1:1. In formulation B, PLA polymer concentration was reduced to 12.75 % (w/w), resulting in a weight polymer to drug ratio of 3:4. In formulation C, weight polymer to drug ratio was again 1:1, but a fraction of the solvent triacetin was replaced by the co-solvent ethanol absolute. Exact compositions of the three formulations are listed in Table 6.1. Beside the formulations, two kinds of placebos were prepared: placebos 1 without active ingredient and placebos 2, containing neither active ingredient nor PLA polymers. All placebos contained an increased amount of triacetin to compensate for the active ingredient or the PLA polymers.

Table 6.1: Compositions [% (w/w)] of formulations A, B and C as well the two kinds of placebos used for stability studies.

Formulation	Component	Concentration of component [% (w/w)]	Placebo 1 (no drug) [% (w/w)]	Placebo 2 (no drug, no polymer) [% (w/w)]
A	NOA449851	17.00	-	-
	PLA	17.00	17.00	-
	triacetin	66.00	83.00	100.00
B	NOA449851	17.00	-	-
	PLA	12.75	12.75	-
	triacetin	70.25	87.25	100.00
C	NOA449851	17.00	-	-
	PLA	17.00	17.00	-
	triacetin	56.00	73.00	90.00
	ethanol	10.00	10.00	10.00

2.3. Storage of formulations

2.3.1. Storage for HPLC and GPC analysis

Formulations A, B and C as well as placebos B1 and C1 were filled up in glass vials and closed with rubber stoppers and aluminum caps. The hermetically closed vials were stored for six months at four different temperatures set at 5°C, 25°C, 30°C and 40°C (Klimazelle Rosenmund, Liestal, CH).

2.3.2. Storage for NIR-screening

Approximately 200 mg of the liquid formulations A, B and C as well as their placebos 1 and 2 were filled in glass sample vials. These were closed with a plastic cap and sealed with an epoxy mixture to prevent contamination. Sample vials were placed for six months in two racks of a Bruker SpecScreen X HTS apparatus heated at 25°C and 40°C.

2.4. Active ingredient and by-products determination

In order to determine stability of the active ingredient and appearance of by-products during storage, HPLC-analysis was carried out directly after manufacturing as well as after 1, 3 and 6 months storage at 5°C, 25°C, 30°C and 40°C for formulations A, B and C.

2.4.1. Preparation of samples for HPLC-analysis

Approximately 250 mg of each formulation were weighed into 100 ml volumetric flasks. The flasks were filled up to calibration line with acetonitrile. The mixtures were shaken manually and put in the ultrasonic bath until complete dissolution. Each active ingredient determination was made in triplicate.

2.4.2. HPLC-method

Content of NOA449851 and of four of its by-products in formulations was determined with HPLC. For this purpose, a LC HP 1100 (Hewlett Packard, Basel, CH) apparatus was used. Column (250 mm length and 4.6 mm internal diameter) was packed with Nucleosil 5 µm, C18 (Macherey-Nagel, Düren, D) and maintained at a temperature of 25°C. Elution medium consisted of a mixture of acetonitrile and an aqueous solution of phosphoric acid 0.05 % (v/v). Composition of mobile phase permanently changed in relation to the time of 90 min. Flow rate was 1.3 ml/min during the whole analyze run. A volume of 15 µl of sample solution was injected per run with an auto-sampler. Samples were detected and analyzed with UV-light at a wavelength of 240 nm.

2.5. Molecular weight analysis of PLA polymers

Molecular weights of the PLA polymers in formulations B and C as well as in the related placebos B1 and C1 were determined with GPC. Samples were analyzed directly after manufacturing as well as after 1, 3 and 6 months storage at 5°C, 25°C and 40°C.

2.5.1. Preparation of samples for GPC-analysis

Formulations and placebos samples containing PLA polymers were diluted with THF to obtain a polymer concentration of 0.3 % (w/v). Before GPC-analysis, the solutions were filtered through 1.0 µm pore size filters.

2.5.2. GPC-method

For molecular weight determination of PLA polymers, an HPLC system with a Spectra-Physics Isochrom HPLC-pump and a Shodex RI-71 differential refractive index detector were used. Three GPC-columns (PSS SDV, styrenedivinylbenzene copolymer, particle size 5 µm, pore size 10^2 Å, ID 8.0 mm x 300 mm; PSS SDV, styrenedivinylbenzene copolymer, particle size 5 µm, pore size 10^3 Å, ID 8.0 mm x 300 mm and PSS SDV, styrenedivinylbenzene copolymer, particle size 5 µm, pore size 10^5 Å, ID 8.0 mm x 300 mm) were connected in series and maintained at a temperature of 23°C. A guard column (PSS SDV, styrenedivinylbenzene copolymer, particle size 5 µm, pore size 10^2 Å, ID 8.0 mm x 50 mm) was connected in front of the three GPC-columns. THF was used as mobile phase at a constant flow rate of 1.0 ml/min. A volume of 100 µl of each sample was injected with an auto-sampler (Spark-Triathlon Autosampler, Techlab GmbH, Erkerode, D). Molecular weights of PLA polymers were calculated from elution volume of polystyrene standards. Calibration line was established using a series of polystyrene standards of narrow molecular weight distribution, with a molecular weight in a range from 162 Da to 2'570'000 Da.

Degradation rate constant k was determined for each sample at storage temperatures of 5°C, 25°C and 40°C. A trendline was obtained for each sample stored at a defined temperature from charts illustrating molecular weight changes verses time. Values of kinetic constants k are obtained from the slopes of the linear trendlines.

2.6. Low field NMR-screening (NIR-screening)

Low field NMR-spectroscopy (NIR-spectroscopy) provides a non-invasive, rapid, reproducible and relatively inexpensive means of characterizing formulations. This vibrational spectroscopic technique was used as complementary alternative method for storage stability determination of the three formulation candidates and corresponding placebos.

2.6.1. NIR-method

Formulations A, B and C as well as their placebos 1 and 2 were scanned by NIR-spectroscopy using a Bruker SpecScreen x HTS apparatus at intervals of 24 h over a period of six months for storage temperatures of 25°C and 40°C. An Infrared Fourier Spectrometer Vector 22/N-T spectrometer was used. Scans were taken at a rate of 5 spectra/second and with a resolution of 8 cm^{-1}.

2.6.2. Low field NMR data evaluation (NIR data evaluation)

Evaluation of low filed NMR spectroscopic data sets was performed by statistical analysis with respect to systematic changes in the spectra during storage period of the samples. Data analysis focused on statistical evaluation of a predetermined spectra range, in order to identify wavelength regions with detectable differences in time. Therefore, data evaluation algorithm was used: it consisted of data pretreatments, principal component analysis and linear regression analysis of the first two principal components regarding systematic modifications in the spectra during storage. Kinetic profiles of changes were calculated and comparison for related samples were made possible after further data pretreatment which fit both arbitrary Y-axis to the same input value. Slopes of the calculated regression lines indicated which samples or which storage conditions lead to better stability [Bruker User's manual, 1996].

2.7. pH measurement of solvent triacetin

The pH value of solvent triacetin was investigated after three months storage at 25°C and 40°C. According to chemical structure of triacetin, degradation would affect first ester bounds of the molecule, leading to acetic acid as degradations product. Therefore, a volume of 25.0 ml of triacetin was mixed to 100.0 ml of water and the pH of this mixture was measured (Metrohm electrode, Type 3M KCl) under mechanical stirring.

2.8. *In-vitro* release of formulation A after six months storage at 40°C

To evaluate impact of stability parameters on drug release performance, a functional control was required. Therefore, after six months storage at 40°C, formulation A was investigated for *in-vitro* drug release, using the same dissolution test method as in Chapter 3, Section 2.2.1.3. Drug release profile of the stored formulation was compared with the one of the identical formulation tested directly after manufacturing (*in-vitro* drug release profile of formulation PLA-AI(1:1), illustrated in Figure 3.3, of Chapter 3).

For comparison of two dissolution profiles, Moore and Flanner have proposed a simple model independent approach using mathematical indices to define difference factor (f_1) and similarity factor (f_2) [Moore et al., 1996]. These factors directly compare differences between percentage of drug dissolved per unit time for a test and a reference formulation. There were defined by Equations 6.1 and 6.2,

Equation 6.1:
$$f_1 = \left\{ \frac{\sum_{t=1}^{n} |R_t - T_t|}{\sum_{t=1}^{n} R_t} \right\} \cdot 100\%$$

Equation 6.2:
$$f_2 = 50 \cdot \log\left\{ \left[1 + \frac{1}{n} \cdot \sum_{t=1}^{n} (R_t - T_t)^2 \right]^{-0.5} \cdot 100 \right\}$$

where R_t is the reference assay at time point t, T_t is the test assay at time point t and n is the number of pull points. Difference factor f_1 is proportional to the average difference between both profiles. The percent error is zero when test and reference profiles are identical and increases with dissimilarity between the two profiles. Similarity factor f_2 measures the closeness between the two profiles. It is a logarithmic transformation of the sum of squared error. It takes the average sums of squares of the difference between test and reference profiles and fits the results between 0 and 100. The similarity factor (f_2) is 100 when the test and the reference profiles are identical and approaches zero as the dissimilarity increases. Similarity factor (f_2) is recommended for dissolution profile comparison in the FDA's Guidance for Industry. FDA has set a public standard of f_2 value between 50-100 to indicate sameness or equivalence of two dissolution profiles [Shah et al., 1998].

3. Results

3.1. Active ingredient content and by-products determination

3.1.1. Active ingredient content

Content of active ingredient in the three formulations was determined after manufacturing with an HPLC-method and the results are depicted in Table 6.2. The declared concentration of active ingredient was 17.00 % (w/w). All active ingredient determinations were done in triplicate. The measurements were made directly after manufacturing and at three different time points during the six months storage at four selected temperatures.

Table 6.2: Active ingredient contents in formulations A, B and C directly after manufacturing. The values are reported in percent [% (w/w)] of the weighted mass (n = 3 ± S.D.).

Formulation	Declared AI content	Real AI content ± S.D.	Percent of declared content
	[% (w/w)]	[% (w/w)]	[% (w/w)]
A	17.00	17.18 ± 0.862	101.06
B	17.00	17.38 ± 0.437	102.24
C	17.00	17.47 ± 0.598	102.76

Active ingredient content determined at four selected time points during the six months storage are depicted in Figures 6.1 to 6.4. Content of active ingredient is reported in percent of the corrected initial amount, 100 % representing the amount of drug at beginning of storage period. Each figure shows the active ingredient content in formulations A, B and C during the six months storage period for a defined temperature.

Active ingredient content in percent of the initial value after six months storage at 5°C was determined to be 101.73 % (w/w) ± 0.84 for formulation A, 99.98 % (w/w) ± 1.76 for formulation B and 102.07 % (w/w) ± 0.39 for formulation C. All results for active ingredient stability study during storage at 5°C are graphically represented in Figure 6.1.

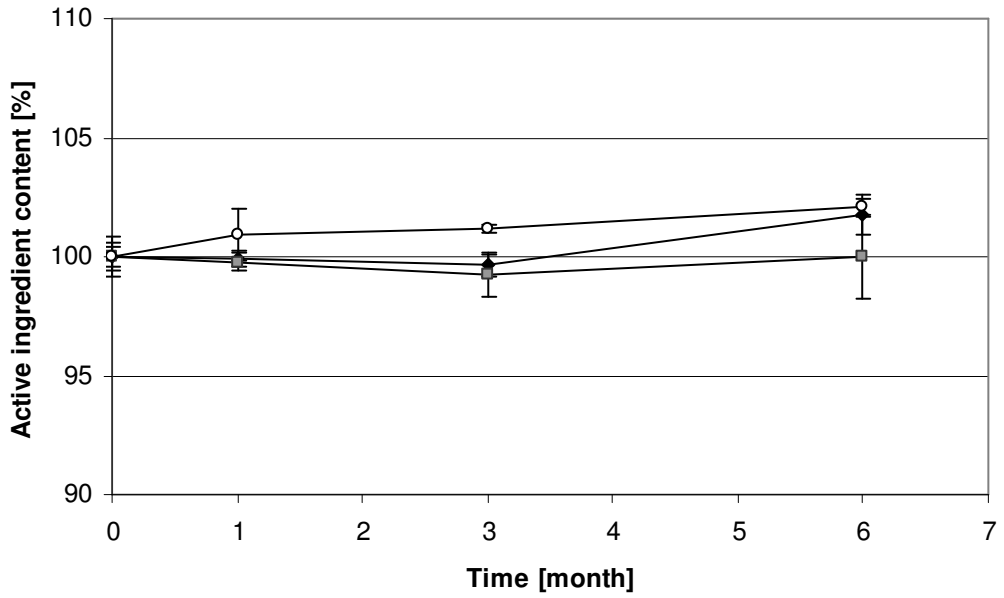


Figure 6.1: Active ingredient content in percent [% (w/w)] of the initial corrected value for formulations A (◆), B (■) and C (O) during six months storage at 5°C (n = 3 ± S.D.).

After six months storage at 25°C, recovery of active ingredient in percentage of initial corrected value represented 100.99 % (w/w) ± 0.81 for formulation A, 100.35 % (w/w) ± 1.01 for formulation B and 99.64 % (w/w) ± 1.28 for formulation C. Stability results of active ingredient concerning storage at 25°C are illustrated in Figure 6.2.

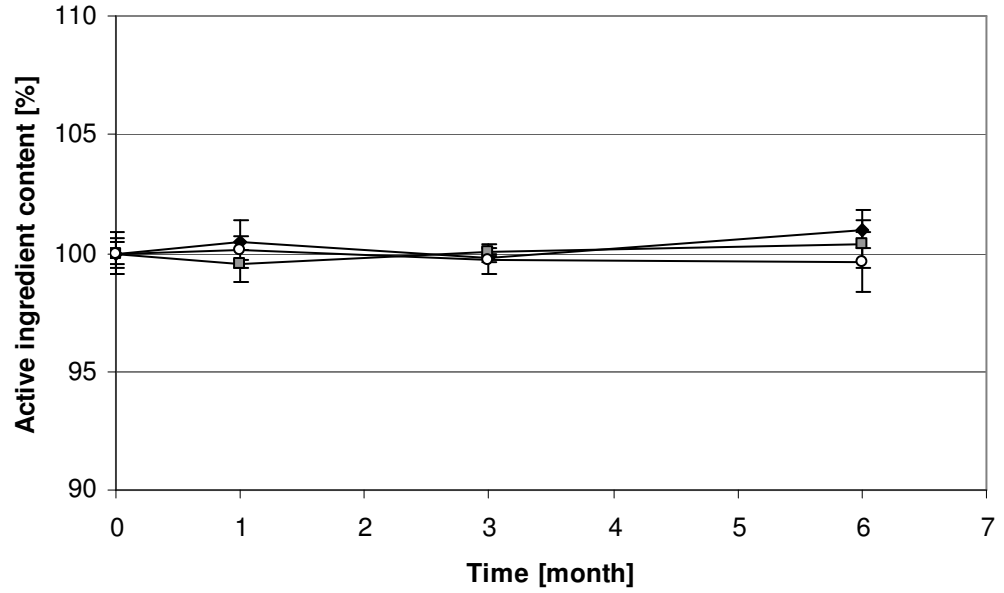


Figure 6.2: Active ingredient content in percent [% (w/w)] of the initial corrected value for formulations A (◆), B (■) and C (O) during six months storage at 25°C (n = 3 ± S.D.).

After six months storage at 30°C, active ingredient content in percent of initial corrected value was 100.41 % (w/w) ± 0.34 for formulation A, 100.19 % (w/w) ± 0.66 for formulation B and 98.51 % (w/w) ± 0.19 for formulation C. Results of active ingredient content during storage at 30°C are illustrated in Figure 6.3.

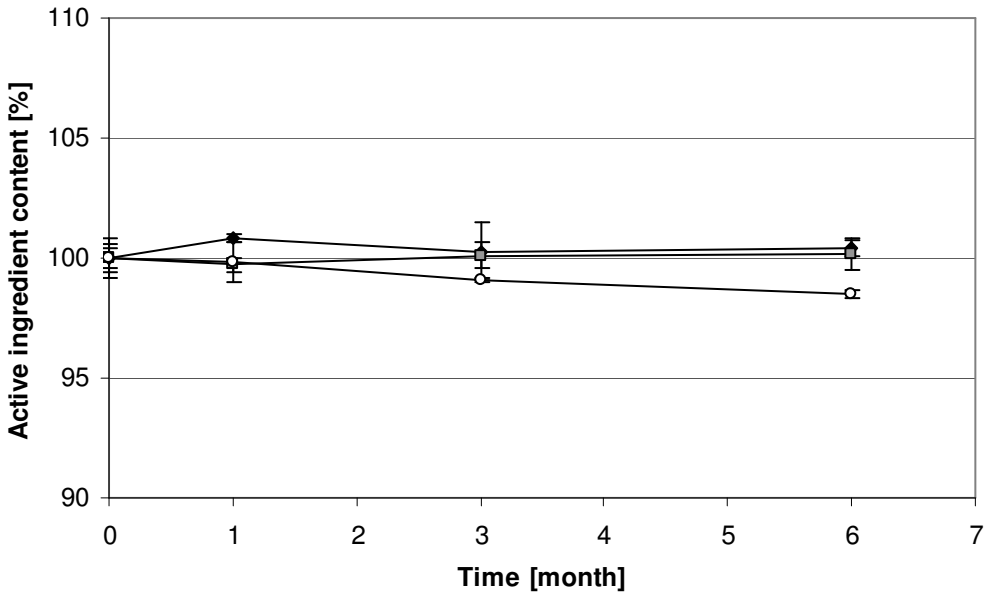


Figure 6.3: Active ingredient content in percent [% (w/w)] of the initial corrected value for formulations A (◆), B (■) and C (O) during six months storage at 30°C (n = 3 ± S.D.).

Active ingredient content decreased in all three formulations after six months storage at 40°C. The main decrease of active ingredient was observed in formulation C with an active ingredient content in percent of the initial corrected value of 92.89 % (w/w) ± 0.35, followed by formulation A with 94.09 % (w/w) ± 0.28 and formulation B with 95.46 % (w/w) ± 0.39. Results for active ingredient stability study during storage at 40°C are graphically represented in Figure 6.4.

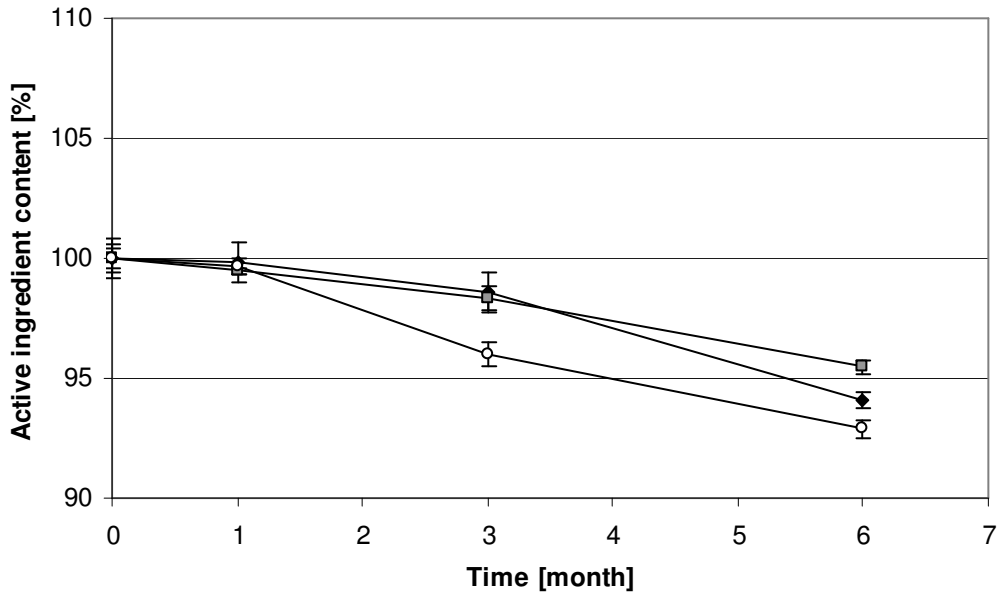


Figure 6.4: Active ingredient content in percent [% (w/w)] of the initial corrected value for formulations A (◆), B (■) and C (O) during six months storage at 40°C (n = 3 ± S.D.).

For storage temperature of 40°C, trendline of each formulation was calculated from the chart illustrated in Figure 6.4 and used to evaluate storage time until degradation of 10 % of the active ingredient. The equations of the linear trendline of type "y = ax + b" are listed in Table 6.3. Time for degradation of 10 % of the active ingredient was obtained by calculating x-value when y-value was set as 90 in the related equation. Time until active ingredient content dropped to 90 % was evaluated to be 10.57 months for formulation A, 13.46 months for formulation B and 8.19 months for formulation C for storage at 40°C.

Table 6.3: Equation of trendline for each formulation stored at 40°C as well as the evaluation of the time [month] to reach a drug content of 90 % (w/w).

Formulation	Equation of trendline [y = ax + b]	Evaluated time until 10 % of AI degraded [month]
A	y = -1.0055 x + 100.63	10.57
B	y = -0.7586 x + 100.21	13.46
C	y = -1.2551 x + 100.28	8.19

3.1.2. By-products analysis

The HPLC-method described in Section 2.4.2 for determination of the active ingredient content also permitted to detect and quantify four known by-products of NOA449851. These substances are designated as BP-1, BP-2, BP-3 and BP-4. Degradation mechanisms of the active ingredient resulting in the various by-products are typical hydrolytic as well as oxidation reactions. None of the by-product is known to have any particular toxic effect. Detection of by-products in formulations A, B and C was performed directly after manufacturing and after 1, 3 and 6 months storage at 5°C, 25°C, 30°C and 40°C. The amount of by-products detected is reported in Figures 6.5 to 6.7, in percent of declared amount of active ingredient.

During six months storage of formulation A at four different selected temperatures, by-products of active substance NOA449851 were detected with HPLC-method described in Section 2.4.2. At storage temperatures of 5°C, 25°C and 30°C, by-products content in percentage of declared amount of active ingredient was below 0.2 % (w/w). After six months storage at 40°C, BP-1 and BP-4, were found to be at concentrations of 0.45 % (w/w) and 0.82 % (w/w), respectively. Results are graphically represented in Figure 6.5.

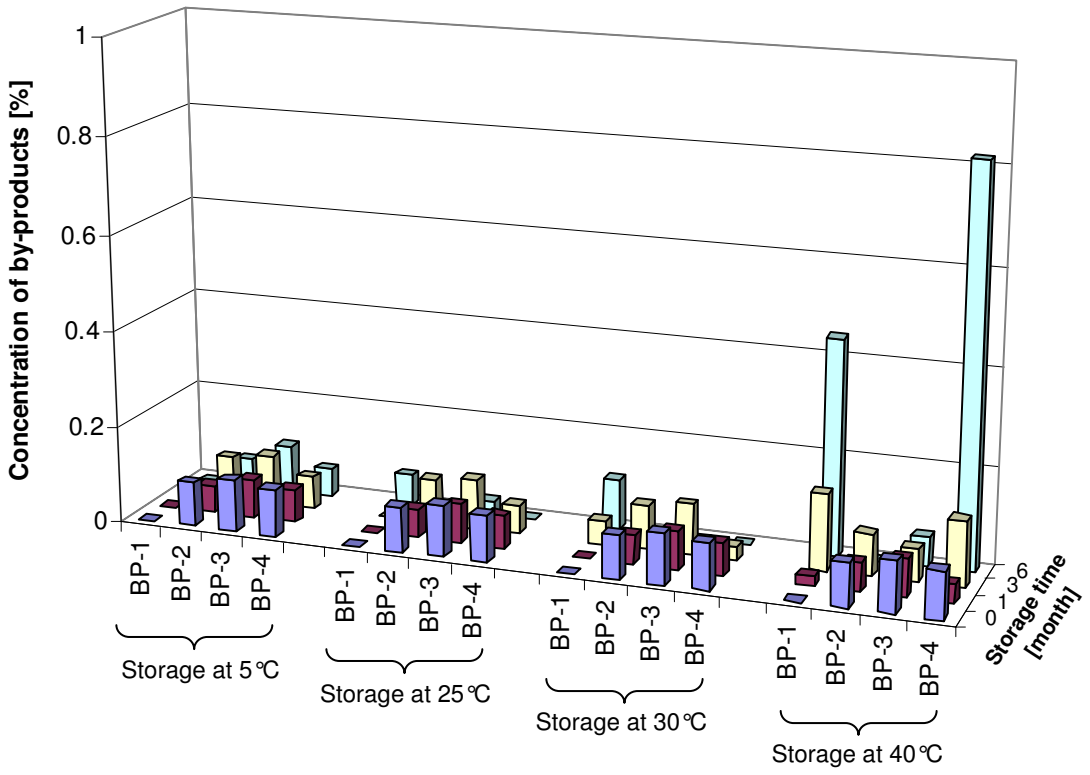


Figure 6.5: Amount of the four by-products of the active ingredient NOA449851 in formulation A during six months storage at 5°C, 25°C, 30°C and 40°C. The by-products concentration was given in percent [% (w/w)] of the declared amount of active ingredient in the formulation.

Four by-products of NOA449851 were detected with HPLC during the six months storage of formulation B. As for formulation A, by-product content in percent of the declared value of active ingredient remained below 0.2 % (w/w) for the storage temperatures 5°C, 25°C and 30°C. After six months storage at 40°C, by-products BP-1 and BP-4 were found to be at concentrations of 0.41 % (w/w) and 0.66 % (w/w), respectively. All results concerning by-products detection in formulation B are represented in Figure 6.6.

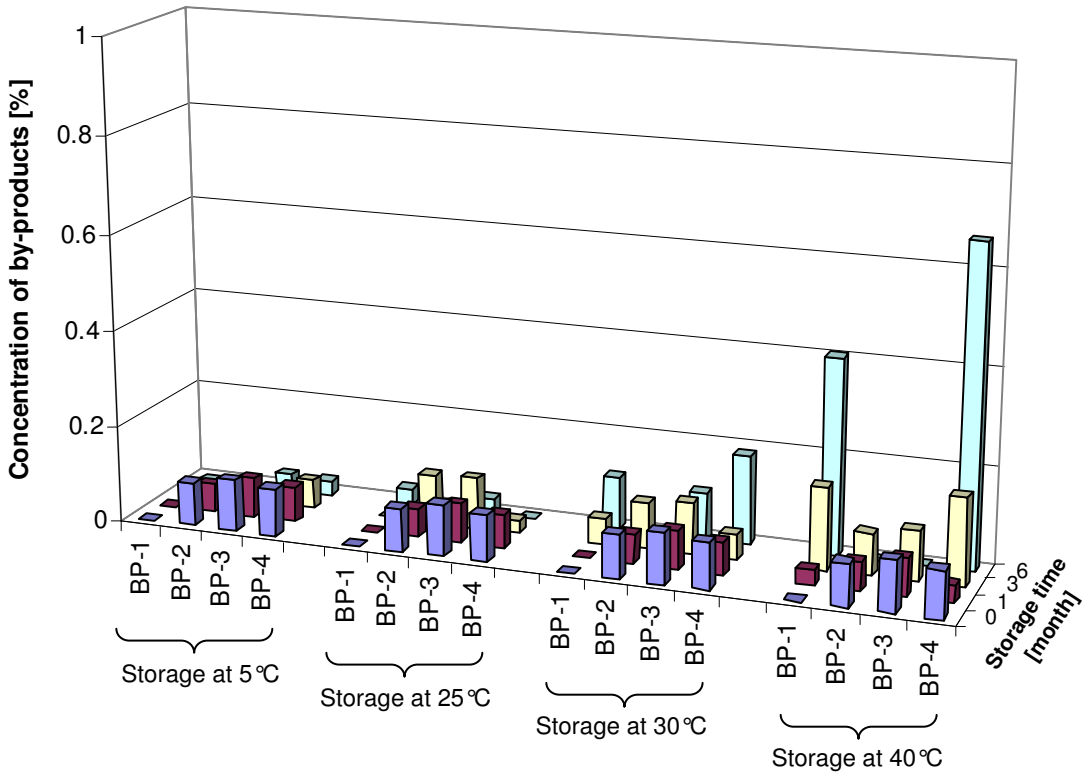


Figure 6.6: Amount of the four by-products of the active ingredient NOA449851 in formulation B during six months storage at 5°C, 25°C, 30°C and 40°C. The by-products concentration was given in percent [% (w/w)] of the declared amount of active ingredient in the formulation.

By-products of the active substance NOA449851 were detected with HPLC-method during the six months storage of formulation C at four different temperatures. At storage temperatures of 5°C and 25°C, all by-products contents in percent of the declared value of the active ingredient, were below 0.2 % (w/w). After six months storage, BP-1 was detected for storage temperature of 30°C in a concentration of 0.21 % (w/w) and for 40°C in a concentration of 0.59 % (w/w). BP-4 reached after six months storage at 40°C a concentration of 0.45 % (w/w). All results concerning by-products analysis during storage of formulation C are represented in Figure 6.7.

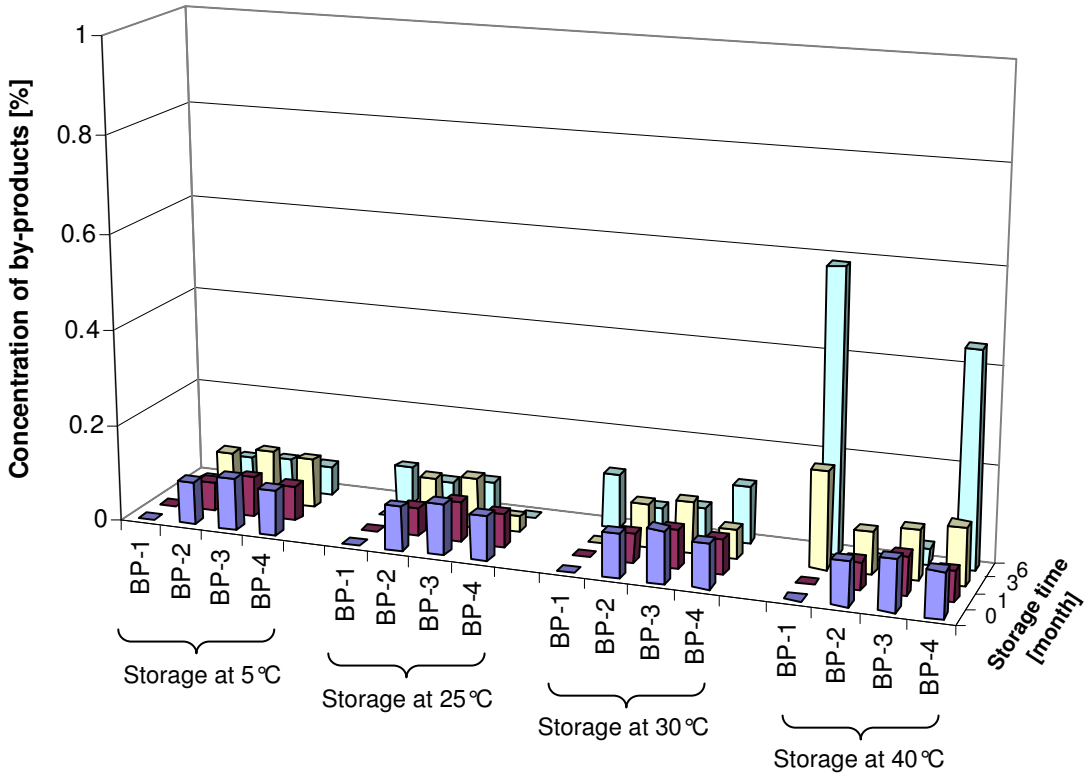


Figure 6.7: Amount of the four by-products of the active ingredient NOA449851 in formulation C during six months storage at 5°C, 25°C, 30°C and 40°C. The by-products concentration was given in percent [% (w/w)] of the declared amount of active ingredient in the formulation.

There were no noticeable differences between formulations A, B and C, concerning by-products appearance during six months storage. By-products concentrations tend to increase with time and temperature storage. In the three tested formulations, particularly BP-1 and BP-4 were present after six months storage at storage temperature of 40°C, but remained at concentrations lower than 1 % of declared amount of active ingredient.

3.2. Molecular weight analysis of PLA polymer

Changes in molecular weight of PLA polymer in formulations B and C as well as in related placebos B1 and C1 were determined using GPC. Molecular weight of PLA polymer were measured at four time points during the six months storage at the selected temperatures of 5°C, 25°C and 40°C.

Molecular weight of PLA polymer measured directly after manufacturing was 69'500 Da for formulations B and C and placebos B1 and C1 (same PLA polymer batch). Changes in average molecular weight of PLA polymer are represented in percent of the initial value, 100 % representing the molecular weight directly after manufacturing.

Reduction of the average molecular weight of PLA polymer in formulation B and its related placebo B1 for storage temperatures of 5°C, 25°C and 40°C are illustrated in Figure 6.8. Changes in molecular weight of the PLA polymers were highly dependent on the temperature storage. The higher the storage temperature the more important was the reduction of molecular weight. After six months storage at 40°C, the molecular weight of PLA polymer in formulation B was reduced to 26.4 % of the initial value, whereby it was reduced during the same period of time to 80.9 % and to 90.6 % of the initial value for storage temperatures of 25°C and 5°C, respectively.

At a determined storage temperature, PLA polymers of the placebo B1 were generally more reduced than PLA polymers of related active ingredient containing formulation B. Molecular weight of PLA polymer in active ingredient containing formulation B was reduced to 26.4 % of the initial value after six months storage at 40°C, whereby it was reduced to 9.5 % of the initial value for the related placebo B1 at the same storage conditions. Similarly, at storage temperature of 25°C, PLA polymers molecular weights were reduced to 80.9 % for formulation B, and to 59.4 % for placebo B1.

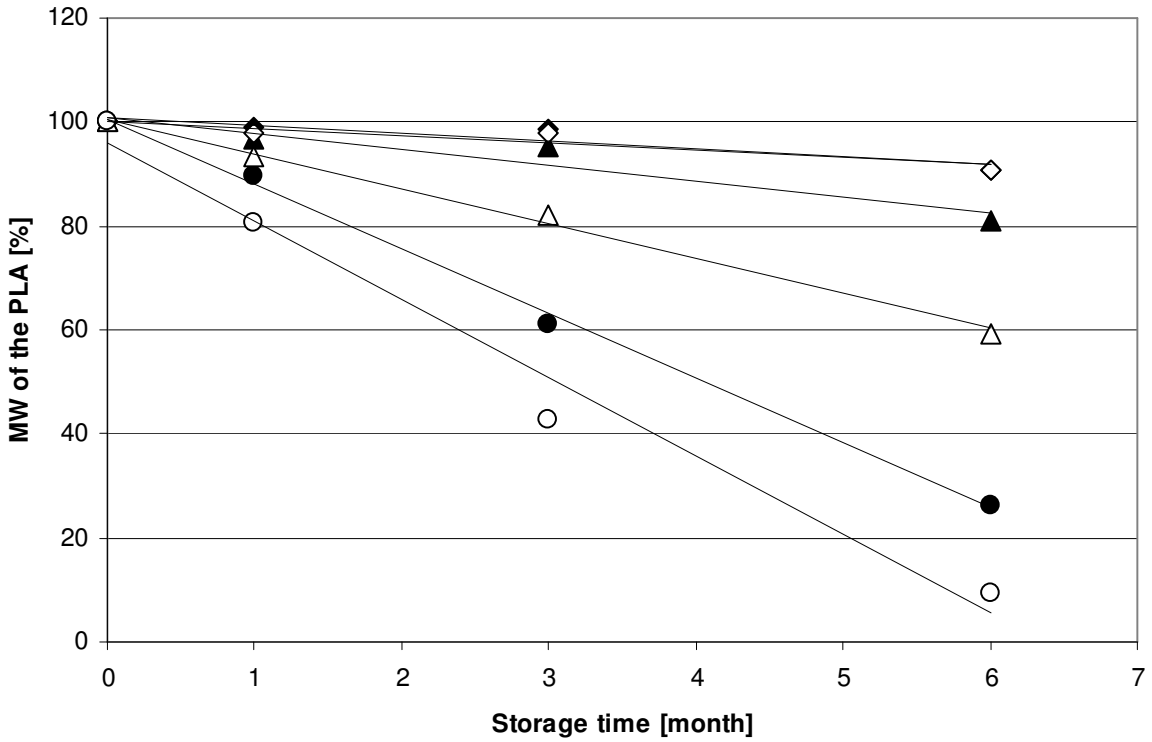


Figure 6.8: Molecular weight (MW) changes of PLA polymer in formulation B (black) and placebo B1 (white) at four selected time points during the six months storage at 5°C (◆,◇), 25°C (▲,△) and 40°C (●,○).

Molecular weight changes of PLA polymer of formulation C and related placebo C1 during the six months storage at the three different temperatures 5°C, 25°C and 40°C are reported in Figure 6.9. As differences with formulation B, formulation C contained 17.00 % (w/w) of PLA polymer instead of 12.75 % (w/w) as well as 10.00 % (w/w) of ethanol absolute as co-solvent. The exact composition of the samples are described in Table 6.1. Changes in molecular weight of PLA polymer in formulation C and its relative placebo C1 are reported in percent of the initial value. Similarly to formulation B, reduction of molecular weight of PLA polymer of the formulation C and its related placebo C1 was highly dependent on storage temperature. Bigger changes were observed for formulations stored at 40°C than for

formulations stored at 25°C or 5°C. After six months storage at 40°C, molecular weight of PLA polymers in formulation C were reduced to 24.0 % of their initial value, whereby it was reduced during the same period of time to 69.5 % and to 85.5 % of their initial value for storage temperatures of 25°C and 5°C, respectively.

Similarly to formulation B, changes in molecular weight of PLA polymers were more important in placebo C1 than in active ingredient containing related formulations C. After six months storage at 40°C, molecular weight of PLA polymers in active ingredient containing formulation C was reduced to 24.0 % of their initial value, whereby it was reduced to 9.5 % of their initial value for related placebo C1 at same storage conditions.

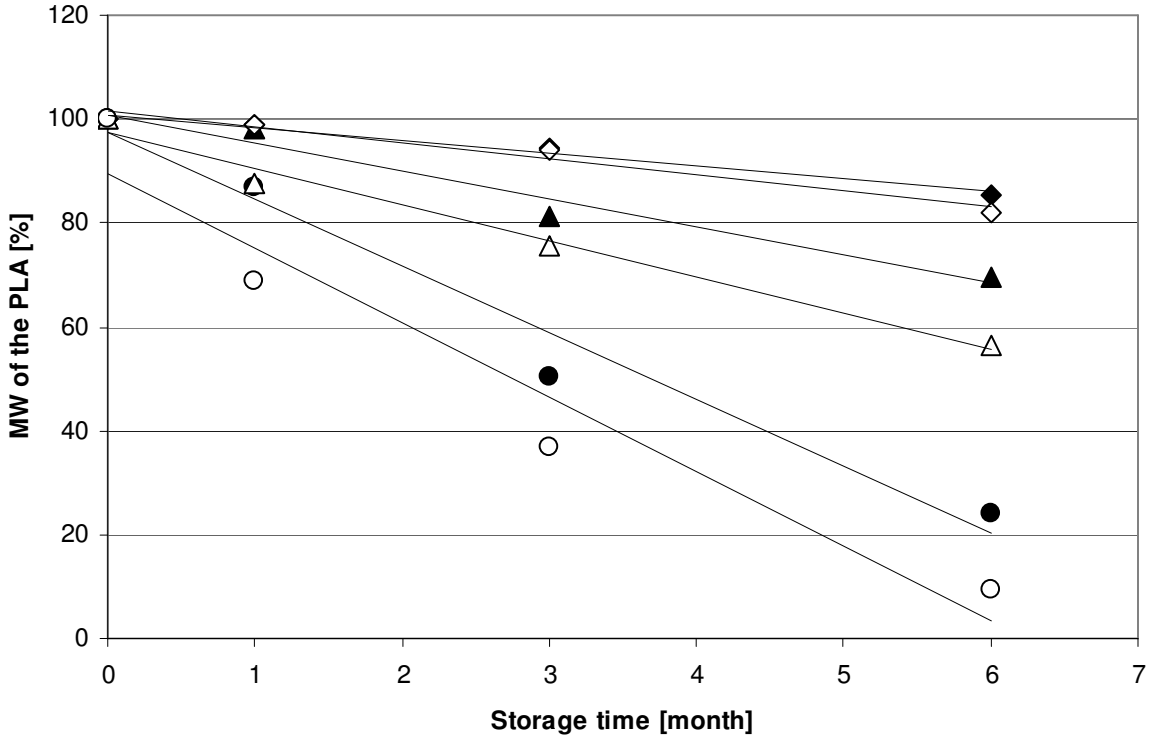


Figure 6.9: Molecular weight (MW) changes of PLA polymer in formulation C (black) and placebo C1 (white) at four selected time points during the six months storage at 5°C (◆,◇), 25°C (▲,△) and 40°C (●,○).

The molecular weight analysis of the PLA polymers in formulations B and C and their related placebos B1 and C1 was carried out during six months for storage temperature of 5°C, 25°C and at 40°C. As illustrated in the charts represented in Figures 6.8 and 6.9, the decrease in molecular weight seemed to follow a zero order kinetic.

A linear trendline was obtained for each sample stored at a defined temperature from the charts illustrating the molecular weight changes verses time (Figures 6.8 and 6.9). The equations of these trendlines of type "y = ax + b" are reported in Table 6.6. The value of the kinetic constant k is obtained from the slope of the linear trendline.

Table 6.4: Equation of the linear trendline and kinetic constant k, obtained for each sample stored at a defined temperature from the charts "molecular weight verses time" represented in Figures 6.8 and 6.9.

	Storage temperature	**Equation of trendline [y = ax + b]**	**Kinetic constant k [kJ/mol]**
formulation B	5°C	y = -1.5182 x + 100.88	1.52
	25°C	y = -3.0808 x + 100.94	3.08
	40°C	y = -12.450 x + 100.47	12.45
placebo B1	5°C	y = -1.4159 x + 100.27	1.42
	25°C	y = -6.7050 x + 100.46	6.71
	40°C	y = -15.067 x + 95.945	15.07
formulation C	5°C	y = -2.4877 x + 100.90	2.49
	25°C	y = -5.3967 x + 100.70	5.40
	40°C	y = -12.900 x + 97.546	12.90
placebo C1	5°C	y = -3.0461 x + 101.39	3.05
	25°C	y = -6.9452 x + 97.359	6.95
	40°C	y = -14.367 x + 89.703	14.37

3.3. Low field NMR-screening (NIR-screening)

Formulations A, B and C and related placebos 1 and 2 were stored in temperature controlled racks of the SpecScreen x HTS apparatus at 25°C and 40°C. NIR spectra were automatically taken during six months at time intervals of 24 h. Evaluation of spectra data sets consisted in determination of process factor and of time profile. Process factor is the slope of the regression line shown in the spectra. It indicates wavelength regions with significant changes during storage period of the samples and support the identification of unstable components in formulations. The calculated time profile shows kinetics of significant processes in formulation.

Absorption of NIR radiation is due to overtone and combination bands primarily of O-H, C-H, N-H and C=O groups. The fundamental molecular stretching and bending of these functional groups absorb in NIR region. These overtones are anharmonic and do not behave in a simple fashion, making NIR spectra complex and not directly interpretable.

In Figure 6.12, prominent absorption bands related to overtone and combination bands of fundamental vibrations occurring in NIR region are depicted. It can be observed that absorbance regions of different functional groups are strongly overlapping. The absorbance bands in NIR spectra are therefore usually composites of different functional groups. In contrast to mid infrared and Raman spectroscopic range, unequivocal assessment of an absorbance band to a functional group is often not possible with NIR spectroscopy. However, despite low specificity, NIR-spectroscopy is used for stability as it bring advantages of high sensitivity in comparison with other vibrational methods.

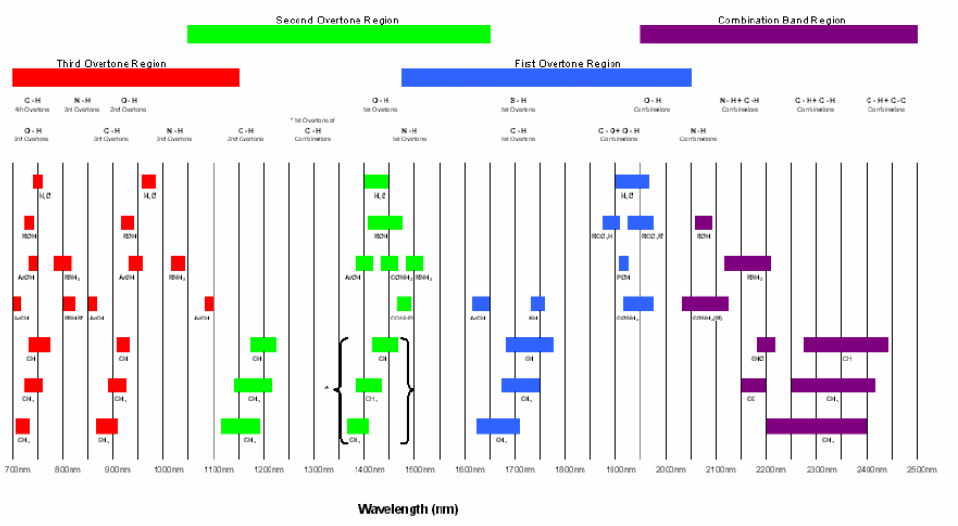


Figure 6.12: Absorbance bands of functional groups in NIR region.

3.3.1. Placebo 2 evaluation: solvent stability

Placebo 2 samples contained neither active ingredient nor PLA polymer. They consist in solvent triacetin for placebos A2 and B2 and in mixture triacetin with 10.00 % ethanol for placebo C2.

3.3.1.1. NIR evaluation for stability of triacetin (placebo A2 and B2)

The solvent triacetin, which corresponds to placebos A2 and B2, was daily analyzed using NIR spectroscopy for a storage period of six months. During this time, the samples were stored in two racks of the SpecScreen x HTS apparatus, heated at 25℃ and 40℃, respectively.

An overlay plot of NIR-spectra for wavelength range from 830 nm to 2500 nm of triacetin, without baseline correction nor variance normalization is illustrated in Figure 6.13. On X-axis of plot, wavelengths are represented in nm while absorbance is illustrated on Y-axis.

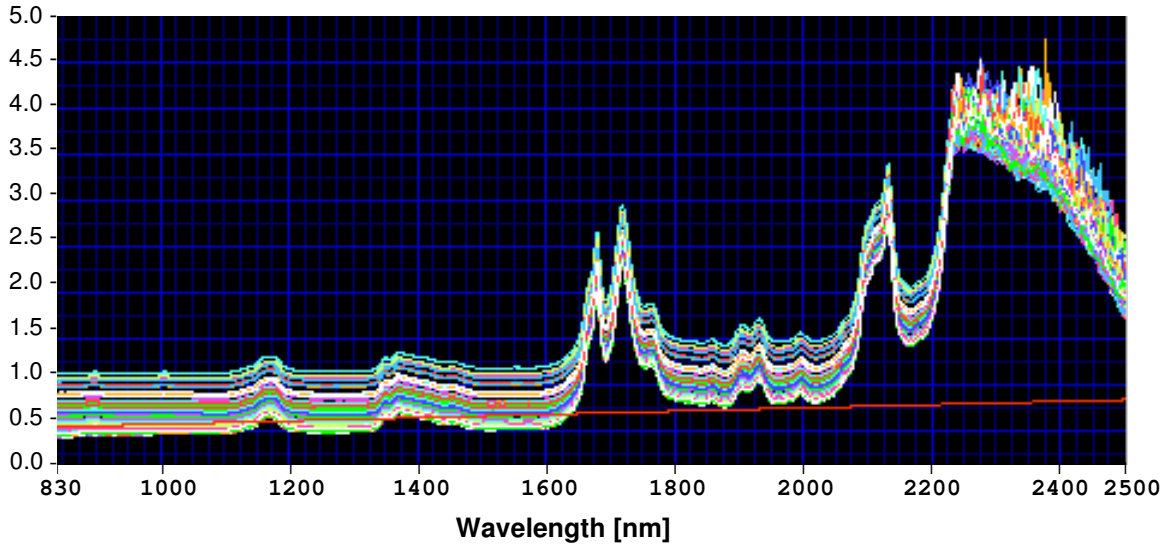


Figure 6.13: Overlay plot of NIR-spectra set of triacetin (placebo A2 and B2) without baseline correction nor variance normalization, in a wavelength range from 830 nm to 2500 nm.

The same data set was used for an overlay plot of NIR-spectra of triacetin, after baseline correction and variance normalization for wavelength range from 830 nm to 2500 nm. As shown on plot of Figure 6.14, it is more convenient to detect the wavelengths region with spectroscopic changes after the procedure of data pre-treatments.

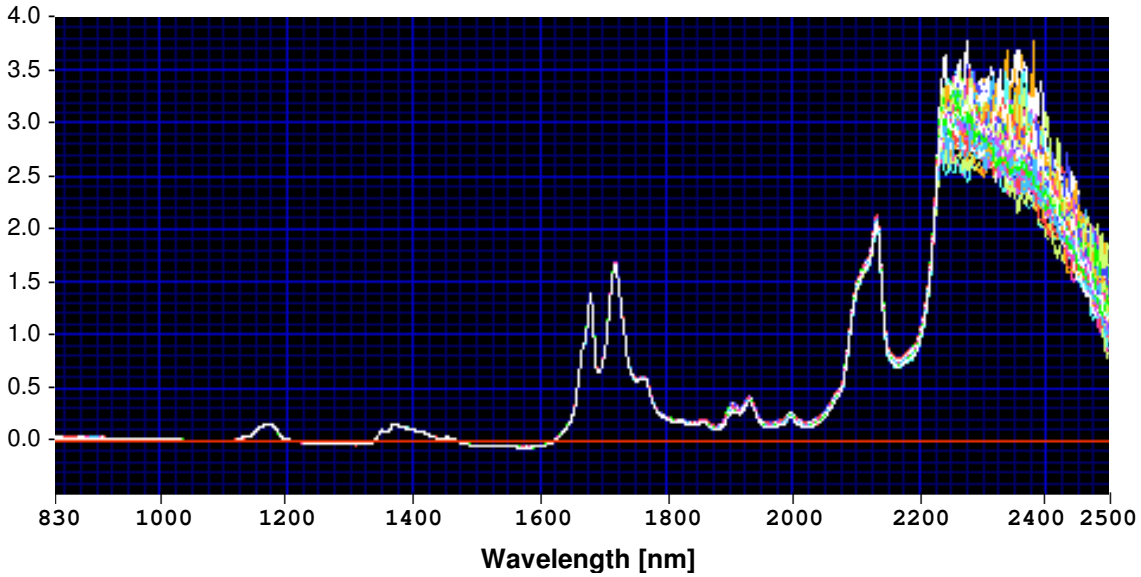


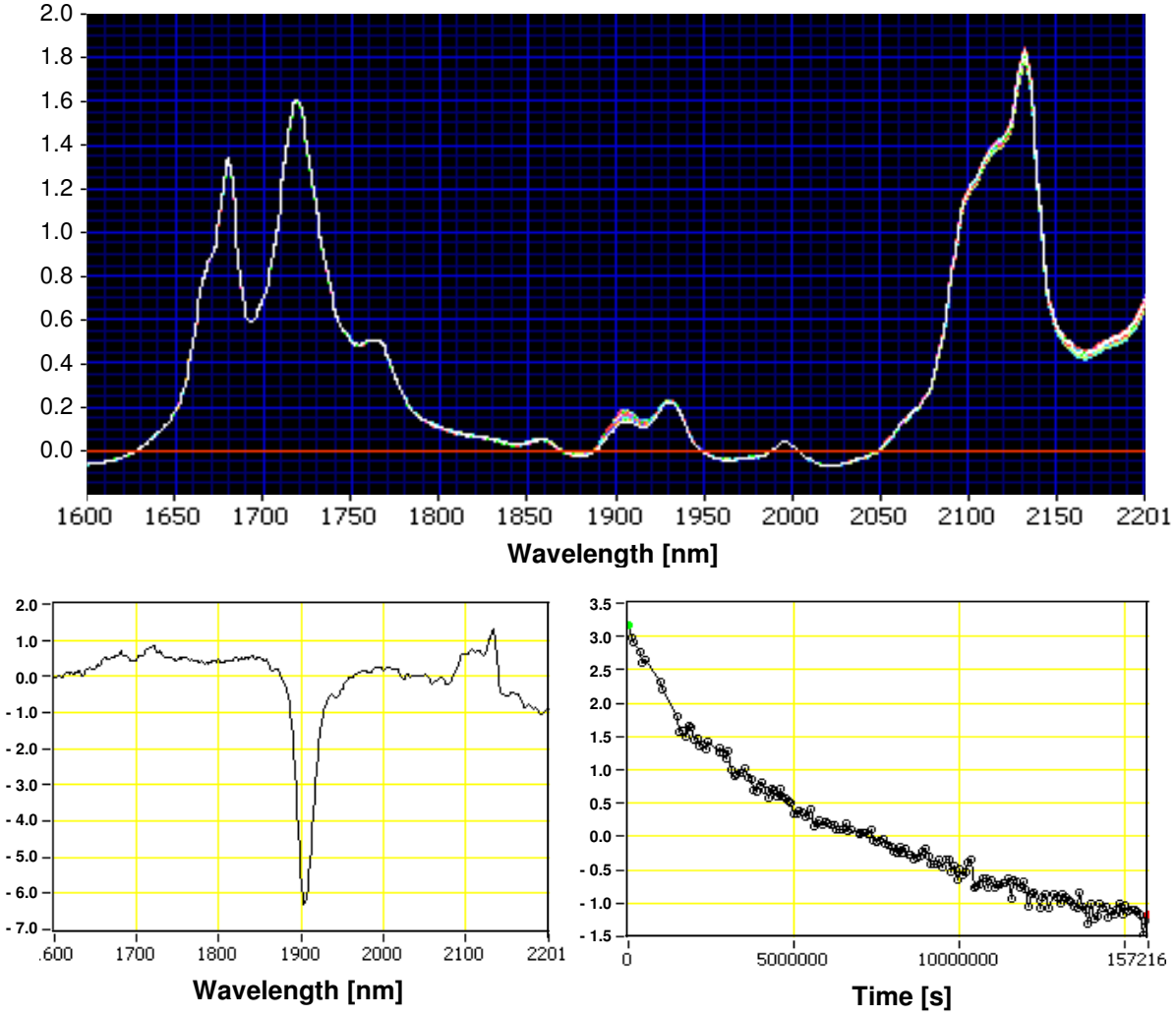
Figure 6.14: Overlay plot of NIR-spectra set of triacetin (placebo A2 and B2), after baseline correction and variance normalization, in wavelength range from 830 nm to 2500 nm.

Not all wavelengths of NIR-spectra are ascertained for stability investigations of samples. Selection of wavelengths region utilized for stability storage investigation depends on the spectra data set as well as on the apparatus used. For further investigations, wavelength regions which may alter the results should be subsequently removed. In this particular case, wavelengths region below 1600 nm and over 2200 nm are systematically removed for stability determination.

Superposition of NIR-spectra of the solvent triacetin taken during storage at 25°C for the wavelength range between 1600 nm and 2200 nm after baseline correction and variance normalization is represented in Figure 6.15a. Process factor calculated from this overlay plot is illustrated in Figure 6.15b. Process factor shows which wavelength regions change significantly on the related overlay plot. The most important changes observed on superposition of NIR-spectra are located at wavelength region around 1900 nm. This was confirmed by the process factor graphic illustrated in Figure 6.15b, which curve shows an important deviation from the X-axis at wavelengths region around 1900 nm.

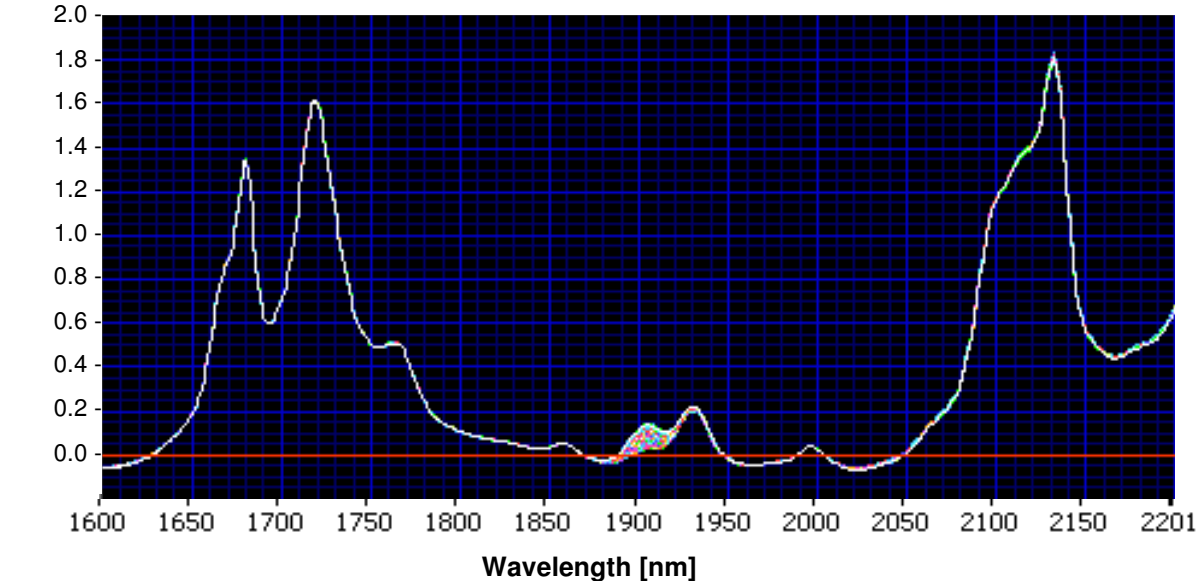
As indicated in Figure 6.12, functional groups with absorbance bands in region of 1900 nm are the first overtones for the RCOOR groups. The chemical structure of triacetin which is a triester of glycerin and acetic acid support this presumption. Other functional entities with absorbance bands in the region around 1900 nm are the groups RCOOH, which may be the degradation products of triacetin as well as $RCONH_2$ and H_2O which are likely not present here.

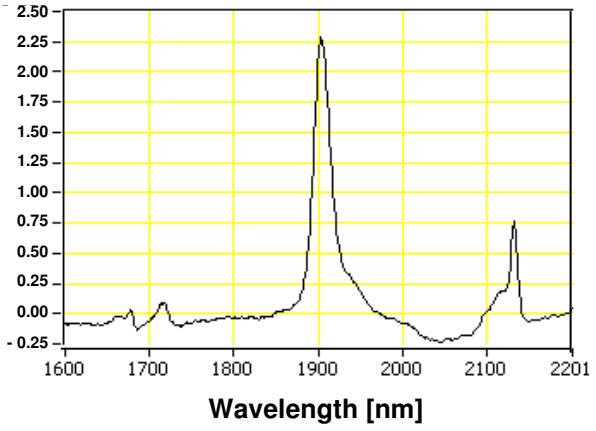
Time profile calculated from the overlay plot of NIR-spectra as well as from process factor is illustrated in Figure 6.15c. X-axis of time profile represents storage time (in seconds) and Y-axis is an arbitrary unit illustrating vector evolution inputs. Each point represents a NIR spectrum and the points are lined in chronological order. Here, the sample shows a continuous process over time which started from spectrum taken on the first day and last until the end of the storage time of six months.

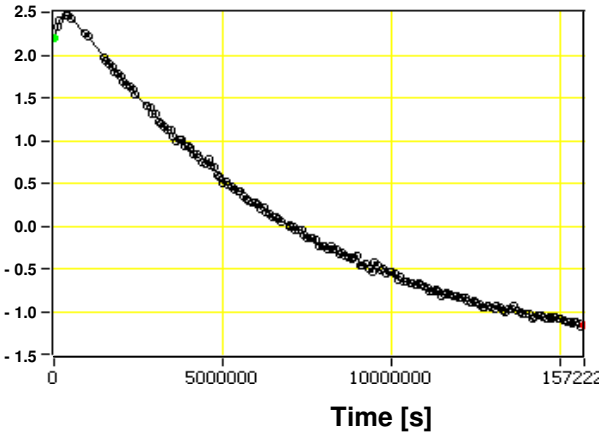


Figures 6.15a-c: (a) Overlay plot of NIR-spectra set of triacetin (placebo A2 and B2) during six months storage at 25℃ after baseline correction and variance normalization for wavelength between 1600 and 2200 nm. (b) Related process factor (c) Related time profile.

Solvent triacetin on its own (placebo A2 and B2), stored at 40°C was also screened on a daily basis with NIR spectroscopy. Superposition of NIR-spectra of solvent triacetin is represented after baseline correction and variance normalization in Figure 6.16a for wavelength range from 1600 nm to 2200 nm. Related process factor and time profile are illustrated in Figures 6.16b and 6.16c, respectively. Analog to the sample stored at 25°C, the main changes detected with NIR screening was located at wavelength 1900 nm as observed from the overlay plot of NIR-spectra set in Figure 6.16a and from process factor represented in Figure 6.16b. Time profile indicates that this effect is a continuous process over storage time.







Figures 6.16a-c: (a) Overlay plot of NIR-spectra set of placebos A3 or B3 during six months storage at 40°C. (b) Process factor. (c) Time profile.

Overlay plots of triacetin show for both temperature storage (25°C and 40°C) changes at wavelength around 1900 cm^{-1}. However, changes in the overlay plot at higher storage temperature (Figure 6.16a) seem to be more important than for lower storage temperature (Figure 6.15a). After proceeding to further data pretreatments which fit both arbitrary Y-axis of time profiles (6.15c and 6.16c) to the same scale for a given reaction, time profiles for storage conditions at 25°C and 40°C could be compared on the same chart for the solvent triacetin during the six months storage.

In Figure 6.17, time profiles of triacetin stored at 25°C and at 40°C are superposed on the same chart, using constant arbitrary scale of Y-axis to examine influence of temperature on degradation kinetic. By comparing deviation of time profile curves from the X-axis, it can be confirmed that degradation rate was higher for formulation stored at 40°C (grey curve) than for the formulation stored at 25°C (black curve). These results are in accordance with expectations and with observations of the related overlay plots (Figures 6.15a and 6.16a).

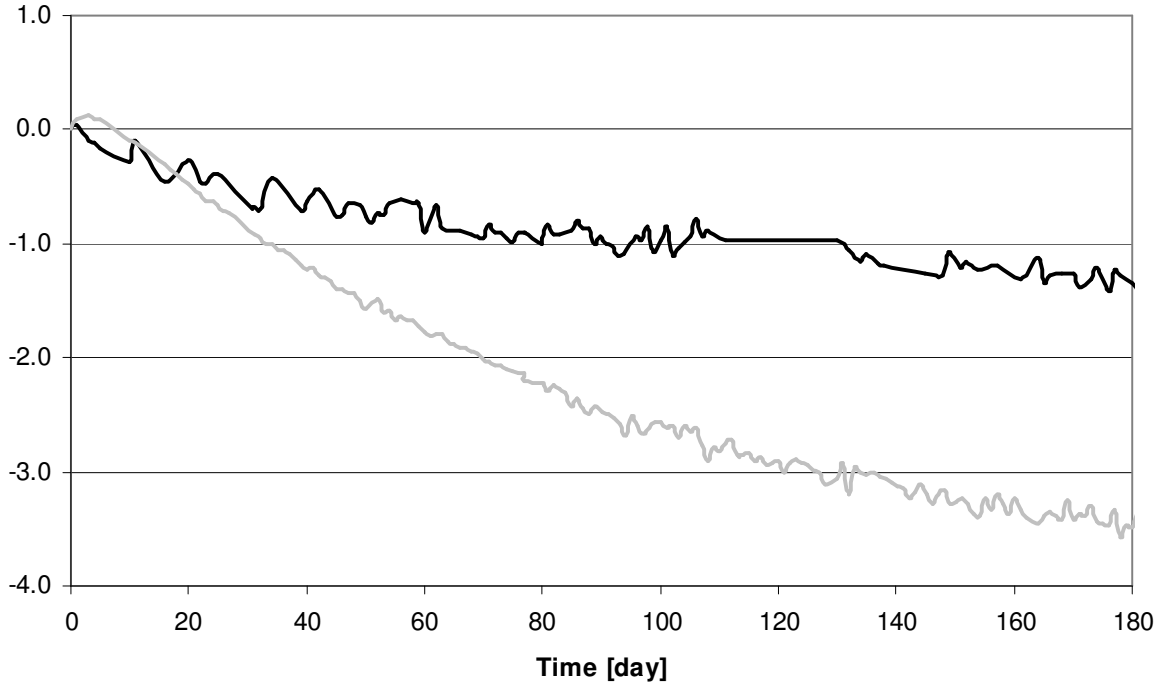
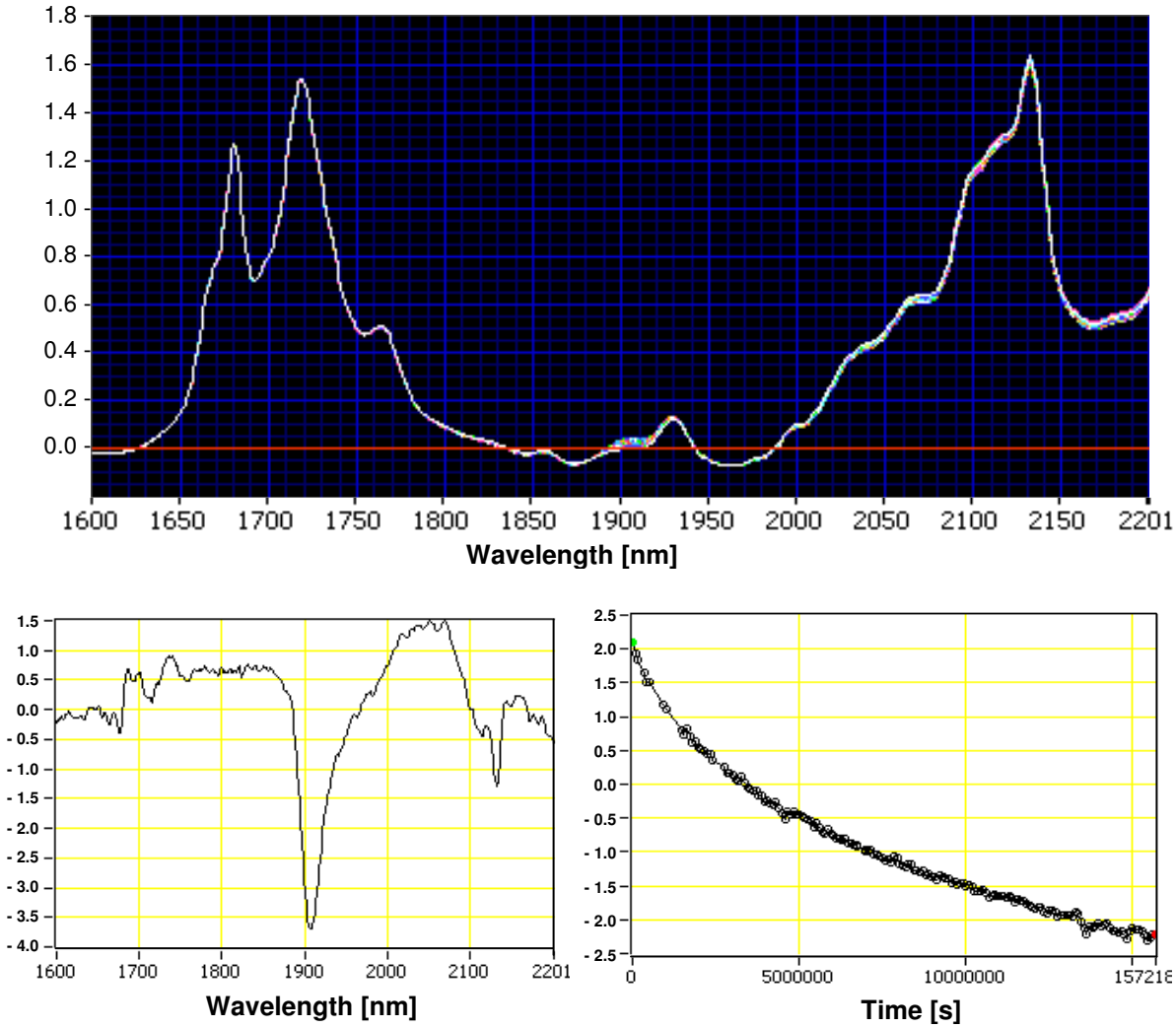


Figure 6.17: Time profile comparison for sample triacetin during six months storage at of 25°C (black curve) and 40°C (grey curve).

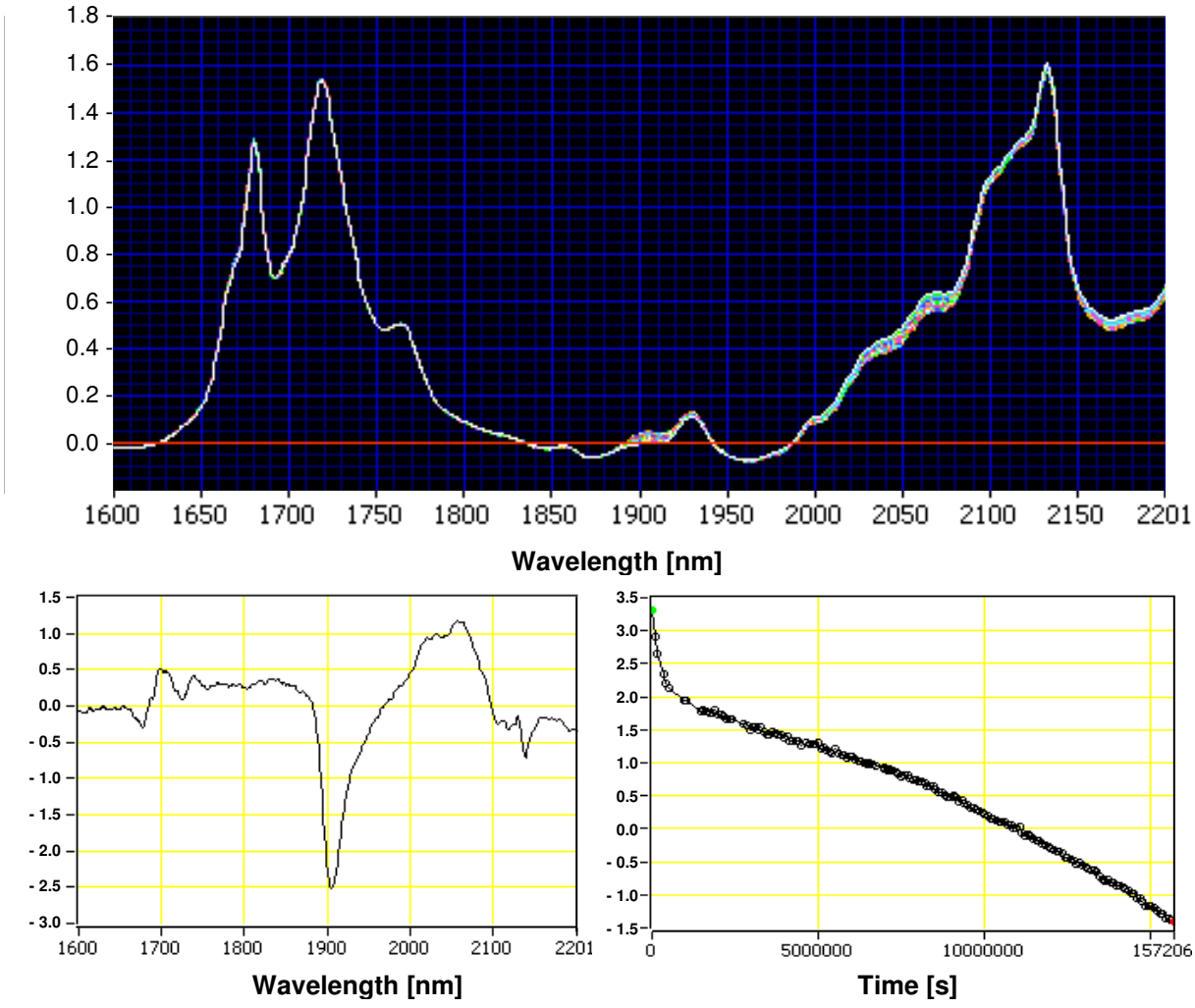
3.3.1.2. Placebo C2 evaluation

Placebo C2 which consist in mixture of triacetin and ethanol absolute in a proportion of 9:1 was screened using NIR-spectroscopy during six months storage at 25℃ and 40℃. Overlay plot of NIR-spectra set of placebo C2 in wavelength range between 1600 nm and 2200 nm after baseline correction and variance normalization is shown in Figure 6.18a. Related process factor represented in Figure 6.18b indicates that the main changes occur at wavelength of 1900 nm as well as in the range between 2000 nm and 2100 nm. As shown in Figure 6.12, functional groups which absorb in the wavelength range around 1900 nm are mainly the groups RCOOR, while absorption between 2000 nm and 2100 nm can be relied to groups such as ROH and $CONH_2(R)$. In this particular case, as the sample is comprised of triacetin and ethanol, absorption detected at wavelengths around 2000 nm are attribute to ROH groups. Time profile illustrated in Figure 6.18c suggest a continuous degradation process over time.



Figures 6.18a-c: (a) Overlay plot of NIR-spectra set of placebo C2 during six months storage at 25℃. (b) Process factor. (c) Time profile.

Placebo C2 comprising the solvents triacetin and ethanol absolute in a weight ratio of 9:1 was also stored at 40°C and daily screened with NIR-spectroscopy. Overlay plot of the NIR-spectra set is represented in Figure 6.19a after baseline correction and variance normalization for a wavelength range between 1600 nm and 2200 nm. From this NIR-spectra set, it can be observed that, similarly to placebo C2 stored at 25°C, changes are mainly located at wavelengths 1900 nm as well as at 2000 nm and 2100 nm. This is confirmed with the related process factor represented in Figure 6.19b. Kinetic of this reaction, as illustrated in Figure 6.19c, was very rapid during the first weeks storage and slowed down to a continuous process for the rest of storage time for this formulation.



Figures 6.19a-c: (a) Overlay plot of NIR-spectra set of placebo C2 during six months storage at 40°C. (b) Process factor. (c) Time profile.

3.3.1.3. Comparison time profile A2 verses C2

Overlay plots of placebo A2 and C2 show for both temperature storage (25°C and 40°C) changes at wavelength around 1900 cm^{-1}. Placebo A2 consisted in the solvent triacetin as placebo C2 consisted in a mixture of triacetin and ethanol in a ratio of 9:1. Influence of co-solvent ethanol on stability of triacetin was investigated by comparing time profiles of both placebo samples. After proceeding to further data pretreatments which fit both arbitrary Y-axis of time profiles to the same scale for a given reaction, time profiles over six months of both placebo samples were compared on the same chart for a given storage temperature.

In Figure 6.20, time profiles of samples A2 (triacetin) and C2 (triacetin and ethanol) during six months storage at 25°C are superposed on the same chart, using constant arbitrary scale of Y-axis to examine influence of the co-solvent ethanol on degradation kinetic. By comparing deviation of time profile curves from the X-axis, it can be observed that degradation rate was similar for both samples. For storage conditions at 25°C, addition of 10.00 % of co-solvent ethanol to triacetin did not seem to influence stability of the samples.

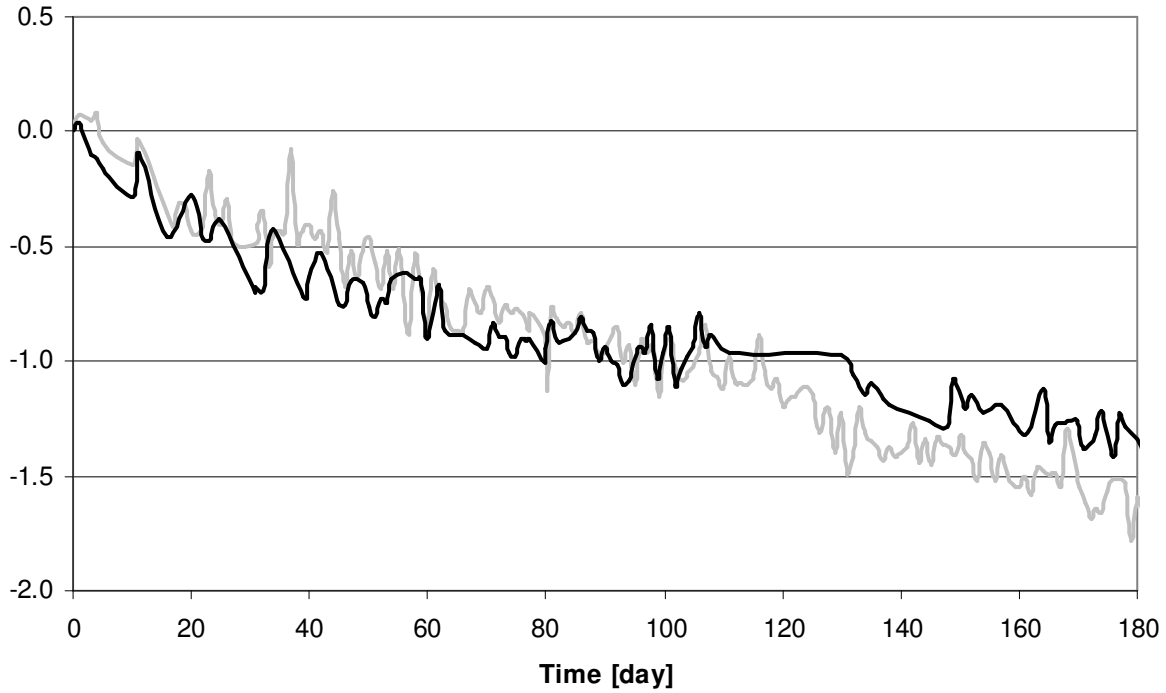


Figure 6.20: Time profiles for placebo A2 (black curve) and for placebo C2 (grey curve) during six months storage at 25°C.

In Figure 6.21, time profiles of samples A2 (triacetin) and C2 (triacetin and ethanol) during six months storage at 40°C are overlaid on the same chart, using constant arbitrary scale of Y-axis to investigate influence of the addition of co-solvent ethanol on degradation of the samples.

Deviation of time profile curves from the X-axis of sample A2 (black curve) was more important than for time profile of sample C2 (grey curve). Addition of 10.00 % of co-solvent ethanol to the solvent triacetin seems to reduce degradation rate of the sample by storage at 40°C.

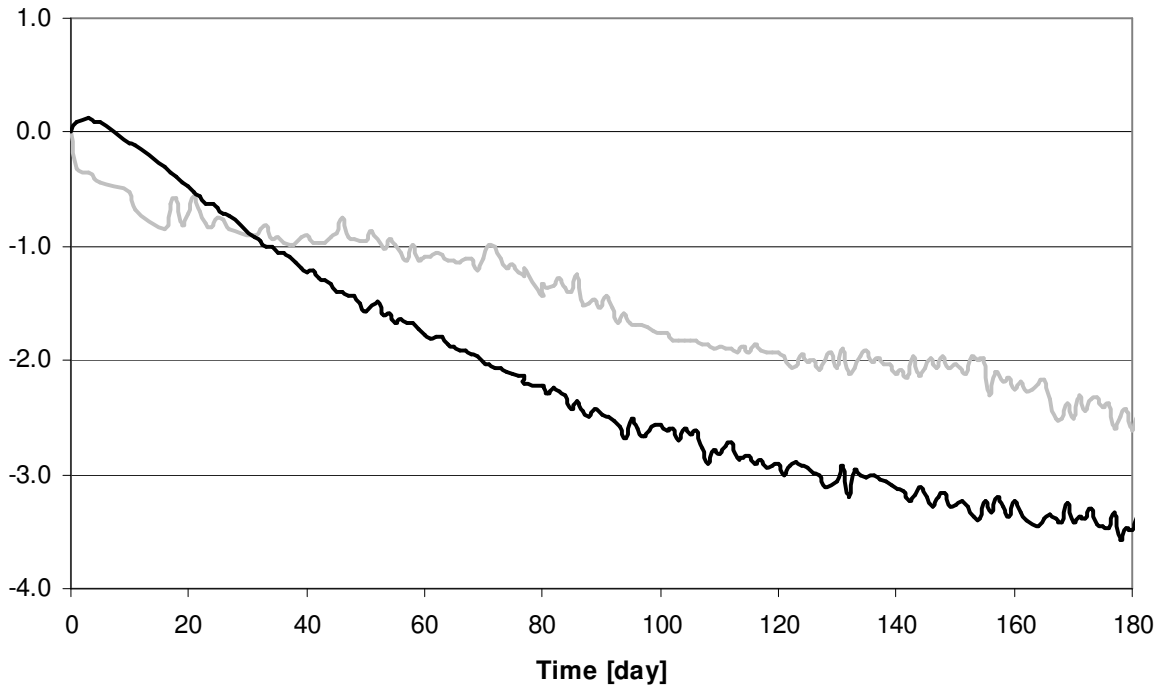


Figure 6.21: Time profiles for placebo A2 (black curve) and for placebo C2 (grey curve) during six months storage at 40°C.

3.3.1. Placebos 1 evaluation

Placebos 1 samples contain PLA polymers dissolved in triacetin or in a mixture of triacetin and ethanol. PLA polymer concentration was 17.00 % in placebos A1 and C1, while it was 12.75 % in placebo B1. Exact compositions of the placebos samples are depicted in Table 6.1.

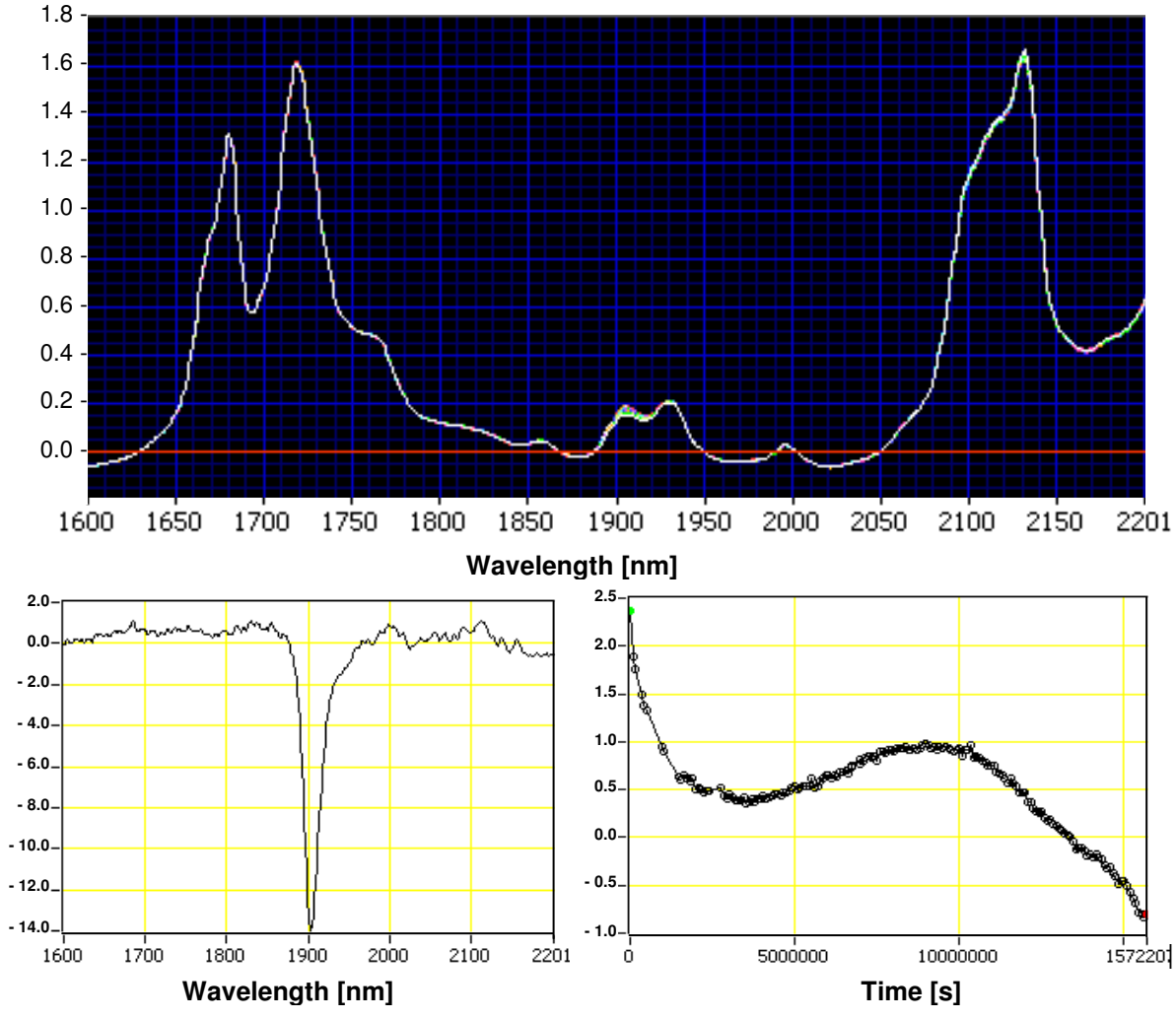
3.3.2.1. Placebo A1 and B1 evaluation

Placebos A1 and B1 contained PLA polymers dissolved in triacetin at concentration of 17.00 % and 12.75 %, respectively. Both samples gave very similar spectroscopic results. Therefore, only spectra data set of the placebo B1 are illustrated and commented here.

Figure 6.22a represents overlay plot of NIR spectra of placebo B1 taken during the six months storage at 25°C for the wavelength range between 1600 nm and 2200 nm, after baseline correction and variance normalization. Changes of NIR spectra were observed at wavelength of 1900 nm. Process factor represented in Figure 6.22b confirmed that this region of the NIR-spectra overlay plot is the only wavelength region which was modified during storage time period.

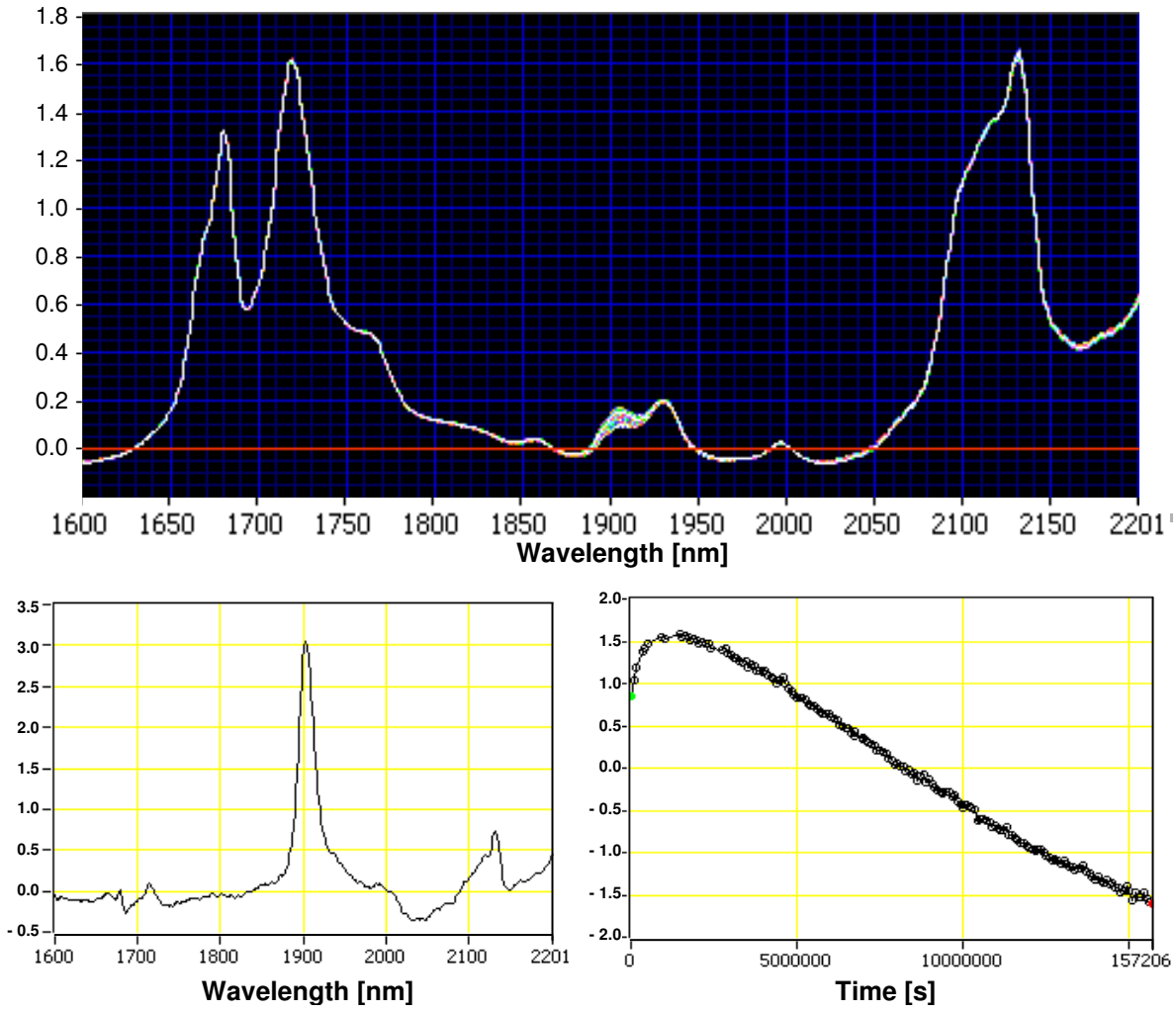
As placebo B1 contains, above PLA polymers, an excess of solvent triacetin, changes at wavelength of 1900 nm are logic. The instability of placebo B2 during storage at 25°C at wavelength of 1900 nm has been illustrated in Figures 6.15a-c. These changes were attributed to the esters groups (RCOOR) of the solvent triacetin. No changes in the NIR-spectra set were specific for presence of PLA polymers in the placebos B1. Modifications of NIR-spectra of placebo B1 were located at the same wavelength region than changes in spectra set of placebo B2. However, GPC analysis carried out in Section 3.1.2 of this chapter for placebo B1 indicated a significant reduction of the molecular weight of PLA polymers, which can be attributed to hydrolysis of their ester functional group. Such modifications would be attested by spectral changes at wavelengths in the regions absorbed by ester functional groups RCOOR. This is precisely the wavelengths region around 1900 nm which is affected by the placebo effect during storage of this particular sample.

Time profile illustrated in Figure 6.22c shows kinetics of degradation reaction of the sample B1, containing 12.75 % of PLA polymers in triacetin. Its includes both alteration of the solvent triacetin observed with the NIR-spectroscopy in Section 3.3.1.1 and concomitant hydrolysis of PLA polymers observed with GPC in Section 3.2.1. Kinetic is very dissimilar to the time profile of the solvent triacetin during storage at the same conditions illustrated in Figure 6.15c. This demonstrate that an additional parameter, in this case PLA polymers, do react during storage period. However, the indications obtained from the time profile of Figure 6.22c must be carefully evaluated. Spectral changes of two chemical reactions located at same wavelength regions should not be considered as cumulative.



Figures 22a-c: (a) Overlay plot of NIR-spectra set of placebo B1 during six months storage at 25°C. (b) Process factor. (c) Time profile.

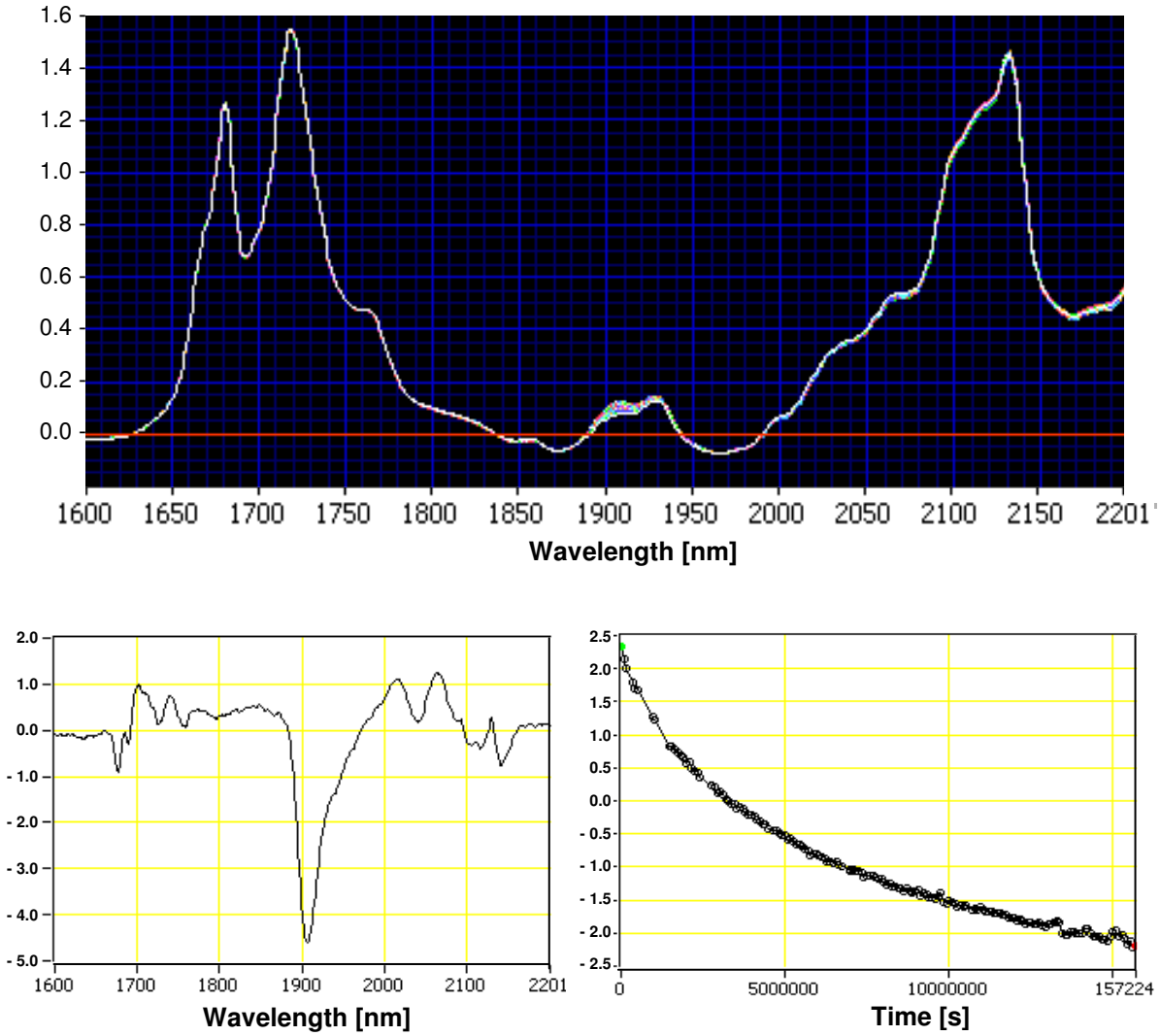
PLA polymers solution in triacetin stored at 40℃ was also screened with NIR-spectroscopy. Overlay plot of NIR-spectra set in wavelengths range from 1600 nm to 2200 nm collected during the six months storage at 40℃ are represented after baseline correction and variance normalization in Figure 6.23a. Changes in spectra can be detected at wavelength region of 1900 nm as also confirmed by the Figure 6.23b, representing the process factor of the related reaction of placebo B1. Time profile is illustrated in chart of Figure 6.23c. Comments made for the spectra data set of the placebo B1 taken at storage temperature of 25℃, concerning lack of specificity for PLA polymers spectral changes can be reiterated here. Time profile of this particular spectral data set should also be prudently estimated.



Figures 6.23a-c: (a) Overlay plot of NIR-spectra set of placebo A1 during six months storage at 40℃. (b) Process factor. (c) Time profile.

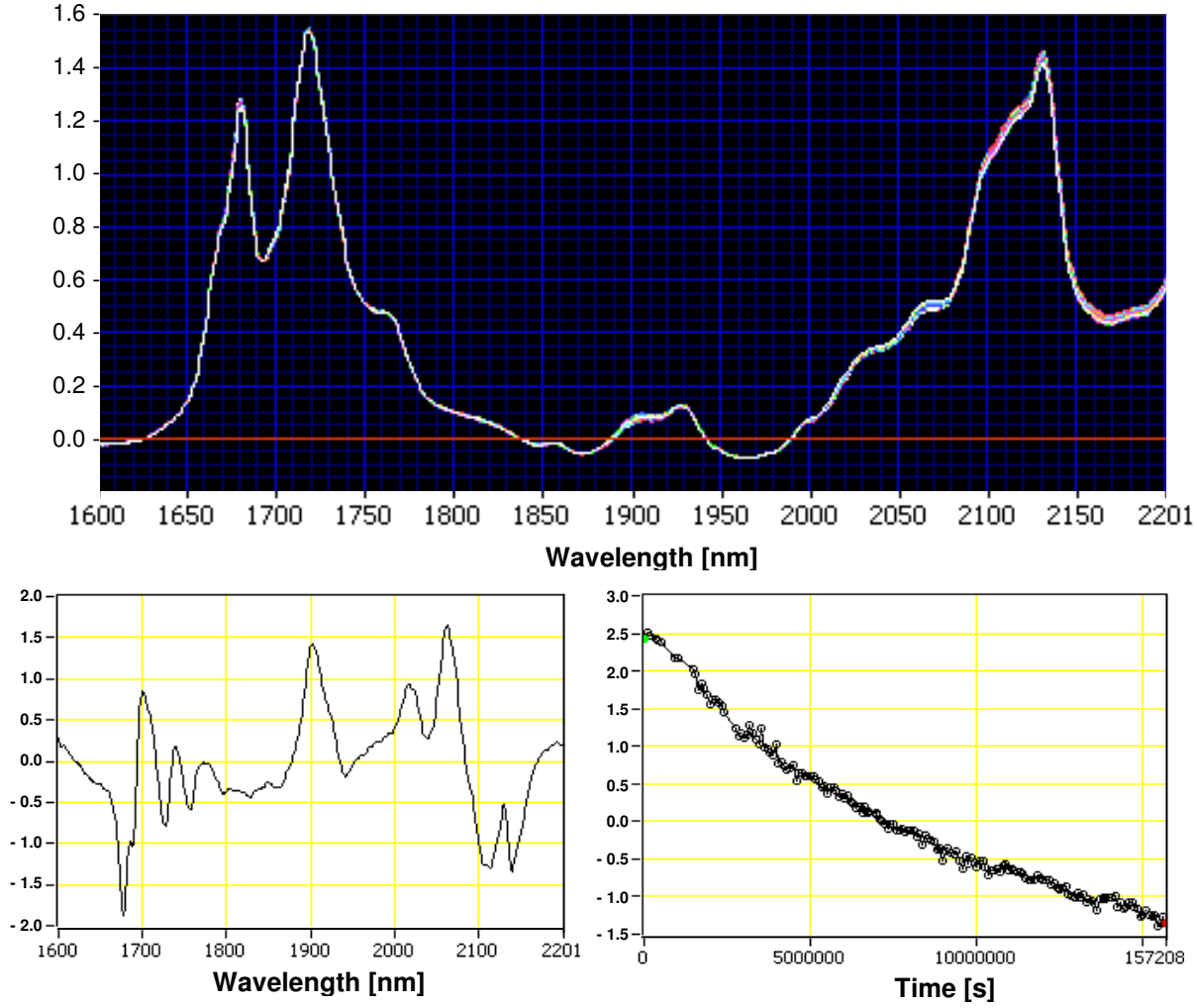
3.3.2.2. Placebo C1 evaluation with NIR-spectroscopy

Placebo C1 consists in 17.00 % of PLA polymers dissolved in a solvent mixture of triacetin and ethanol. Overlay plot of NIR-spectra set obtained from placebo C1 during six months storage at 25°C is represented in Figure 6.24a, after baseline correction and variance normalization for wavelengths region between 1600 nm and 2200 nm. Process factor illustrated in Figure 6.24b shows main changes at wavelength region of 1900 nm. As illustrated in Figure 6.12, the functional groups absorbing in this region are mainly RCOOR groups. These changes can be attributed to ester groups of the solvent triacetin and of PLA polymers. Because of poor specificity of NIR-spectroscopy, the obtained indications should be wisely considered.



Figures 6.24a-c: (a) Overlay plot of NIR-spectra set of placebo C1 during six months storage at 25°C. (b) Process factor. (c) Time profile.

Placebo C1, consisting in PLA polymers dissolved in triacetin and ethanol was stored also at 40°C and was screened with NIR-spectroscopy on a daily basis. Overlay plot of NIR-spectra set in wavelengths range from 1600 nm to 2200 nm collected during the six months storage at 40°C are represented after baseline correction and variance normalization in Figure 6.25a. Changes in the spectra can be detected at wavelength region of 1900 nm 2000 nm and 2100 nm as also confirmed by process factor represented in Figure 6.25b. Time profile is illustrated in the chart of Figure 6.25c and indicates a continuous process over storage time.



Figures 6.25a-c: (a) Overlay plot of NIR-spectra set of placebo C1 during six months storage at 40°C. (b) Process factor. (c) Time profile.

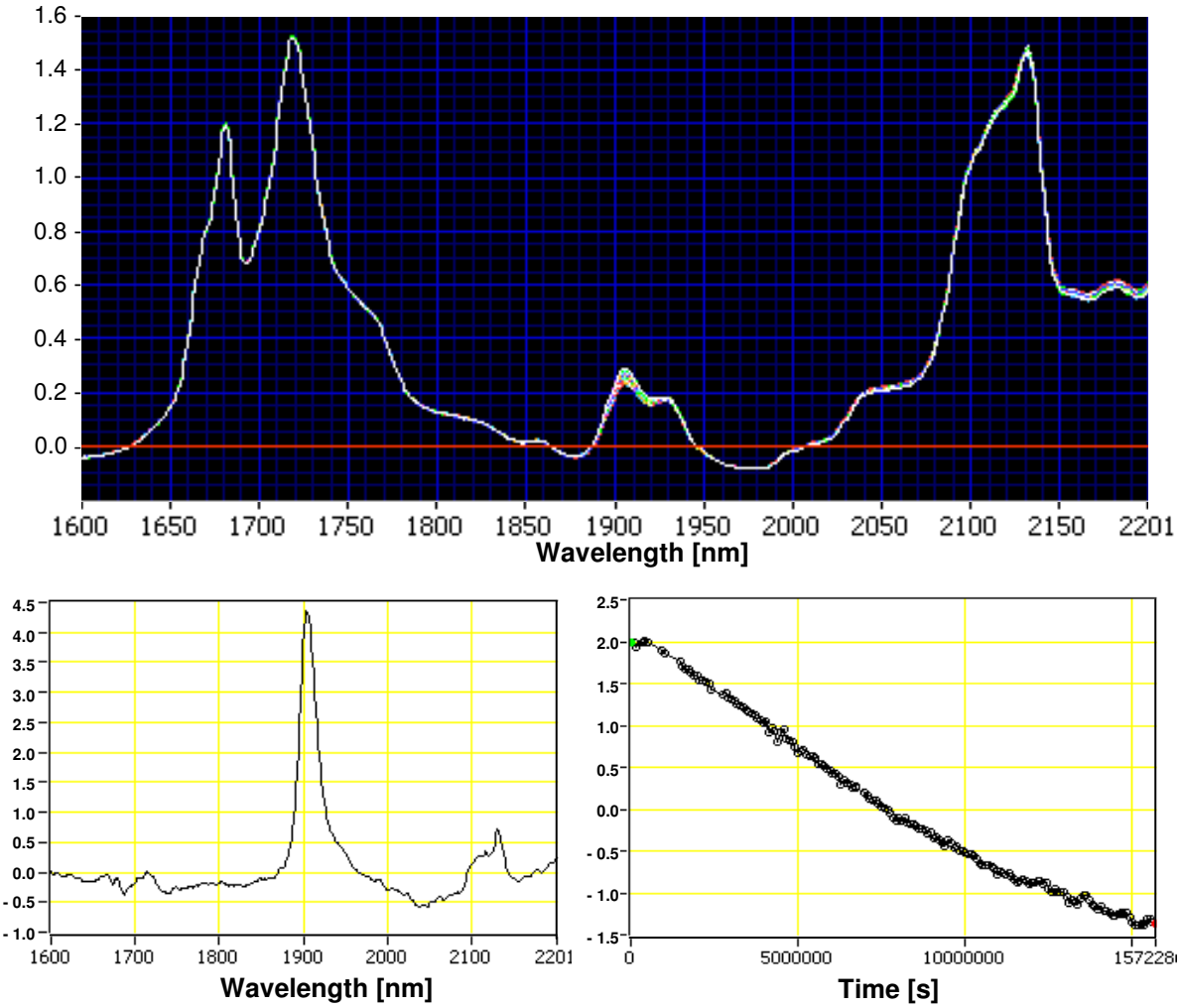
3.3.3. Formulation evaluation

Formulations containing both the active ingredient NOA449851 and the PLA polymers were evaluated using the SpecScreen x HTS apparatus.

3.3.3.1. Formulation A and B

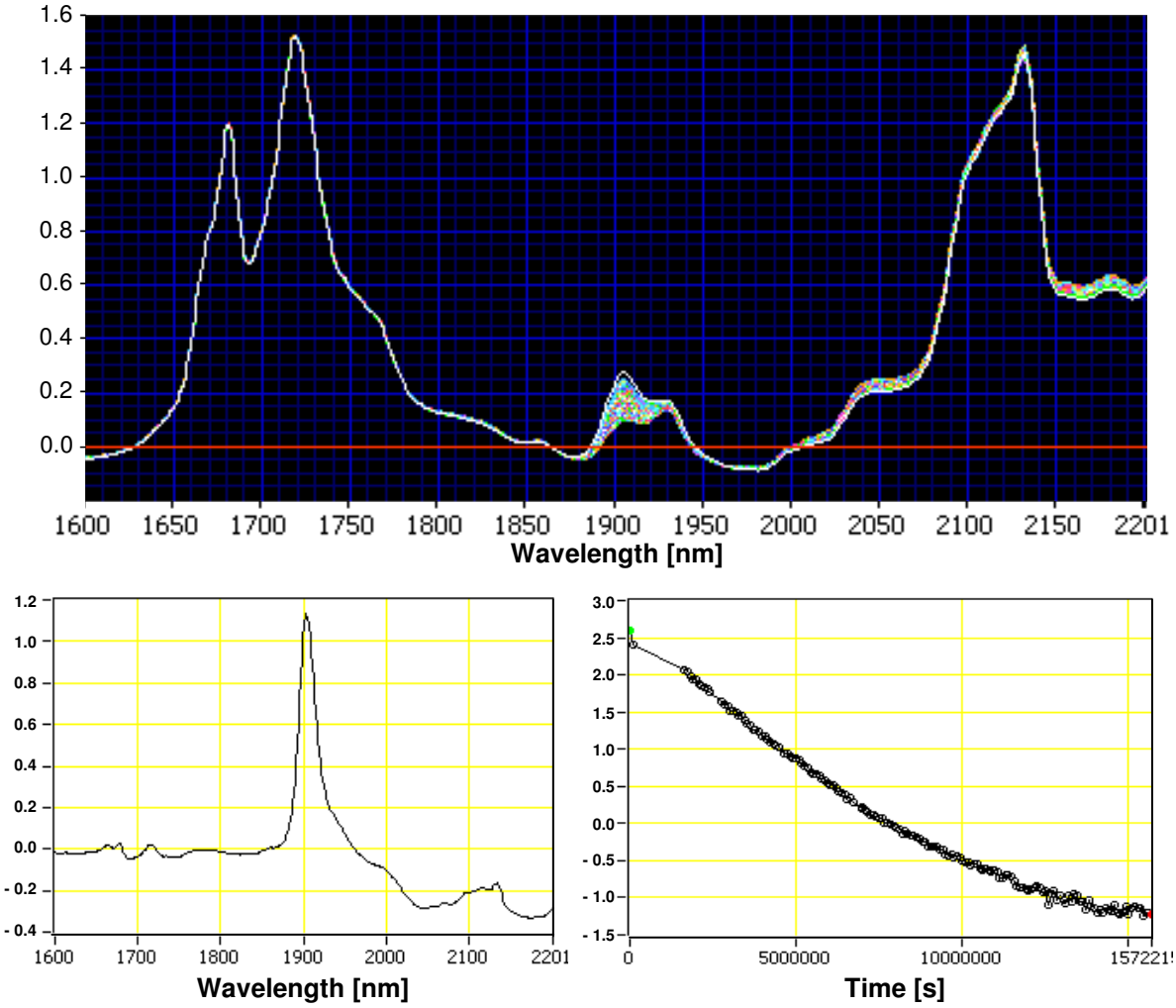
Formulations A and B contained both 17.00 % of the active ingredient NOA449851 and 17.00 %, respectively 12.75 % of PLA polymers solved in triacetin. Due to the similar results of both samples A and B, only graphics and spectra of formulation B are shown and commented here.

Overlay plot of NIR-spectra set for wavelength range between 1600 nm and 2200 nm taken during the six months storage at 25℃ are represented in Figure 6.26a after procedure of baseline correction and variance normalization. Changes in various spectra can be observed at wavelength region of 1900 nm, which is confirmed by process factor illustrated in Figure 6.26b. Time profile illustrated in Figure 6.26c indicate a continuous kinetic over time.



Figures 6.26a-c: (a) Overlay plot of NIR-spectra set of formulation B during six months storage at 25℃. (b) Process factor. (c) Time profile.

Superposition of NIR-spectra at wavelengths between 1600 nm and 2200 nm and taken from formulation B during the six months storage at 40°C is represented after baseline correction and variance normalization in Figure 6.27a. Process factor illustrated in Figure 6.27b indicates that the degradation reaction effects functional groups which absorption occurs at wavelength of 1900 nm.

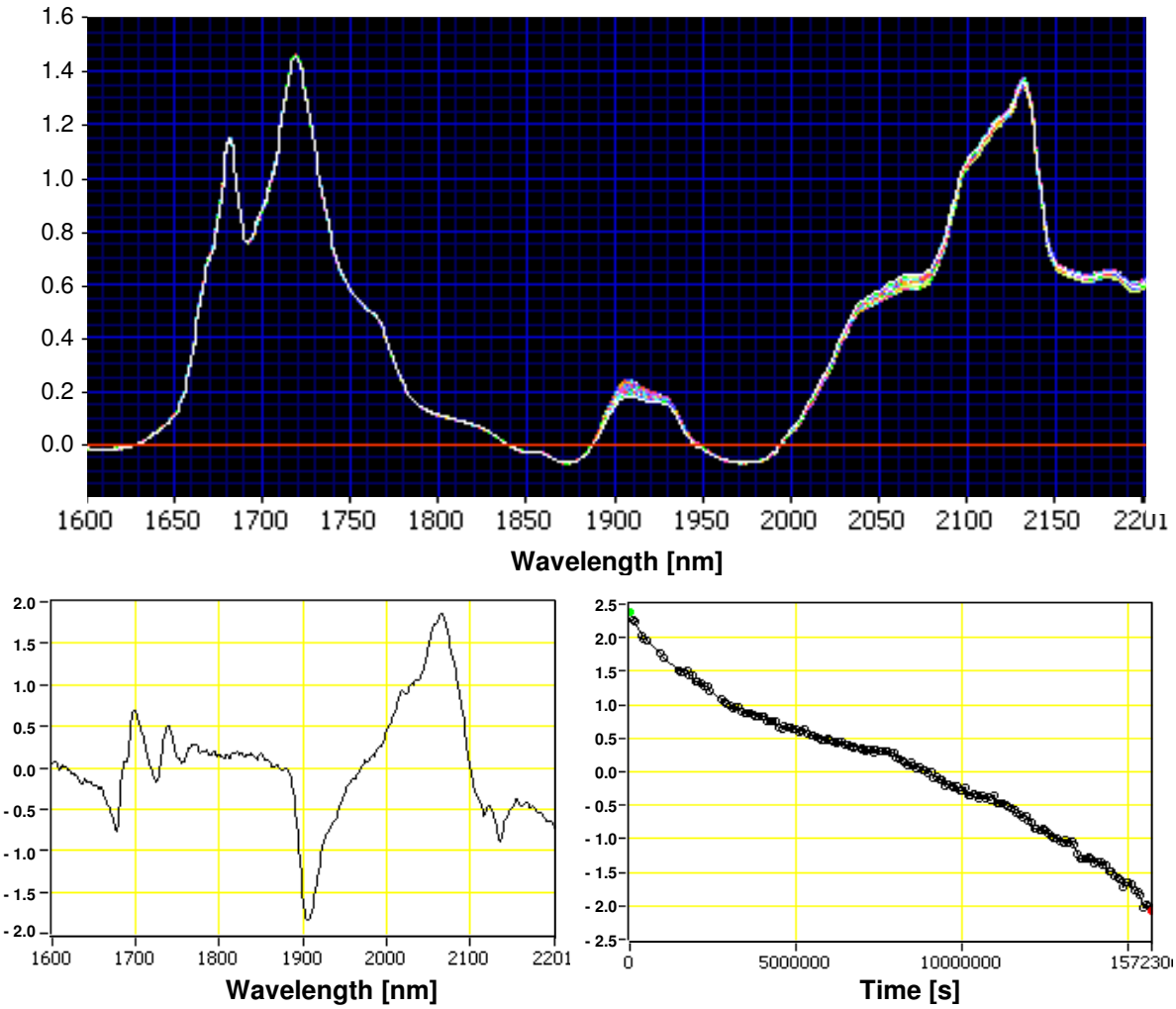


Figures 6.27a-c: (a) Overlay plot of NIR-spectra set of formulation B during six months storage at 40°C. (b) Process factor. (c) Time profile.

3.3.3.2. Formulation C

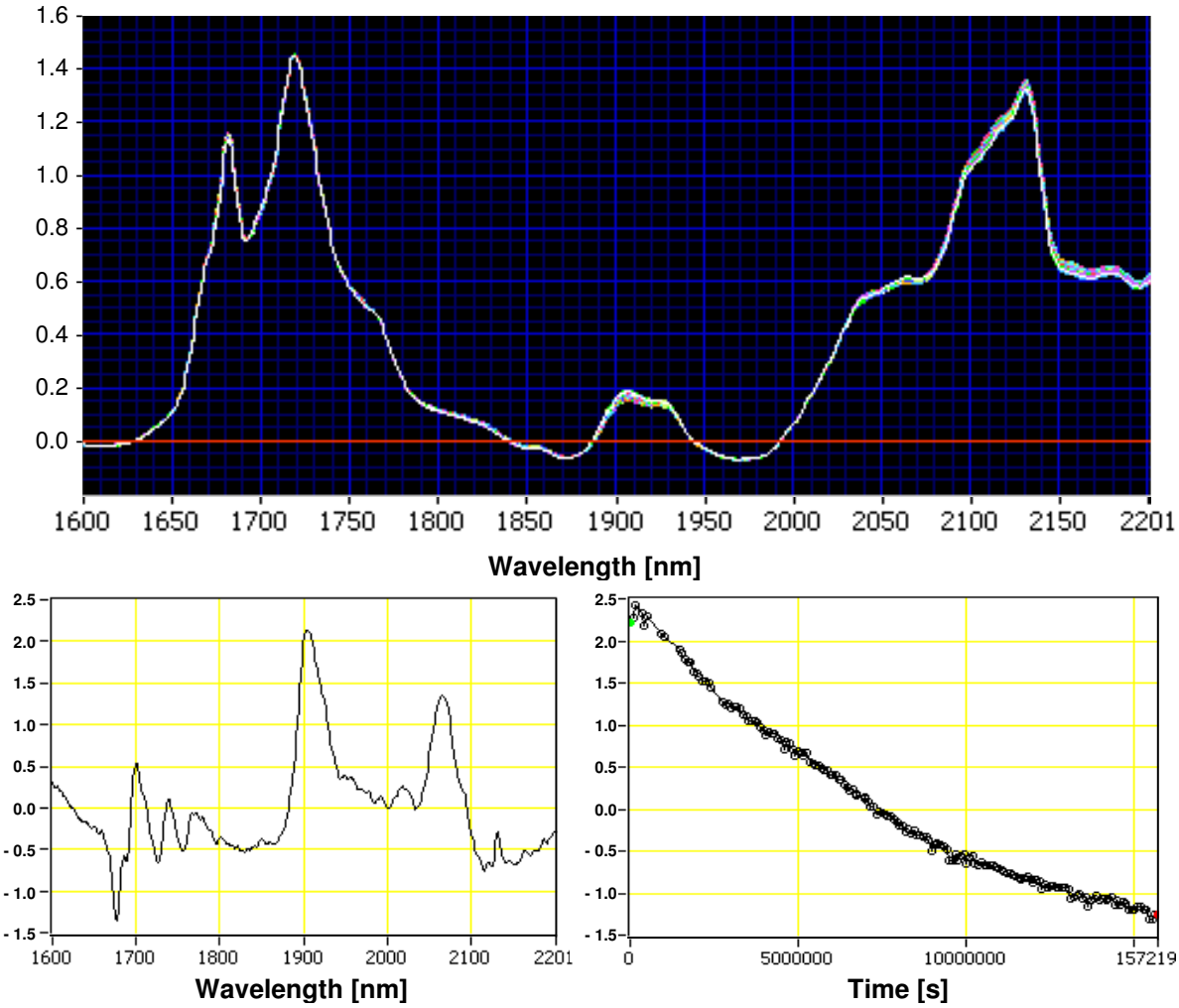
Formulation C was stored for six months at 25℃ and 40℃ and screened using NIR-spectroscopy. Formulation C contained, additionally to solvent triacetin, 10 % of co-solvent ethanol absolute. Exact composition of formulation C can be found in Table 6.1.

Figure 6.28a, which represents overlay plot of NIR-spectra taken during storage at 25℃, shows wavelength region such as 1900 nm, 2000 nm - 2100 nm and 2200 nm with poor superposition. Changes at these wavelengths were confirmed by process factor represented in Figure 6.28b. Absorbance in regions between 2000 nm and 2100 nm can be attribute to ROH functional groups. Solvent ethanol absolute as well as active ingredient NOA449851 comprise such functional groups.



Figures 6.28a-c: (a) Overlay plot of NIR-spectra set of formulation C during six months storage at 25℃. (b) Process factor. (c) Time profile.

Formulation C, consisting in 17.00 % of active ingredient and 17.00 % of PLA polymers dissolved in triacetin and ethanol was stored also at 40℃ and was screened with NIR-spectroscopy on a daily basis. Overlay plot of NIR-spectra set in wavelengths range from 1600 nm to 2200 nm collected during the six months storage at 40℃ are represented after baseline correction and variance normalization in Figure 6.29a. Changes in the spectra can be detected at wavelength region of 1900 nm, 2000-2100 nm and 2200 nm as also confirmed by process factor represented in Figure 6.29b. Time profile is illustrated in the chart of Figure 6.29c and indicates a continuous process over storage time



Figures 6.29a-c: (a) Overlay plot of NIR-spectra set of formulation C during six months storage at 40℃. (b) Process factor. (c) Time profile.

3.4. pH measurements of the solvent triacetin

To determine stability of triacetin, pH-values of a mixture of triacetin and water were measured. The measurements were done in triplicate after 1 and 3 months storage at 25°C and 40°C. No significant change in pH-value could be measured, indicating good stability of the solvent triacetin during storage.

3.5. *In-vitro* drug release from formulation A after six months storage at 40°C

In-vitro drug release performances of formulation A after six months storage at 40°C were investigated. Active ingredient concentration in formulation A after storage period of six months was determined in Section 3.1.1 to be 94.09 % (w/w). Molecular weight of PLA polymer as measured with GPC, decreased during storage period to 26.0 % of its initial value (Data not shown).

Drug release was expressed in percent of cumulative amount of active ingredient released, reported to the corrected refunded value. *In-vitro* releasing test parameters were set as for dissolution test method carried out in Chapter 3, Section 2.2.1.3. Therefore, drug release profile of formulation A after six months storage at 40°C can be compared to release kinetic of formulation *"PLA-AI(1:1)",* tested directly after manufacturing in Chapter 3 (Figure 3.2).

Release profiles of formulation A investigated directly after manufacturing and after six months storage at 40°C were very similar, as showed in Figure 6.30. Drug release after 40 days dissolution test was 43 % for formulation investigated directly after manufacturing and 38 % for formulation tested after six months storage. Both formulations showed a substantial initial burst corresponding to 17.00 % of active ingredient released within 6 days form formulation tested directly after manufacturing and 18 % from formulation tested after six months storage at 40°C.

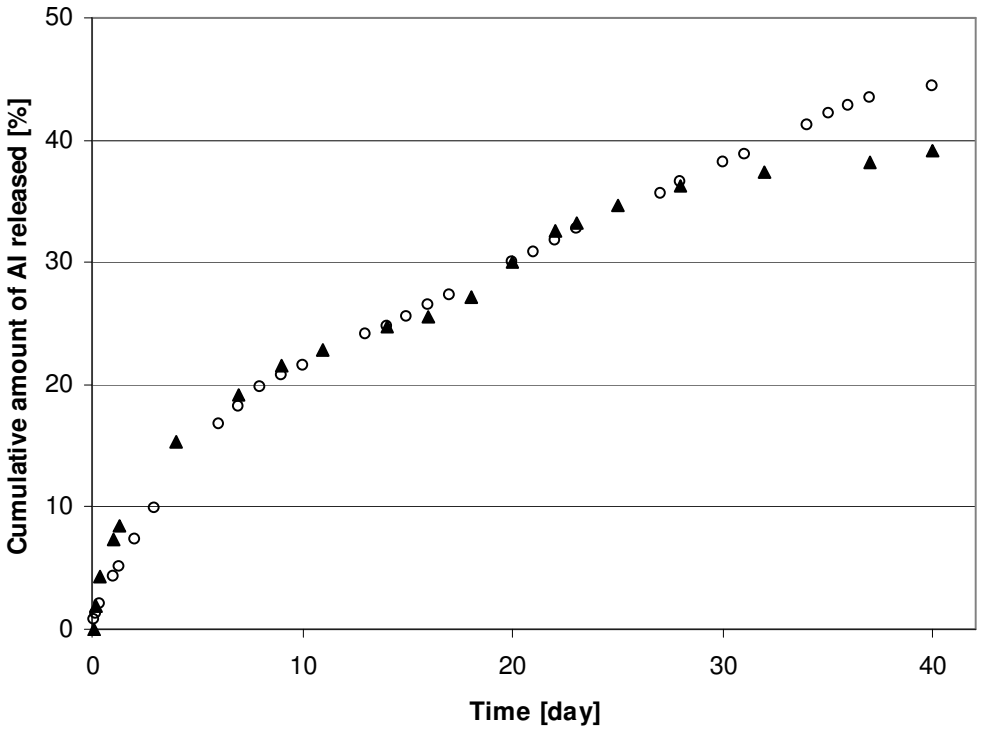


Figure 6.30: Influence of storage on the release of NOA449851 [% (w/w)] from sustained release injectables formed *in-situ,* related to time. Formulation A was tested directly after manufacturing (O) as well as after six months storage at 40°C (▲).

For comparison of two dissolution profiles, Moore and Flanner have proposed a simple model independent approach using mathematical indices to define difference factor (f_1) and similarity factor (f_2) [Moore et al., 1996]. These factors directly compare difference between percentage of drug dissolved per unit time for a test and a reference formulation and can be defined by Equations 6.1 and 6.2. The results are reported in Figure 6.5.

Table 6.5: Difference factor and similarity factor for drug release form formulation A measured directly after manufacturing and after six months storage at 40°C.

		Results for formulation A
Difference factor	f_1	7.01 %
Similarity factor	f_2	61.76 %

Factor f_1 is proportional to average difference between both profiles. Percent error is zero when test and reference profiles are identical and increases with dissimilarity between the two profiles. Similarity factor f_2 measures the closeness between the two profiles. It is a logarithmic transformation of the sum of squared error. It takes the average sums of squares of the difference between test and reference profiles and fits the results between 0 and 100. Similarity factor (f_2) is 100 when test and reference profiles are identical and approaches zero as dissimilarity increases. Similarity factor (f_2) is recommended for dissolution profile comparison in FDA's Guidance for Industry. FDA has set a public standard of f_2 value between 50-100 to indicate sameness or equivalence of two dissolution profiles [Shah et al., 1998].

4. Discussion

Stability properties of three selected formulations were investigated with various analytical methods during a storage period of six months at four determined temperatures. First, content of active ingredient and of by-products in the three selected formulations was determined with an HPLC-method. Then, molecular weight of PLA polymers was measured using GPC for formulations B and C and relative placebo B1 and C1. Finally, NIR-spectroscopy was used as a vibration spectroscopic screening system for supporting storage stability studies of new formulation candidates.

4.1. Active ingredient and by-products analysis with HPLC

HPLC is a commonly used method to analyze drug content and by-products. HPLC-analysis are specific for substance identity and very accurate for quantitative measurements. As disadvantages, samples preparation and analysis run need both large amount of organic solvents and time.

HPLC-method used in this chapter has the advantage, compared to HPLC-method described in Chapter 2, Section 2.2.1, to separate additionally to the peaks of the active ingredient, four of its by-products. Therefore, time of the HPLC-run was increased from 30 min to 90 min per run.

Content of active ingredient during six months storage at temperatures of 5°C, 25°C and 30°C was found to be stable in a range of ± 2.5 % (w/w) for all three formulations. At storage temperature of 40°C, content of drug in the three formulations decreased to reach a content in percent of the initial corrected value of 94.09 % for formulation A, 95.46 % for formulation B and 92.89 % for formulation C. For storage temperatures of 5°C and 25°C, no difference between the three formulations was observed. At storage temperatures of 30°C and 40°C, the active ingredient contents in formulation C containing the co-solvent ethanol absolute were found to be slightly lower than for the two others formulations containing only triacetin as solvent.

For storage temperature conditions of 40°C, trendline of each formulation was calculated from the chart and used to evaluate storage time until degradation of 10 % of the active ingredient. This represented a storage time of 10.6 months for formulation A, 13.5 months for formulation B and 8.2 months for formulation C.

After six months storage, by-products were found to be in a concentration below 1 % of the content of active ingredient, with a tendency to appear after long storage duration at higher temperatures. The by-products concentrations were acceptable as no substance had a specific toxicity. Degradation mechanisms of the various by-products are typical hydrolytic as well as oxidation reactions. As investigated in Section 3.1.2, there was no significant differences between the three formulations A, B and C concerning the by-products appearance during the six months storage.

4.2. PLA polymer analysis with GPC

GPC also called size exclusion chromatography uses porous particles to separate molecules of different sizes. Its most important use is to determine molecular weights and molecular weight distributions of polymers. Polymer molecules that are smaller than the pore sizes in the particles of the column can enter the pores and therefore have a longer path and transit time than larger molecules that cannot enter the pores. Movements in and out of the pores is governed by Brownian motion. Small particles which are entering the pores have a longer way to pass through the column and elute later than large molecules which do not enter the pores and have shorter way. GPC is a relative molecular weight method and such instrumentation needs to be calibrated. Therefore, synthetic anionic polystyrenes beads of narrow molecular weight distributions are used for calibration [Sperling, 2001].

GPC results indicated a reduction of the weight-average molecular weight of PLA polymers highly dependent on storage temperature. Decrease in molecular weight of PLA polymers in solution was much more important for formulations stored at 40°C than for formulations stored at 5°C or 25°C. Kinetic constant k give good indication of the magnitude of the influence of storage temperature: at storage temperature of 40°C, kinetic constant of all investigated samples were between 12.45 kJ/mol and 15.07 kJ/mol, while they were between 3.08 kJ/mol and 6.95 kJ/mol for storage temperature of 25°C and even between 1.41 kJ/mol and 3.05 kJ/mol for storage temperature of 5°C. This temperature dependent effect is usual for chemical reactions like hydrolysis, which probably occurred to the PLA polymers during storage.

Decrease in molecular weight of PLGA polymers dissolved in benzyl benzoate was already described as a function of time and temperature [Wang et al., 2003]. The unstable character of the ester bounds is an inconvenient property of the PLA polymers, especially regarding storage of formulations. However, this is a necessary aspect of these excipients to allow biodegradation of the solid matrix at physiological conditions [Shih, 1995; Wang et al., 1997; Li et al., 1999].

In formulation C and related placebo C1, the substitution of a fraction of triacetin with ethanol absolute did slightly increase degradation rate of the PLA polymers. Examination of kinetic rate constants k indicates a value of 2.5 kJ/mol for formulation C stored at 5°C, while it was only 1.5 kJ/mol for formulation B stored at the same conditions. As an other example, kinetic rate constant k was 3.1 kJ/mol for placebos C1 formulation stored at 5°C and only 1.4 kJ/mol for placebo B1, the sample without the co-solvent ethanol absolute.

Furthermore, results of the GPC indicated that, when stored at same temperature, PLA polymers of placebo solutions degraded faster than PLA polymers of active ingredient containing formulations. The active ingredient NOA449851 may have played a role of stabilizator, by competitively attracting water molecules responsible for polymer hydrolysis.

Polymeric dosage forms are often used to provide sustained release of various pharmaceutical entities. When polymer acts as a solid matrix, release rate is controlled by physicochemical property of the polymer. It is therefore essential to define specifications for PLA polymers and for storage conditions.

The charts obtained with GPC data indicate evident influence of storage temperature on PLA polymers stability. However, with a data set of only four molecular weight measurements for each samples at defined storage conditions, kinetic of reactions cannot be reliably determined. In these experiments, kinetic of the reactions seemed to be linear; however, it was not possible to exclude totally other kinetic orders. Furthermore, mechanism or pathway of the chemical breakdown can change with storage temperature.

Dependence of the drug release as function of the molecular weight of the PLA polymers should be carefully investigated. An *in-vitro* dissolution test was performed with a formulation stored for six months at 40°C and compared with drug release parameter of a formulation tested directly after manufacturing.

4.3. low field NMR-screening (NIR-screening)

Low field NMR (NIR) is a rapid, non-destructive and non-invasive analytical tool which is gaining acceptance in pharmaceutical industry as a technique for qualitative and quantitative analysis. The NIR applications in pharmaceutical industry include qualitative identification of raw materials, quantitative analysis of drug content in tablets, determination of moisture content as well as detection of degradation products within a solid. Low field NMR-spectroscopy offers a lot of options for fast and easy analyses of liquids, powder and solids without time consuming sample preparation. This method is non destructive and therefore the same sample is used for all spectral analysis, circumventing the problem of samples collection [Blanco et al., 2002; Reich, 2002].

Low field NMR region covers a wavelength range from 700 nm to 2500 nm (1400 cm^{-1} - 4000 cm^{-1}). It contains primarily overtones and combinations band of C-H, N-H and O-H stretching frequencies which are adequate for studying many organic compounds. Because absorbance regions of different functional groups are strongly overlapping, absorbance bands in NIR spectra are usually composites of different functional groups. In contrast to mid infrared and Raman spectroscopic range, unequivocal assessment of an absorbance band to a functional group is often not possible. Therefore, requirements of spectroscopic selectivity are normally not fulfilled and the selectivity must be generated by experimental study design.

Evaluation of the spectra data sets was performed with different software tools. Efficient data pre-treatment algorithm reduces contribution of the instrument and the experimental variance without having significant effects on the variance caused by systematic changes in the samples. As examples of data pre-treatment algorithm, baseline correction and variance normalization can be mentioned. Baseline correction of NIR-spectra reduces light diffractive effects caused by surface of the glass sample vial. Variance normalization eliminates the noise in the spectra by weighting absorbance at each wavelength with the corresponding noise level. For illustrating the effect of the data pre-treatments, Figure 6.13 should be compared to Figure 6.14.

The first level for data evaluation performs statistical data analysis of each test sample stored in the SpecScreen X HTS apparatus and informs about ongoing degradation processes in the sample. After baseline correction, variance normalization and wavelength selection, an overlay plot of NIR-spectra set is obtained from which process factor can be calculated. This represents the slope of the regression line shown in the spectra space. Process factor indicated the wavelength regions with significant spectral modifications during the storage of the samples. Additionally, time profile of the process is calculated and shows the kinetic of significant processes in the formulations.

The second level of data analysis focuses on comparison of different related test samples. Based on further data pre-treatments and evaluation algorithms which fit magnitude of Y-axis value of the time profiles to a same scale, allowing direct comparison. Therefore, it was possible to evaluate the impact of storage temperature for samples B2 (Figure 6.17). Further, the influence of the addition of ethanol on stability for a given storage temperature was investigated (Figures 6.20 and 6.21).

One of the concept of low field NMR screening method is to save time by avoiding development of a specific method for each compound. Therefore, no calibration is performed and in case of modifications in the NIR spectra set, stability of the sample is defined by comparison of the various data sets and their related time profiles. Stability assumptions are therefore more qualitative than quantitative. In the present case, this concept could not be successfully applied because of an extensive placebo effect of the solvent triacetin. The instability of the solvent triacetin was detected at same wavelength region on the NIR spectra as the predicted labile functional group of the PLA polymer. Because there was no previous experience with the NIR screening method for PLA polymer solutions, it was necessary to compare the NIR-screening results with a reliable method. Therefore, results obtained with NIR screening method were systematically compared to GPC results obtained in section 3.2.

The overlay plot of the NIR-spectra set of the solvent triacetin (Figures 6.15 and 6.16) showed spectral changes for wavelengths at 1900 nm. As no calibration of this specific component was previously made, it could not be determined if the degradation process of the solvent was significant or not. Therefore pH-measurements of solvent triacetin were performed to detect eventual degradation products such as acetic acid. No changes in the pH-values could be measured, confirming a very high sensitivity of the NIR-screening method, which probably do not have any consequence for formulation storage conditions.

Technically, instability of triacetin was very incommoding for the next NIR-spectroscopy investigations of samples using this excipient as solvent. This excipient was in excess in each further analyzed sample, as it was the selected solvent for the PLA polymers. Spectral changes at wavelengths of 1900 nm were consistent in every analyzed spectra sets (Figures 6.15 to 6.27).

According to GPC investigations performed in Section 3.2.1, weight average molecular weight of PLA polymers decreased dramatically during storage. Reduction of molecular weight of PLA polymers is due to hydrolysis of ester bounds of polymer chains. Such effects should be detected with NIR-spectroscopy, as functional groups RCOOR and RCOOH absorb in the wavelength region around 1900 nm (Figure 6.12). However, this region correspond to the wavelength affected by degradation of the solvent triacetin (Figures 6.15 and 6.16) and changes due to the polymer hydrolysis are masked by the solvent effect (Figures 6.20 and 6.21). The influence of the solvent effect could not be removed by study design, as no specific wavelength could be attributed to the PLA polymers parameter when the solvent triacetin was used.

When two different chemical reactions interfere at the same wavelength region in a spectra, comparison of modifications should be carefully considered. Spectral changes are not cumulative and it is not possible to compare quantitatively two unstable formulations with more than one variable influencing one unique wavelength region. Comparison would be possible with a stable placebo (solvent system) or with an unstable placebo which changes are located at other specific wavelengths than the new investigated parameter.

Spectroscopic selectivity requires that at least one relevant absorbance band of the test substance does not interfere with absorbance bands of the other excipients in formulation. In such cases, data evaluation is simple and determination of degradation of the tested substance can be directly performed. However, this requirement is usually not fulfilled for NIR spectroscopy and alternative should be evaluated. Selectivity should therefore be generate by experimental design of the study. The first assumption is that different samples with similar compositions will have same changes in spectra during storage at same conditions. This is a successful approach for investigation of the effect for a large number of variables. However, the result of comparison of data evaluation of different sample is more qualitative than quantitative.

Unfortunately, it must be concluded that this new analytical screening method is suitable for stability investigations, only when changes in NIR spectra can be properly interpreted. A direct interpretation of the NIR spectral changes was here not possible because of the lack of specificity of the changes attributed to the solvent triacetin and to the PLA polymers, which were located at same wavelength regions in the NIR spectra. Evaluation of a spectroscopic data set should give clear evidence about the stability of a substance in a formulation. However, this requirement makes high demands on the spectra properties with respect to selectivity and specificity. However, this pointed out the labile stability of the solvent triacetin, which was a neglected aspect with the other traditional analytical methods. Due to the missing calibration step, this effect was not quantified and it could not be determined if the solvent degradation was a significant issue with regards to storage stability.

Both PLA polymer and solvent triacetin have labile ester bounds which are likely degraded during storage. The solvent triacetin has a molecular weight of 218 g/mol (Pharmacopoe) and comprises three ester bounds in the chemical structure (triester of glycerin). As determined with GPC (Section 3.2.1), PLA polymers used have, directly after manufacturing, a molecular weight of about 70’000 Da. The lactic units of the polymers, which each represents a molecular weight of about 90 g/mol are joined with ester bounding. So the ester functional groups is present in a similar frequency for both components, PLA polymers and solvent triacetin. However, the labile ester bound of the lipophilic and long polymer chains are probably less accessible to water molecules than the ester bounding of small solvent molecule, according to steric hindrance as well as hydrophilic interactions. Furthermore, in all tested formulations, the solvent triacetin was in excess compared to the amount of PLA polymer which was at maximum in a concentration of 17.00 % (w/w). Theoretically, hydrolysis of one unique ester bounding in the middle of a polymeric PLA chain reduces molecular weight of PLA from 70’000 Da to 35’000 Da. Average molecular weight of the polymer decrease drastically, while the physico-chemical properties of the polymeric chains are not affected as fundalmentally as it could seem.

4.4. *In-vitro* drug release from formulation A after six months storage at 40°C

Dissolution test was performed for formulation A after six months storage at 40°C. As reported in previous sections, refund of active ingredient was 94.09 % and molecular weight of the PLA polymers dropped to 26.0 % of its initial value. In order to investigate the impact of the decrease of the polymer molecular weight on drug release performances, *in-vitro* drug release was compared to the one of the same formulation tested directly after manufacturing.

Formulations containing lower molecular weight polymers were able to form implants after injection in aqueous milieu. Drug release was surprisingly similar to release from formulations tested directly after manufacturing. Difference factor f_1 and similarity factor f_2 were calculated for a quantitative comparison of both dissolution profiles. Difference factor f_1 was measured to be 7.41 % and similarity factor f_2 had a value of 60.60. The f_2 value is between 50 and 100 and therefore within the standard set by FDA to indicate equivalence [Shah et al., 1998].

As already mentioned in the previous chapters, drug release is assumedly mainly diffusion controlled and not erosion controlled. Therefore, molecular weight of polymer did not influence drug release during the 40 days of the dissolution test. However, in various published papers, the release is reported to be function of molecular weight of polymers [Hatefi et al., 2002; Yewey et al., 1997; Eliaz et al., 2002]. Yewey et al. investigated *in-vitro* release of myoglobin from injectables implants based on PLA polymers of various molecular weight (inherent viscosity of 0.05 dl/g and 0.33 dl/g). Initially, the two formulations released the protein at similar rates, but with time, the lower molecular weight formulation released at higher rates [Yewey et al., 1997]. Further, release of plasmid DNA from implants formed with polymer of various molecular weight (inherent viscosity of 0.24 dl/g and 0.59 dl/g) were investigated by Eliaz et al.. Drug release was more rapid from the low molecular weight polymer but in both cases, the DNA was released before complete erosion of the polymeric matrix [Eliaz et al., 2002].

5. Conclusion

Different analytical methods were used for stability studies of sustained release injectables formed *in-situ* during six months storage at four selected temperatures. Formulations were based on PLA polymers, active ingredient NOA449851 and solvent triacetin. Co-solvent ethanol was added in formulation C.

An HPLC-method was utilized for determination of active ingredient content and detection of by-products. No differences between the three formulations in active ingredient content and in apparition of by-products were observed. Content of active ingredient NOA449851 decreased with time and temperature. Concomitantly, four degradation products of the NOA449851 were found to increase.

Molecular weights of the PLA polymers were determined with GPC. It was found that the decrease in molecular weight of the PLA polymers was significant increased with storage temperature. No significant influence of the co-solvent ethanol absolute on the changes of the molecular weight of the PLA polymers in the formulation could be measured. However, the presence of the active ingredient in the formulation seemed to decrease hydrolysis process of PLA polymer.

NIR data analysis of solvent triacetin showed spectral changes for wavelengths at 1900 nm. Spectral changes at these wavelengths were consistent in every analyzed spectra sets as the solvent triacetin was in excess in all samples. The influence of the solvent effect could not be removed by study design, as no specific wavelength could be attributed to the PLA polymers parameter.

Drug release from formulation A tested directly after manufacturing and after six months storage at 40°C were found to be similar for the investigated 40 days, despite reduction of molecular weight of PLA polymers. This is surprising, as the molecular weight of the polymers is reported to influence drug release form matrix. However, this confirm a drug release mechanism mainly diffusion controlled and not erosion controlled.

Chapter 7

Exploration of microspheres as comparison technology for sustained release injectables formed *in-situ*

1. Introduction and objectives

Polymeric microspheres are a thoroughly investigated and well described drug delivery technology with regard to manufacturing process and drug release mechanisms [Jalil et al., 1990; Choi et al., 2002; Mandal et al., 2001; Dalpiaz et al., 2002; Berckland et al., 2002; Sinha et al., 2003; Zhou et al., 2003]. They provide sustained release of active ingredient after subcutaneous or intramuscular application. The excipients needed are water insoluble polymers with biodegradable and biocompatible properties. Therefore, polymers such as PLA or PLGA are often used as excipients to form the matrix of microspheres. Formulations of PLA/PLGA microparticles containing active ingredients such as triptorelin (Decapeptyl® Depot, Ferring), leuprorelin (Enantone® Gyn Monats-Depot, Takeda Pharma), bromocriptin (Parlodel® LAR, Sandoz), risperidon (Risperdal Consta®, Janssen-Cilag) or octreotide (Sandostatin® LAR-Monatsdepot, Novartis Pharma) are on the market for human use. For veterinarian use, Proheart® 6 (Fort Dodge), which was recently removed from the marketplace because of severe side effects, consisted in hydroxypropylmethylcellulose based microparticles with the active ingredient moxidectine and was used for prevention of heartworm disease caused by *Dirofilaria immitis*.

Sustained release injectables formed *in-situ* and microspheres are both technologies intended for parenteral application, designed to achieve a long acting release of the drug. In both technologies, the sustained effect is caused by the slow biodegradable PLA/PLGA polymer matrix in which the active ingredient is embedded. In order to better understand the release mechanisms of the implants formed *in-situ*, microspheres were manufactured, using

the same polymer types and the same drug to polymer ratios as for formulations investigated in Chapter 3. The microsphere batches were analyzed with regard to yield, drug content, encapsulation efficiency, particle size distribution and *in-vitro* drug release properties. Additionally, microspheres were used as simplified two-components models, containing polymers and active ingredient NOA449851, but lacking the solvent in comparison to the sustained release injectable formed *in-situ*. Raman and NIR spectroscopy were used to determine conformation/polymorphism of the active ingredient within the polymer matrix and also to detect possible interactions between polymer and active ingredient. Vibrational spectroscopy has already been successfully used for investigations of the conformation of active ingredients within various polymeric matrices as well as for detection of polymer/drug interactions [Bolton et al., 1984; Taylor et al., 1997; Breitenbach et al., 1999].

2. Materials and methods

2.1. Materials

The active ingredient NOA449851 was obtained from CarboGen Laboratories, Aarau, CH. The polymers PLA, 85/15 PLGA and 75/25 PLGA (inherent viscosity of 0.68 dl/g, 0.63 dl/g and 0.67 dl/g in trichloromethane at 30°C, respectively) were provided by Birmingham Polymers Inc., Birmingham, AL, USA. PVA 15000 was purchased from Fluka Chemie AG, Buchs, CH. The solvents acetonitrile, methanol, methylene chloride and water for chromatography were supplied by Merck Inc., Darmstadt, D. Solutol HS 15® was purchased from BASF, Ludwigshafen, D. All solvents were of at least reagent analytical grade.

2.2. Methods

2.2.1. Preparation of the microspheres

Twelve microspheres batches were manufactured using three types of polymer (PLA, 85/15 PLGA and 75/25 PLGA) and four different concentrations of the active ingredient (placebo, 0.99 %, 16.67 % and 50.00 %), corresponding to weight polymer to drug ratios of 100:1, 5:1 and 1:1, as already used for the sustained release injectables formed *in-situ* investigated in Chapter 3.

Microspheres were prepared using an O/W emulsion solvent evaporation method according to Conti et al. (Conti et al., 1992). The polymers were dissolved in methylene chloride to give a 20 % (w/v) solution. An accurate amount of active ingredient (listed in Table 7.1) was added to the polymer solution, which was stirred until complete dissolution. The solution was then emulsified in 100 ml of water containing 5 % PVA 15000 for 3 min under mechanical stirring with 1200 rpm (Merck Eurolab, Typ Euro-ST D, Staufen, D). The resulting O/W emulsion was poured into a 20 times larger volume of distilled water preheated at 40°C. The diluted O/W emulsion was stirred for 30 min at 40°C to remove the organic solvent methylene chloride. The resulting hardened microspheres were collected by filtration, washed with distilled water and dried under reduced pressure at room temperature for two days (Salvis Trockenschrank, Typ KVTS11, Reussbühl, CH). The exact compositions of the twelve microsphere batches are listed in Table 7.1.

Table 7.1: Composition in percent [% (w/w)] of microsphere batches.

Microspheres	**Polymer to drug weight ratio**	**Components**	**Content of components [% (w/w)]**
PLA-placebo	placebo	PLA	100.00
85/15 PLGA-placebo	placebo	85/15 PLGA	100.00
75/25 PLGA-placebo	placebo	75/25 PLGA	100.00
PLA-(100:1)	100:1	PLA NOA449851	99.01 0.99
85/15 PLGA-(100:1)	100:1	85/15 PLGA NOA449851	99.01 0.99
75/25 PLGA-(100:1)	100:1	75/25 PLGA NOA449851	99.01 0.99
PLA-(5:1)	5:1	PLA NOA449851	83.33 16.67
85/15 PLGA-(5:1)	5:1	85/15 PLGA NOA449851	83.33 16.67
75/25 PLGA-(5:1)	5:1	75/25 PLGA NOA449851	83.33 16.67
PLA-(1:1)	1:1	PLA NOA449851	50.00 50.00
85/15 PLGA-(1:1)	1:1	85/15 PLGA NOA449851	50.00 50.00
75/25 PLGA-(1:1)	1:1	75/25 PLGA NOA449851	50.00 50.00

2.2.2. Yield

The total microsphere yield was calculated gravimetrically on the basis of the polymer/drug recovery after the final drying step.

2.2.3. Determination of active ingredient content in the microspheres

The drug content of each microsphere batch was analyzed in triplicate after manufacturing. Approximately 100 mg of microspheres were weighed into a 100 ml volumetric flask. The flasks were filled up to the calibration line with acetonitrile. The mixtures were shaken manually and put into the ultrasonic bath until complete dissolution of the microspheres. The concentration of NOA449851 in the solution was determined using the HPLC-method as described in Chapter 2, Section 2.2.1.

2.2.4. Size distribution of microspheres

Size distribution of microspheres was determined before and after dissolution test (Section 2.2.7) using a laser particle size analyzer (Granolomètre 715™, Cilas, Paris, F). Approximately 500 mg of microspheres were dispersed in the water bath of the analyzer by stirring and use of ultrasound. During measurements, a circulating pump transported the particles from the water bath via a flexible tube into a glass cuvette. Each batch was analyzed in triplicate.

2.2.5. Raman spectroscopy

Raman spectroscopy was used for determination of the different spectra related to each copolymer used to form the microspheres matrices. In addition, the following qualitative aspects of the incorporated drug were studied: crystallinity, presence of other polymorphic structures and possible interactions with the polymer. Raman measurements were performed according to the method described in Chapter 2, Section 2.2.5.

2.2.6. IR spectroscopy

IR spectroscopy was used for investigation of possible active ingredient interactions with the polymorphic matrices, especially regarding its capability of building hydrogen bonds. Therefore, the three microspheres batches with the weight polymer to drug ratio 1:1 were analyzed with IR spectroscopy according to the method described in Chapter 2, Section 2.2.4.

2.2.7. X-ray analysis

Powder X-ray diffraction patterns of the drug loaded microspheres batch PLA-(1:1) were analyzed to study the physical state of both components, the PLA polymer and the active ingredient NOA449851, when formulated as microspheres. The test was carried out according to the method described in Chapter 2, Section 2.2.6.

2.2.8. *In-vitro* drug release from microspheres

The *in-vitro* drug release from microspheres was determined by dissolution testing in triplicate of the nine active ingredient containing microsphere batches. The influence on the *in-vitro* drug release of different polymer types and of different drug loadings was investigated.

An amount of 500 mg microspheres, corresponding to a drug load of about 5 mg for the three microspheres batches with the polymer to drug ratio 100:1, 83 mg for the microspheres batches with the polymer to drug ratio 5:1 and of 250 mg for the microspheres batches with the polymer to drug ratio 1:1 was accurately weighed in felt bags and placed into the basket of the USP dissolution test apparatus (AT 7 Smart™, Sotax AG, Basel, CH). The same experimental conditions were applied as for the dissolution tests of the sustained release injectables formed *in-situ* (Chapter 3, Section 2.2.1.3). The dissolution medium consisted of one liter of a 5 % solution of Solutol HS 15® in distilled water which was replaced weekly by a fresh solution to keep sink conditions. The temperature was maintained at 37°C, the rotation speed of the baskets at 100 rpm. Drug concentration in the dissolution medium was determined by HPLC-analysis according to the method described in Chapter 2, Section 2.2.1. As for the dissolution tests of the sustained release injectables formed *in-situ*, the experiments were stopped after 40 days.

3. Results

Twelve microsphere batches were manufactured using three different polymer types and four different drug concentrations. They were analyzed with regard to yield, drug content, encapsulation efficiency, particles size distribution, release properties, cristallinity and drug/polymer interactions. The microsphere technology was studied to better understand the *in-vitro* sustained release characteristics of the implants formed *in-situ* and to investigate the interaction between the drug and the polymer matrix, using these simplified two-components models. Therefore, special attention was paid to produce microspheres with the same excipients and with the same polymer to drug ratios as for the sustained release injectables formed *in-situ* which have already been investigated. Whenever possible, the same test conditions were applied for analysis to assure a valid comparison with the sustained release injectables formed *in-situ*.

3.1. Yield, drug content and encapsulation efficiency

The yield of the microsphere preparation was found to be around 90 % in average (from 88.8 % to 95.0 %) for all twelve batches. The drug content, measured in triplicate, was 0.99 % in average for the three batches with a polymer to drug ratio of 100:1, 16.35 % for the batches with a ratio of 5:1 and 47.32 % for the batches with a ratio of 1:1. This represented an encapsulation efficiency of 99.02 %, 98.11 % and 94.65 %, respectively. The encapsulation efficiency is defined as the ratio of the effective measured content compared to the theoretical drug content of the microspheres. The results concerning yield, drug content and encapsulation efficiency of the twelve microsphere batches are listed in Tables 7.2 to 7.5.

Table 7.2: Yield of the three placebo microsphere batches.

Microspheres	Yield [% (w/w)]
PLA-placebo	91.8
85/15 PLGA-placebo	88.9
75/25 PLGA-placebo	89.2
Average	90.0

Table 7.3: Yield, active ingredient content (n = 3) and encapsulation efficiency of the three microsphere batches with the weight polymer to drug ratio 100:1.

Microspheres	Yield [% (w/w)]	Drug content ± S.D. [% (w/w)]	Theoretical drug content [% (w/w)]	Encapsulation efficiency [%]
PLA-(100:1)	89.3	0.99 ± 0.46	0.99	99.01
85/15 PLGA-(100:1)	90.7	0.99 ± 0.21	0.99	98.97
75/25 PLGA-(100:1)	95.0	0.99 ± 0.03	0.99	99.09
Average	91.7	0.99 ± 0.26	0.99	99.02

Table 7.4: Yield, active ingredient content (n = 3) and encapsulation efficiency of the three microsphere batches with the weight polymer to drug ratio 5:1.

Microspheres	Yield [% (w/w)]	Drug content ± S.D. [% (w/w)]	Theoretical drug content [% (w/w)]	Encapsulation efficiency [%]
PLA-(5:1)	89.3	16.43 ± 0.13	16.67	98.58
85/15 PLGA-(5:1)	90.7	16.60 ± 0.78	16.67	99.60
75/25 PLGA-(5:1)	95.0	16.05 ± 4.27	16.67	96.15
Average	91.7	16.35 ± 2.64	16.67	98.11

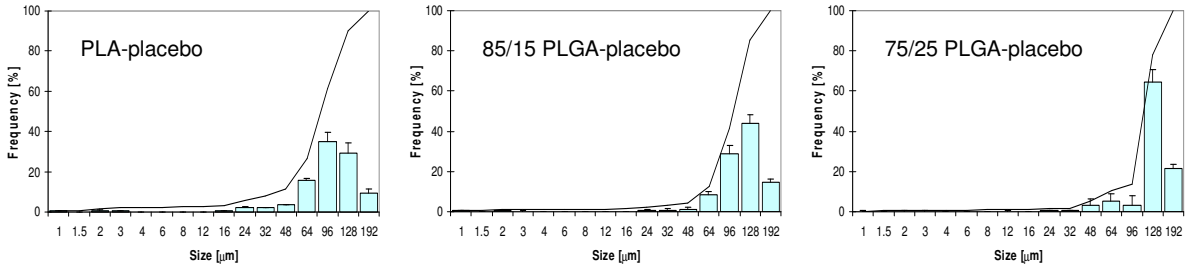
Table 7.5: Yield, active ingredient content (n = 3) and encapsulation efficiency of the three microsphere batches with the weight polymer to drug ratio 1:1.

Microspheres	Yield [% (w/w)]	Drug content ± S.D. [% (w/w)]	Theoretical drug content [% (w/w)]	Encapsulation efficiency [%]
PLA-(1:1)	91.6	47.04 ± 2.31	50.00	94.08
85/15 PLGA-(1:1)	88.8	47.43 ± 0.11	50.00	94.86
75/25 PLGA-(1:1)	90.9	47.49 ± 4.15	50.00	95.01
Average	90.4	47.32 ± 2.87	50.00	94.65

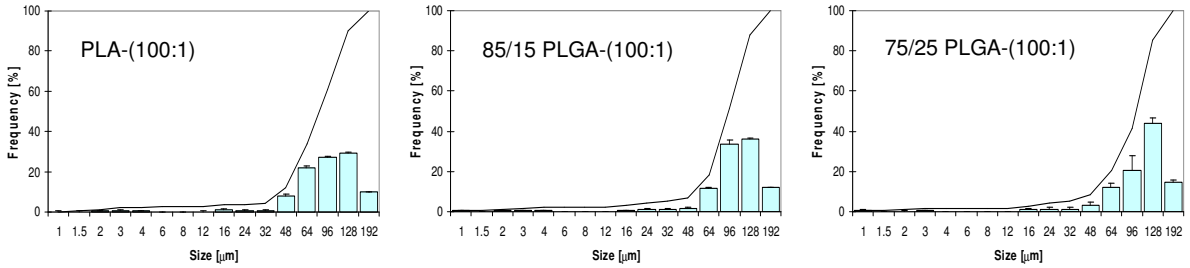
3.2. Granulometrie

The particle size distribution of the microsphere batches was determined in triplicate, using a laser particle size analyzer, immediately after manufacturing as well as after dissolution tests. Immediately after manufacturing, the microspheres based on PLA polymers had a diameter

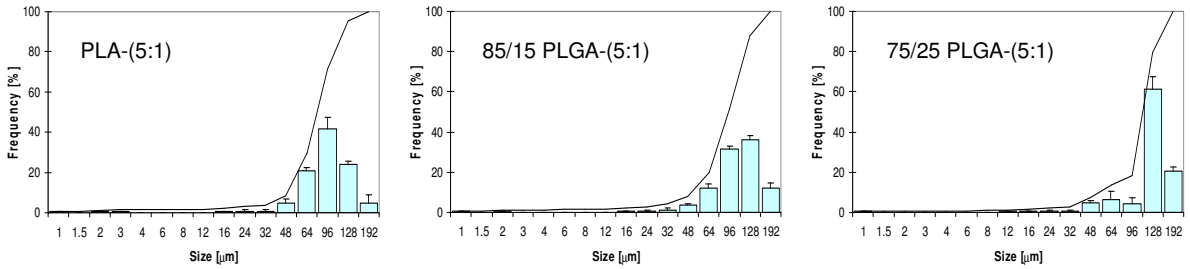
of 86.62 µm in average, whereas for 85/15 PLGA and 75/25 PLGA microspheres, the diameters were 100.93 µm and 110.50 µm in average. The particle size distribution histograms of the twelve different microsphere batches as measured after manufacturing are depicted in Figures 7.1 to 7.12.



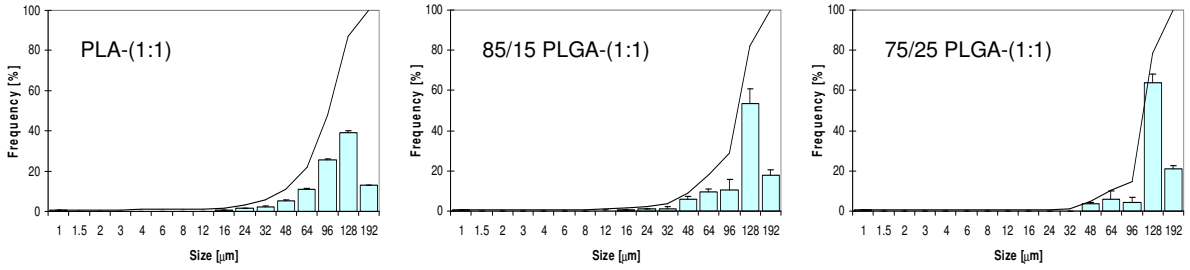
Figures 7.1-7.3: Particle size distribution histograms of the three placebo microsphere batches determined after manufacturing.



Figures 7.4-7.6: Particle size distribution histograms of the three microsphere batches with the polymer to drug ratio 100:1 determined after manufacturing.



Figures 7.7-7.9: Particle size distribution histograms of the three microsphere batches with the polymer to drug ratio 5:1 determined after manufacturing.



Figures 7.10-7.12: Particle size distribution histograms of the three microsphere batches with the polymer to drug ratio 1:1 determined after manufacturing.

The median sizes of the twelve microsphere batches determined after manufacturing as well as after dissolution test are listed in Table 7.6. Additionally, the differences of both measurements are represented in percent of the initial value.

After dissolution tests, the diameter of the microspheres was reduced by 0.99 % on average (0.37 % to 2.07 %) for microspheres with the polymer to drug ratio 100:1, by 1.4 % on the average (0.63 % to 2.01 %) for the microspheres with the polymer to drug ratio 5:1 and by 5.1 % on the average (4.75 % to 5.22 %) for the microspheres with the polymer to drug ratio 1:1.

Table 7.6: Median size [µm] of the twelve microsphere batches after manufacturing and after dissolution test (n = 3). The differences of the median size are represented in percent of the initial value.

Microspheres	**Median size after manufacturing [µm]**	**Median size after dissolution test [µm]**	**Difference [%]**
PLA-placebo	86.07	-*	-
85/15 PLGA-placebo	106.30	-*	-
75/25 PLGA-placebo	113.80	-*	-
PLA-(100:1)	83.07	82.70	- 0.37
85/15 PLGA-(100:1)	94.47	94.00	- 0.47
75/25 PLGA-(100:1)	102.27	100.20	- 2.07
PLA-(5:1)	79.57	77.97	- 2.01
85/15 PLGA-(5:1)	94.53	93.93	- 0.63
75/25 PLGA-(5:1)	112.37	110.73	- 1.46
PLA-(1:1)	97.77	92.67	- 5.22
85/15 PLGA-(1:1)	108.43	102.80	- 5.19
75/25 PLGA-(1:1)	113.53	108.14	- 4.75

* No dissolution test performed.

3.3. Raman spectroscopy

Raman spectra were obtained from the microspheres batches in order to study the difference related to each copolymer as well as to characterize the drug incorporated into the polymer matrices. Figure 7.13 represents the Raman spectra of the three placebo microsphere batches in the wavenumber region between 300 cm^{-1} and 2000 cm^{-1}. Each placebo microspheres batch was based on a different polymer type such as PLA, 85/15 PLGA and 75/25 PLGA polymers. The curve (a) represents the PLA-placebo microspheres, (b) the 85/15 PLGA-placebo microspheres and (c) the 75/25 PLGA-placebo microspheres.

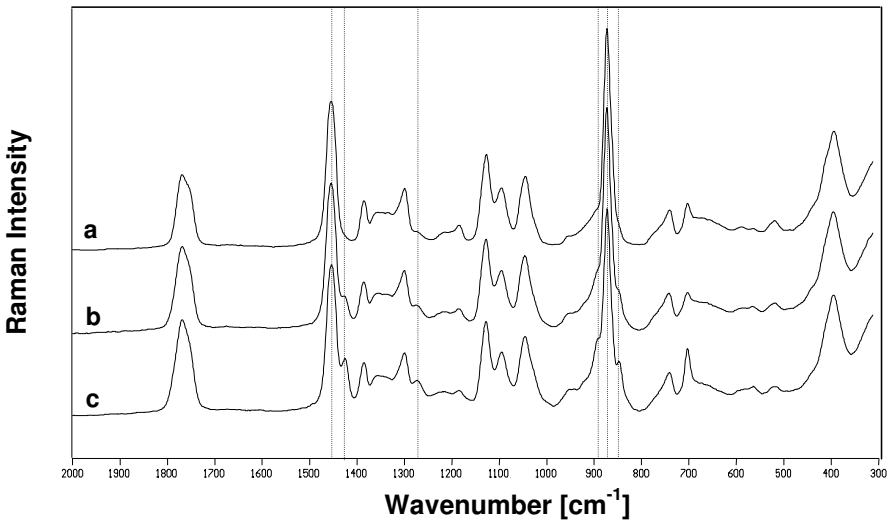


Figure 7.13: Raman spectra of the placebo microspheres plotted in the wavenumber region between 300 cm^{-1} and 2000 cm^{-1}: (a) PLA-placebo microspheres, (b) 85/15 PLGA-placebo microspheres, (c) 75/25 PLGA-placebo microspheres.

Some bands of the Raman spectra can be specifically attributed to the lactate units and to the glycolate units of the copolymers [Kister et al., 1997; Kister et al., 1998 (1), (2)]. These assignments are listed in Table 7.7.

Table 7.7: Assignment of bands specific for the lactate units and for the glycolate units from the Raman spectra of the placebo microspheres.

Lactate units [cm^{-1}]	Assignment	Glycolate units [cm^{-1}]	Assignment
1773-1749	$\nu C=O$	1760	$\nu C=O$
1455	$\delta_{as}CH_3$	1426	$\delta_{as}CH_2$
1386	$\delta_{s}CH_3$	1274	$twCH_2$
1300	δCH	890	νCC
873	νCC	848	rCH_2

The $\nu_{as}CH_2$ deformation band observed at 1426 cm^{-1} and the $\nu_{as}CH_3$ deformation band located at 1455 cm^{-1} in the Raman spectra (dotted lines) allow differentiation of copolymers with different compositions in lactate and glycolate units. The variations of the intensity ratio for the Raman lines change as a function of content in glycolate units and are in agreement with the expectations.

The νCC stretching modes appear as sharp Raman lines of high intensity. Raman spectra of the PLA and PLGA copolymers show a strong and sharp line at 873 cm^{-1} (dotted lines) typical for the lactate units, and well identified shoulders at 848 cm^{-1} and at 890 cm^{-1} (dotted lines) due to the glycolate units. The intensity ratio is different for the various proportions in lactate and glycolate units in the polymer. This again allows the determination of the proportion of monomers within the polymer chains.

Raman spectroscopy was also applied for qualitative and semi-quantitative characterization of the drug incorporated within the different polymeric matrices. Four Raman spectra are represented in Figure 7.14. The spectrum of the amorphous configuration of the active ingredient NOA449851 is represented as curve (a). The amorphous configuration of the active ingredient was confirmed with X-ray analysis in Chapter 2, Section 3.4. Curves (b) and (c) represent spectra of PLA-microspheres with a drug loading of 50 % and 16.67 %, respectively (batches PLA-(1:1) and PLA-(5:1)). The spectrum of the PLA placebo microspheres is represented as curve (d). The normation of the three spectra of the microspheres was made in reference to the νCC band at 890 cm^{-1}.

Analogue representations of the four Raman spectra for the microspheres with polymer matrices 85/15 PLGA and 75/25 PLGA are shown in Figures 7.15 and 7.16, respectively.

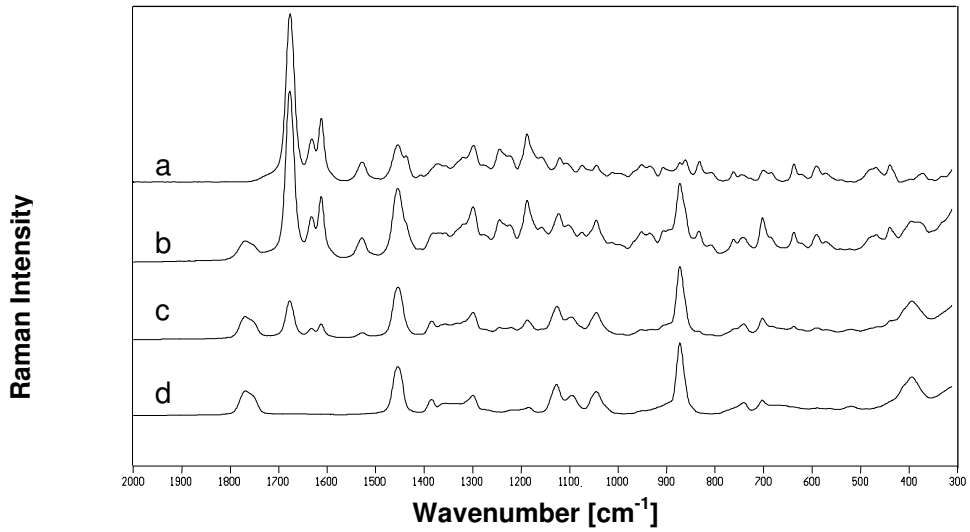


Figure 7.14: Raman spectra of amorphous drug substance and PLA-microspheres loaded with drug substance plotted in the region between 300 cm^{-1} and 2000 cm^{-1}: amorphous active ingredient NOA449851 (a), PLA-(1:1) (b), PLA-(5:1) (c) and PLA-placebo (d).

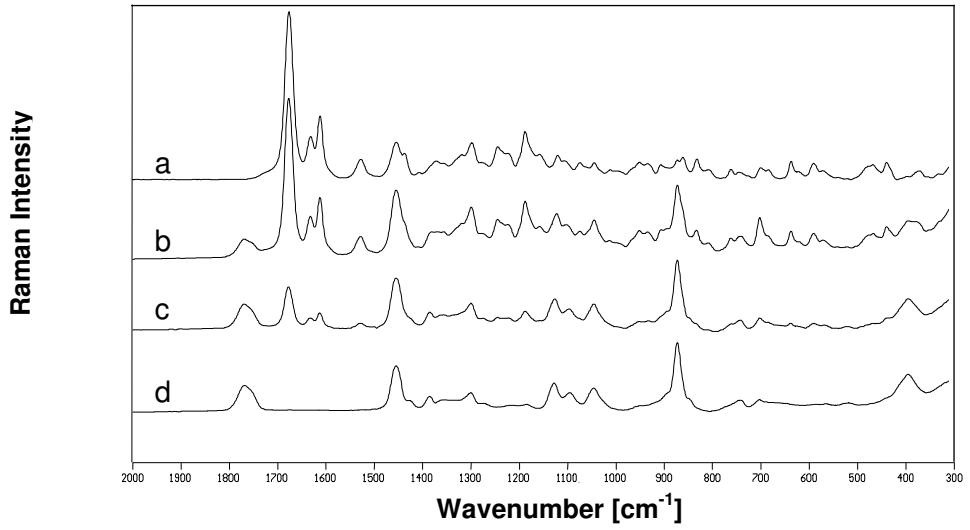


Figure 7.15: Raman spectra of amorphous drug substance and 85/15 PLGA microspheres loaded with drug substance plotted in the region between 300 cm^{-1} and 2000 cm^{-1}: amorphous active ingredient NOA449851 (a), 85/15 PLGA-(1:1) (b), 85/15 PLGA-(5:1) (c) and 85/15 PLGA-placebo (d).

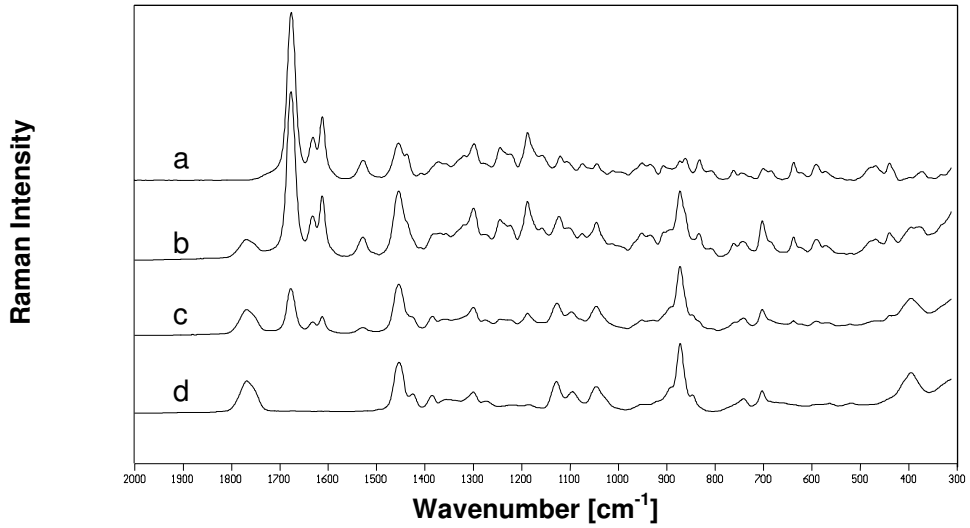


Figure 7.16: Raman spectra of amorphous drug substance and 75/25 PLGA microspheres loaded with drug substance plotted in the region between 300 cm^{-1} and 2000 cm^{-1}: amorphous active ingredient NOA449851 (a), 75/25 PLGA-(1:1) (b), 75/25 PLGA-(5:1) (c) and 75/25 PLGA-placebo (d).

In Figures 7.14 to 7.16, the curves (b) and (c) represent the spectra of NOA449851 loaded microspheres with the polymer to drug ratios 1:1 and 5:1, respectively. They logically contained both drug and polymer bands whereas the intensity of the bands reduced with the relative concentration of the components. The spectra did not reveal any noticeable change as compared with their respective model Raman patterns (curve (a) for the amorphous active ingredient and curve (d) for the placebo microspheres). Therefore, no indication either of structural modification of the active ingredient NOA449851 or PLA/PLGA matrix or of their interactions could be discerned in the studied wavelength range.

The band positions and shapes in the spectra indicate the absence of crystalline drug substance and the presence of an amorphous form of the active ingredient in each microspheres batch, independently of active ingredient concentration and of polymer matrix. As a control, the Raman patterns of the active ingredient NOA449851 were previously investigated in Chapter 2, Section 3.3. Figure 2.5 of Chapter 2 illustrates the relative Raman spectra of the active ingredient for a crystalline (curve a) and an amorphous (curve b) sample.

3.4. IR spectroscopy

The overlay plot of the IR spectra of the three microsphere batches with the polymer to drug ratio 1:1 is illustrated in Figure 7.17, plotted in the wavenumber region between 2500 cm^{-1} and 4000 cm^{-1}. As already investigated in Chapter 2, Section 3.3.1, the wavenumber region between 3100 cm^{-1} and 3700 cm^{-1} is typical for the –NH and –OH signals of the active ingredient and therefore very sensitive to the external environment, with which these chemical groups can build hydrogen-bonds. Figure 2.4 of Chapter 2 illustrates the changes in the wavenumber region between 2500 cm^{-1} and 4000 cm^{-1} of the IR spectra of the active ingredient, depending on the polarity of the solvent used, and therefore confirms the capability of the active ingredient NOA449851 to build hydrogen bonds with the external milieu.

IR spectra of the three microspheres batches with the polymer to drug ratio 1:1 were identical in this particular region (not shaded wavenumber region between 3100 cm^{-1} and 3700 cm^{-1}). This indicates that there were no detectable interaction of the nature of hydrogen bond in the micrsopheres between polymer matrix and active ingredient NOA449851, independent of the polymer type used.

The spectra variations at wavenumber 2997 cm^{-1} are specific for $\nu_{as}CH_3$ group of the lactic units in the matrix poylmers. These differences confirm that three different polymer types were used as matrix for the microspheres.

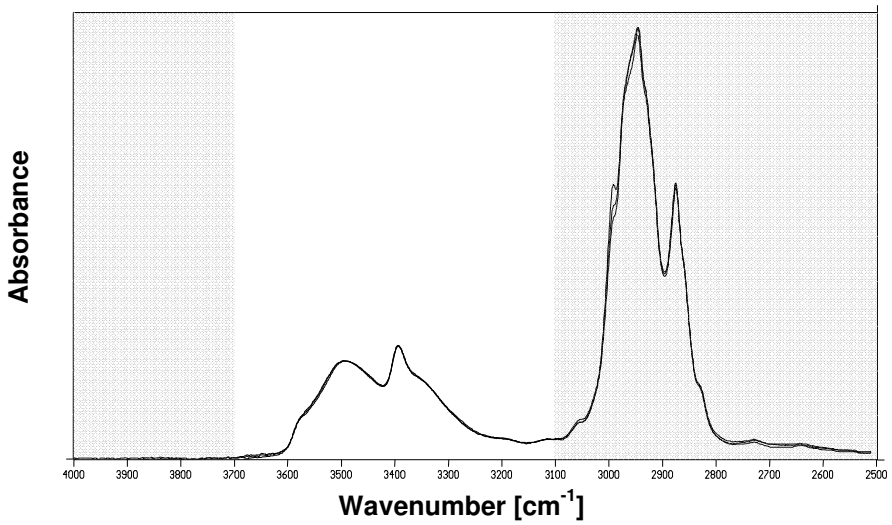


Figure 7.17: Overlay plot of IR spectra of the three microspheres batches with polymer to drug ratio 1:1, plotted in the wavenumber region between 2500 cm^{-1} and 4000 cm^{-1}.

3.5. X-ray analysis

Powder X-ray diffraction was carried out for the microsphere batch PLA-(1:1), directly after its manufacture. The diffractogram represented in Figure 7.18 shows a halo pattern. This indicates that both the active ingredient NOA449851 and the PLA polymer were in amorphous state in the microsphere batch PLA-(1:1).

As a control, diffraction patterns of active ingredient NOA449851 without polymer matrix were previously investigated in Chapter 2, Section 3.4. Figures 2.6 and 2.7 of Chapter 2 illustrate the relative diffractograms of the active ingredient for a crystalline and an amorphous sample, respectively.

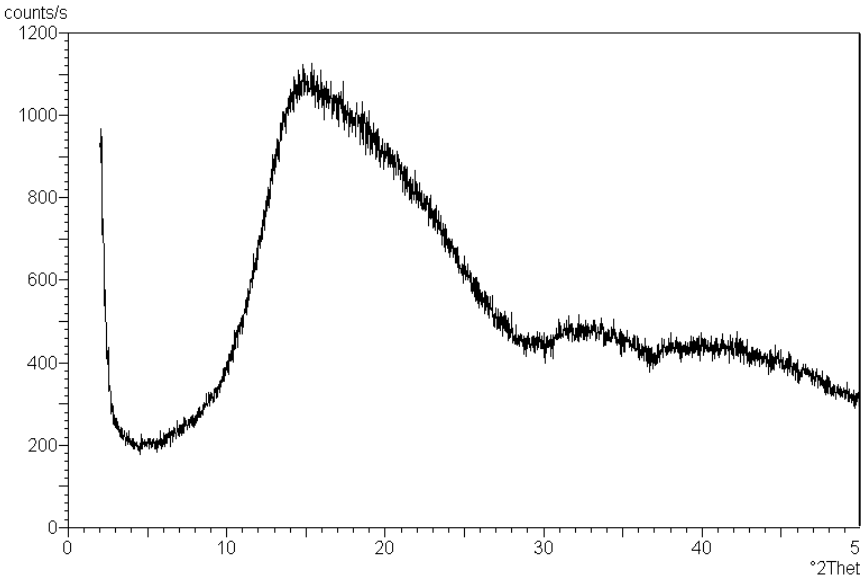


Figure 7.18: X-ray diffractogram of microsphere batch PLA-(1:1). The broad diffusion band indicates that both components of the microparticles were in the amorphous state.

3.6. In-vitro drug release from microspheres

In-vitro drug release profiles from microspheres were determined in triplicate using a modified USP basket dissolution test method. Release studies were performed over a period of 40 days, under the same test conditions as described for the sustained release injectables formed *in-situ* (Chapter 3, Section 2.2.2).

The release profiles of NOA449851 from the three microsphere batches based on different polymer types with the polymer to drug ratio 100:1 are represented in Figure 7.19. During the 40 days of dissolution testing, no active ingredient could be detected in the dissolution medium, keeping the cumulative released amount at 0 during the whole experiment for all three microparticle batches.

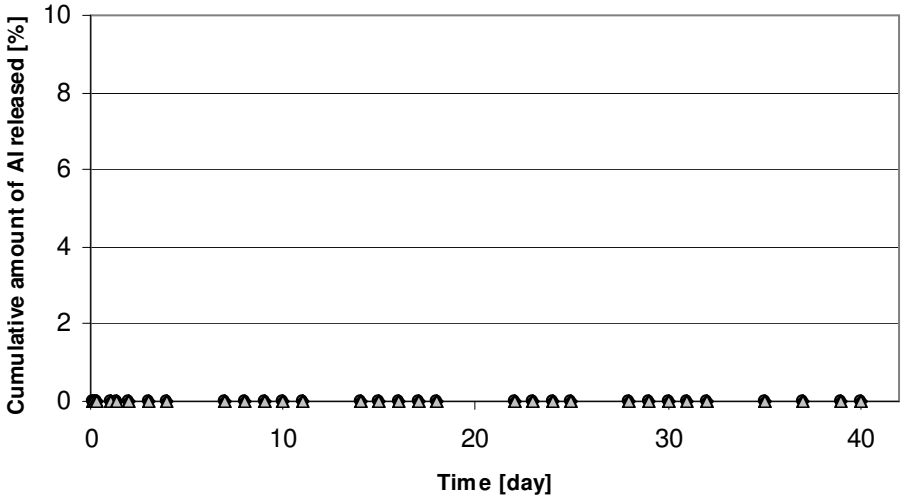


Figure 7.19: *In-vitro* cumulative release profiles of NOA449851 from PLA (O), 85/15 PLGA (▲) and 75/25 PLGA (◆) microspheres (n = 3) with a polymer to drug ratio 100:1.

The release profiles of NOA449851 from the three microsphere batches based on different polymer types with the polymer to drug ratio 5:1 are represented in Figure 7.20. The drug release profiles suggest a small and instantaneous burst of 1.02 % of the total amount of active ingredient for batch PLA-(5:1), of 1.21 % for batch 85/15 PLGA-(5:1) and of 1.91 % for batch 75/25 PLGA-(5:1), followed by a lag phase. At the end of the experiment, the total amount of active ingredient released in the medium was 1.04 % for the batch PLA-(5:1), 1.31 % for batch 85/15 PLGA-(5:1) and 2.23 % for batch 75/25 PLGA-(5:1).

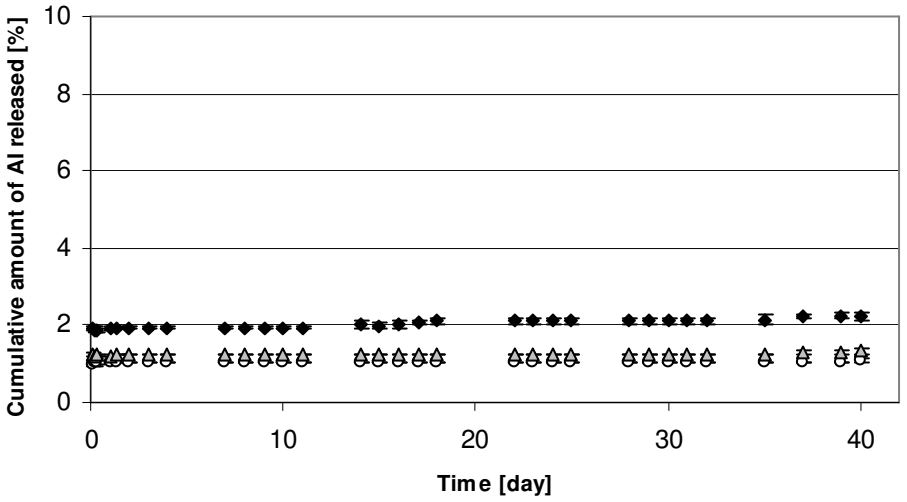


Figure 7.20: *In-vitro* cumulative release profiles of NOA449851 from PLA (O), 85/15 PLGA (▲) and 75/25 PLGA (◆) microspheres (n = 3) with a polymer to drug ratio 5:1.

The release profiles of NOA449851 from the three microsphere batches based on different polymer types with the polymer to drug ratio 1:1 are represented in Figure 7.21. The drug profiles indicate an initial burst release of 3.46 % of the total amount of active ingredient for batch PLA-(1:1), of 3.38 % for batch 85/15 PLGA-(1:1) and of 3.13 % for batch 75/25 PLGA-(1:1) during the first two days of dissolution testing. During the second phase of the profiles, very little active ingredient was released in the medium. After 40 days the total amount of active ingredient released from the microspheres was 3.87 % for batch PLA-(1:1), 3.56 % for batch 85/15 PLGA-(1:1) and 3.47 % for batch 75/25 PLGA-(1:1).

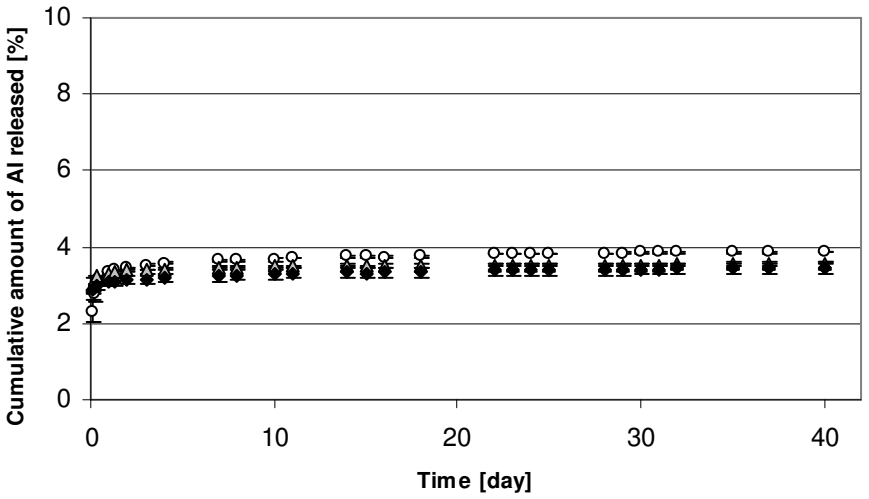


Figure 7.21: *In-vitro* cumulative release profiles of NOA449851 from PLA (O), 85/15 PLGA (▲) and 75/25 PLGA (◆) microspheres (n = 3) with a polymer to drug ratio 1:1.

4. Discussion

4.1. Preparation of microspheres and encapsulation efficiency

A conventional method for preparation of O/W emulsions [Conti et al., 1992; Jalil et al., 1990; Jain, 2000] was successfully applied to manufacture the twelve microsphere batches with a drug load up to 50 % (w/w). A loss of 10 % in weight is common for this manufacturing method [Jalil et al., 1990; Choi et al., 2002].

The active ingredient NOA449851 is a hydrophobic drug, highly soluble in methylene chloride which was the solvent for the PLA/PLGA polymers. The physicochemical properties of the active ingredient designated it as an ideal candidate for incorporation into microspheres by a O/W emulsion solvent evaporation method [Jalil et al., 1990; Conti et al., 1992; Jain, 2000]. The active ingredient NOA449851 was very efficiently entrapped in the PLA/PLGA microspheres: the encapsulation efficiency was 99.02 % for the microspheres with the polymer to drug ratio 100:1, 98.11 % for the microspheres with the polymer to drug ratio 5:1 and 94.65 % in average for the microspheres with the polymer to drug ratio 1:1. Independent of the polymer type used, the encapsulation efficiency slightly decreased when the amount of polymer was reduced (Tables 7.3 to 7.5). These observations are in agreement with the finding of Choi et al., who investigated microspheres loaded with the slightly water soluble drug fentanyl, as well as Schlicher et al., who investigated microspheres loaded with the water soluble drug desferrioxamine. Both reported that an increased polymer concentration in the microspheres composition resulted in an enhanced encapsulation efficiency [Choi et al., 2002; Schlicher et al., 1997].

4.2. Granulometry

Particle size of microspheres is an important formulation parameter that influences pharmaceutical properties of the particulate delivery system. Decreasing particles size leads to a large increase of total surface area per unit volume and therefore facilitates exchanges between polymer matrix and medium. Further, the drug release profile type depends strongly on microparticle diameter. The release of small molecule drugs can be varied from typical diffusion controlled profiles to slower sigmoid profiles as microsphere diameter is increased in the range of 10-100 µm [Berkland et al., 2002; Siepmann et al., 2004].

Analysis of particle sizes revealed a median size of 86.62 µm for microspheres based on PLA polymers, of 100.93 µm for 85/15 PLGA polymers and of 110.50 µm for 75/25 PLGA polymers. The median diameter size changed with regard to the type of polymer used: the higher the glycolate units in the polymer matrix, the larger the microspheres size. This can probably be attributed to the different surface tensions of the polymer emulsions. A change in the polymer to drug ratios from placebo to 100:1, 5:1 or 1:1 did not affect the median particle size. In a similar experimental set up, Mandal et al. found that microparticles formulated with 50/50 PLGA polymer and the water soluble active ingredient pentamidine had similar median particles size in all manufactured batches, independent of the polymer to drug ratio used. However, these authors did only vary the polymer to drug ratio in a tight range from 2:1 to 8:1 [Mandal et al., 2001].

After immersion in an aqueous medium for 40 days at 37°C for the dissolution tests, a decrease of the median particle size of 0.9 % in average for the microspheres batches with the polymer to drug ratio 100:1, of 1.4 % in average for the microspheres batches with the polymer to drug ratio 5:1 and of 5.1 % in average for the microspheres batches with the weight polymer to drug ratio 1:1 was observed. Surprisingly, the decrease in diameter was independent of the polymer type. PLA and PLGA polymers degrade via random chain scission of ester linkage in the polymer backbone. The extent of hydrolytic degradation depends on many physicochemical characteristics of the polymers, including molecular weight, proportion of monomer units and hydrophilicity.

It can be hypothesized that no significant erosion of the polymers could occur during the dissolution test and therefore no differences were observed between the polymer types. The diminution of the median particle size may be attributed to the dissolution of the active ingredient from the surface of the microspheres. The diameter reduction of the microspheres can be correlated with the magnitude of the initial burst release observed during the *in-vitro* dissolution tests.

4.3. Spectroscopy methods

IR and Raman spectroscopy are complementary methods for studying molecular vibrations. They both give information if the vibration is accompanied by a variation of electric dipole moment, however Raman scattering is only observed if a variation of molecular polarizability appears during the vibration [Dean, 1995]. Vibrational spectroscopy has been reported to be suitable for investigation of polymeric materials used in biomedical applications, particularly for the analysis of morphology, chemical composition and configurational structure of the homopolymers and copolymers based on poly(lactic acid), poly(glycolic acid) and poly(ε-caprolactone) [Kister et al.,1997, 1998 (1), (2), 2000; Qin et al., 1998; Taddei et al., 2001].

The various polymer types can be differentiated with Raman spectroscopy. The distinction of PLA/PLGA copolymers with various compositions in monomer units was possible considering the CH and CC stretching regions of the Raman spectra of the placebo microspheres [Kister et al., 1997; Kister et al., 1998 (1), (2)]. Assignments of these bands with localization of the corresponding vibrations at lactate or glycolate units were proposed in Table 7.7. The specific bands for distinguishing the different copolymers were located at the wavenumbers 1455 cm^{-1}, 1426 cm^{-1}, 890 cm^{-1}, 873 cm^{-1} and 848 cm^{-1} [Kister et al., 1998 (1), (2)].

Characterization of drugs within a polymer support is an important prerequisite to understand physicochemical mechanisms which control the release of drugs from a biomedical polymer. Raman spectroscopy has been used for investigation of various drugs embedded in polymer extrudates such as sulfathiazole in povidone [Bolton et al., 1984], diclofenac in sodium alginate [Tudor et al., 1990; Davies et al., 1990], ibuprofen in PVP [Breitenbach et al., 1999] or indomethacin in PVP [Taylor et al., 1997]. Also, drugs embedded in polymer matrices formulated as microspheres were investigated using Raman spectroscopy [Watts et al., 1991; Roman et al., 1999; Geze et al., 1999].

The physicochemical properties of the embedded active ingredient helps to determine the nature of the release process and the rate of diffusion of the drug into the environment. Several factors are considered to contribute to the increase in release rates of drugs including the greater solubility of the amorphous drug relative to the crystalline material as well as the absence of drug interaction with the polymeric matrix. In the spectroscopic investigations of the sulfasalazine containing microspheres described by Watts [Watts et al.,

1991], the authors presume a drug/polymer interaction, as a shift of an active ingredient band was detected in Raman spectra.

IR and Raman spectroscopy were used to characterize microspheres produced from pharmaceutical PLA/PLGA polymers containing a range of concentrations of the drug NOA449851 from 0 % to 50 %. The qualitative information included the analysis of the spectral shape, the band position and their relative intensity. This allowed the molecular characterization of the samples and therefore detection of probable structural changes of the drug or of the polymer matrix induced by interaction.

The Raman spectra bands of the active ingredient NOA449851 can clearly be distinguished from the PLA/PLGA polymer bands in these two-components systems. The Raman spectra show that the active ingredient NOA449851 had always the same conformation when integrated in microspheres, independent of active ingredient concentration and polymer type. As previously investigated with Raman spectroscopy (Chapter 2, Section 3.3) the major change in the active ingredient spectra between the crystalline and the amorphous conformation was illustrated with the shift of the band from 1520 cm^{-1} to 1528 cm^{-1}. As previously determined in Chapter 2, Section 3.2, this band corresponds to the amid II and the benzene ring of the side chain of the active ingredient molecule. Therefore it can be concluded that the active ingredient was always in the amorphous conformation when integrated into the microspheres. These results are in agreement with the predictions of Taylor who assumes that drugs are most of the time present in the amorphous conformation when embedded in a polymeric matrix [Taylor et al., 1997]. However, this was not the case for the microspheres studied by Mandal et al., where the active ingredient pentamidine was found to be mostly present in the crystalline form in the PLGA microparticles [Mandal et al., 2001].

No changes were detected for any band related to the different copolymers, independent of presence of the active ingredient. After investigation of all Raman spectra, there was no evidence for any drug/polymer interaction. Despite the capability of the active ingredient to form hydrogen bonds with external medium (Figure 2.4, Chapter 2), the substance does not interact with the polymeric matrix, most probably because of superior thermodynamic stability.

As already investigated in Chapter 2, Section 3.5, the active ingredient NOA449851 showed strong modifications in the IR spectra, which depend on the external solvent medium, particularly concerning the wavenumber region between 3100 cm^{-1} and 3700 cm^{-1} of the IR

spectra, where the hydrogen bonding of -OH and -NH groups with other molecules can easily be detected. This implies that the active ingredient NOA449851 is able to form H-bonds. (Chapter 2, Figure 2.4). The IR spectra of the three microsphere batches with the drug to polymer ratio 1:1 showed identical spectra for the active ingredient in this region (between 3100 cm^{-1} and 3700 cm^{-1}), indicating no detectable interaction with the surrounding polymers (Figure 7.17).

With both Raman and IR spectroscopy, no evidence for any drug/polymer interaction in microspheres was found. If there was interaction between the drug and the polymer matrix drug release and stability would be influenced. A significant polymer/drug interaction could give rise to a decreased release rate and possibly stabilization of an amorphous state [Sinha et al., 2003].

4.4. X-ray

X-ray measurement of microsphere batch PLA-(1:1) permitted to determine that both active ingredient and PLA polymer of the measured batch are in an amorphous conformation. This is in agreement with the finding of the Raman spectroscopy investigations of this chapter. The cross-comparison of Raman measurements confirmed that the active ingredient was in amorphous conformation in all microsphere batches, independent on drug content and polymer type.

4.5. *In-vitro* drug release from microspheres

The drug release profiles from microspheres was determined using a modified USP basket method over a period of 40 days. No active ingredient could be detected in the medium during the dissolution test of microspheres batches with the polymer to drug ratio 100:1, independently of the polymer used. For microsphere batches with the polymer to drug ratio 5:1 and 1:1, the observed drug release occurred in two distinct phases: first, a small initial burst release in a range of 1 % to 5 % occurred, followed by a second phase characterized by a negligible release until the end of the experiment after 40 days. The initial burst release was slightly more important for microspheres containing a higher amount of active ingredient. The use of three different polymer types for the microspheres matrix did not affect drug release profiles. The total amount of drug released was found to be particularly low for all microsphere batches tested. Microspheres with the polymer to drug ratio 1:1 showed a

higher cumulative percent drug release (from 3.47 % to 3.87 %) compared to microspheres with the polymer to drug ratio 5:1 (from 1.07 % to 2.22 %) within the 40 days of the experiment.

Release mechanisms of microspheres have been widely studied and can be summarized in four main processes: release from the microspheres surface, diffusion through the polymer barrier, polymer erosion and bulk degradation. Particularly the two last mechanisms are affected by the rate of hydrolysis of polymer chains [Jalil et al., 1990]. As a consequence for these various release mechanisms, a typical triphasic release profile for lipophilic drug has been reported in the literature. This effect has been observed for microspheres with low drug loads, i.e., from 1 % to 5 % [Puri et al., 2000; Wang et al., 1996; Cleland et al., 1997], as well as for microspheres with higher drug load, i.e. from 16 to 25 % [Singh et al., 1997 (2)]. The second phase has also been reported to vary in a lag time from one week [Singh et al., 1997 (2)] to six weeks [Hampl et al., 1996], depending on the polymer matrix type and on the physicochemical characteristics of the active ingredient. Diverse authors have further discussed parameters influencing the triphasic release such as diameter of microparticles, influence of additives and porosity of the matrix [Berkland et al., 2002; Hora et al., 1990; Cleland, 1998].

The triphasic release profile is characterized by an initial burst followed by a lag phase and finally a secondary burst [Singh et al., 1997 (3)]. The initial burst is caused by the release of drug that is loosely bound to the surface or embedded in a superficial region of the microspheres [Choi et al., 2002; Berkland et al., 2002; Shah et al., 1992]. The lag period may be due to the medium being diffused into the polymer matrix. The second burst effect occurs when the matrix becomes more water soluble which results in erosion and collapse of the matrix. Initial burst release followed by a period of no or low drug release from PLA/PLGA matrices is a major problem for the microsphere technology. In the present case, only the two first phases were observed, since the experiments were stopped after 40 days.

As observed in Figures 7.19 to 7.21, the release patterns of the active ingredient from the microspheres were not affected by the polymer type. Generally, the various properties of the polymeric drug carrier are used to control the drug release. As widely described in the literature [Middleton et al., 2000; Wang et al., 1997; Sinha et al., 1998], the different polymers are characterized by various erosion rates in an aqueous medium. After evaluation of the release properties and of the granulometry data, it was concluded that not the polymer types but much rather the ratio of polymer to drug influenced the drug release properties.

The very low active ingredient amount released from the microspheres was very surprising. With an active ingredient concentration up to 50 % (w/w), a significant faster drug release was expected. The hydrophobic characteristics of the active ingredient NOA449851 may have prevented penetration of water molecules of the dissolution medium into the polymeric microsphere matrix. This would have resulted in a reduced rate of hydrolysis of the ester bounds of the polymer and consequently in a delay of the secondary burst of the triphasic drug release profile. The polymer bulk may therefore need more than the 40 days of the duration of the dissolution test for significant hydrolytic degradation. However, reduction of the lipophilic active ingredient concentration in the microspheres from 50.00 % to 16.67 % or even 0.99 % did not increase the relative drug release. This can be attributed to the concomitant reduction of the concentration gradient which is the major driving force for the assumed primarily drug release mechanism, the passive diffusion of the drug molecules through the polymeric matrix.

The microsphere batches as investigated in the present experiment are not suitable as injectable sustained release technology for this active ingredient. The release profiles did not fulfill the required drug release characteristics for a sustained release parenteral drug delivery system. Changes in composition or in manufacturing method of the microspheres are necessary to improve the drug release rate. The formulation variables include decrease of polymer amount, use of lower molecular weight polymers, increase of porosity of polymer matrix, decrease of diameter, addition of releasing modifying factors such as manitol or fatty acid esters [De Rosa et al., 2000; Urata et al., 1999; Mandal et al., 2001; Jain et al., 2000 (2); Dalpiaz et al., 2002; Conti et al, 1992]. As this technology was primarily investigated for comparison with the sustained release injectable formed *in-situ* and for detection of drug/polymer interaction, no further optimization of the microsphere formulation was planed. Comparison of drug release from microparticles and from the related sustained release injectables formed *in-situ* will be discussed in Chapter 8.

5. Conclusion

Microspheres were evaluated as an alternative technology for sustained release injectable dosage form for the active ingredient NOA449851. Microspheres were manufactured with the same types of polymer as for the sustained release injectables formed *in-situ*. Attention was paid for keeping the same polymer to drug ratios as for the injectable implants. The lipophilic active ingredient NOA449851 was very efficiently incorporated in the microspheres, when prepared with a conventional O/W solvent evaporation method.

No active ingredient was detected in the external medium during the dissolution test of the microparticles with a polymer to drug ratio 100:1. For microspheres with weight polymer to drug ratios of 5:1 and 1:1, the *in-vitro* drug releases were between 1 % and 5 % during the first two days, probably corresponding to the drug loosely bound at the surface of the microspheres. After this small initial burst, drug releases were close to zero, independent of polymer type and of the polymer to drug ratio of the microspheres. These *in-vitro* drug release profiles, combined with granulometry data, indicate that, most probably, the mechanism for active ingredient release from the polymeric matrices was primarily diffusion controlled. Polymer erosion appeared not to play an important role in active ingredient release from the microspheres during the 40 days of the *in-vitro* dissolution testing. Drug release profiles from the microparticles investigated in this chapter will be compared in Chapter 8 with the ones from the related sustained release injectables formed *in-situ* as investigated in Chapter 3.

Raman and IR spectroscopy as well as X-ray investigations revealed that the active ingredient was incorporated in amorphous conformation in all microsphere batches, independent of the polymer type and of the active ingredient concentration. No evidence for any interaction between active ingredient and polymer matrix could be detected. The microspheres were used as a simplified two-components model, lacking the solvent in comparison to the sustained release injectables formed *in-situ*.

The NOA449851 loaded microspheres did not meet the drug release requirements for a sustained release delivery system. The release profiles could probably be improved by changing the composition of the microspheres or by modifying the method of preparation. As this technology was primarily investigated for comparison with the sustained release injectable formed *in-situ* and for detection of drug/polymer interaction, no further optimization of the microsphere formulations was performed.

Chapter 8

General Discussion and Outlook

1. Introduction

The aim of this PhD thesis was to investigate properties of sustained release injectables formed *in-situ* for use in dogs, comprising an organic solvent or solvents mixture in which the biodegradable PLA/PLGA polymers and the lipophilic anti-infective NOA449851 were dissolved. Dissolution tests were performed to investigate *in-vitro* drug release characteristics of various formulations. Tolerability and pharmacokinetic properties were explored after subcutaneous administration to experimental animals. Additionally, stability of formulations at different storage conditions over a period of six months was investigated and a comparison with microspheres technology was made with regard to manufacturing method and *in-vitro* release properties.

The new sustained release injectables formed *in-situ* showed a prolonged active ingredient release under *in-vitro* as well as *in-vivo* conditions, confirming that this drug delivery technology is suitable to achieve a controlled release of the selected active ingredient NOA449851. The technology used has several attractive features such as the simplicity of concept, ease of manufacturing as well as the use of FDA approved polymers. However, the limitations of this technology include the use of organic solvents, its high viscosity and its release variability, particularly with respect to the initial burst. These difficulties need to be addressed in the future in more detail, in order to allow a safe use of this injectable technology in the market.

In the next section, the findings of the previous chapters are re-discussed in relation with each other. In particular, the correlation of *in-vitro* and *in-vivo* active ingredient release parameters as well as comparison between *in-situ* forming implant technology and microparticles technology are discussed.

Finally, the principal critical issues related to the use of this new technology are pointed out, especially with regard to the adaptations still needed to make this studied *in-situ* forming implant suitable for market. To achieve a marketable product accepted by regulatory authorities and end-users, additional investigations and improvements have to be performed. The main concerns are the high viscosity and the consequently inferior syringeability of formulation, the choice of an adequate active ingredient as well as the stability of biodegradable polymers during storage of formulation.

2. Relationship between the findings of the various chapters

2.1. Comparison of the *in-vitro* and the *in-vivo* drug release profiles

Drug release properties from *in-situ* forming implants were already discussed in Chapter 5 which was especially dedicated to *in-vitro/in-vivo* correlation of drug release. The comparison is, logically, only possible for formulations which were tested *in-vitro* as well as *in-vivo*. This concerned six formulations, varying in polymer content and in solvent composition. Particularly the influence of the presence of hydrophilic co-solvents such as glycerol formal, anhydrous glycerol and ethanol absolute in the formulation on the drug release characteristics was investigated.

The *in-vitro* dissolution tests reported in Chapter 3 showed a significant reduction of initial burst when concentration of PLA polymer was increased in the composition of the formulation as well as when a fraction of the main solvent triacetin was substituted by hydrophilic co-solvents. During the first week of the *in-vitro* dissolution test, 60.13 % of the active ingredient integrated in the formulation was released from the sustained release injectable formed *in-situ* containing 12.75 % (w/w) of PLA polymers while only 17.57 % of the active ingredient was released from the formulation containing 17.00 % (w/w) of the PLA polymers during the same period of time (Chapter 3, Figure 3.4). Substitution of 10.00 % of the main solvent triacetin reduced initial drug release during the first week of the *in-vitro* dissolution test from 17.57 % to 15.86 %, to 11.41 % and to 9.82 % by using the hydrophilic

co-solvents glycerol formal, anhydrous glycerol and ethanol absolute, respectively (Chapter 3, Figures 3.8 to 3.10).

The formulations applied subcutaneously to the dogs, as described in Chapter 4, tend to act in an analog manner. Reduction of polymer content in the formulations gave rise to a higher initial burst and use of the various hydrophilic co-solvents reduced maximum drug burst concentration of the dog blood profiles. Maximal blood concentrations were 89.2 ng/ml and 87.6 ng/ml for the dogs to which formulation containing 17.00 % of the PLA polymer was applied (Chapter 4, Figure 4.5). By reducing the concentration of biodegradable polymer to 12.75 %, higher maximal blood concentrations of 92.7 ng/ml and 117.2 ng/ml were reached (Chapter 4, Figure 4.6). In contrast, lower maximal blood concentrations were obtained after subcutaneous injection of formulations containing the hydrophilic co-solvents ethanol absolute (44.2 ng/ml and 47.5 ng/ml), glycerol formal (35.6 ng/ml and 37.4 ng/ml) or anhydrous glycerol (35.0 ng/ml and 40.6 ng/ml) in comparison to the formulation containing triacetin as sole solvent (89.2 ng/ml and 87.6 ng/ml), (Chapter 4, Figures 4.7, 4.9 and 4.10).

To evaluate how far a correlation between the *in-vitro* drug release and the *in-vivo* release could be found, further investigations were carried out. Correlation between *in-vitro* dissolution parameters and *in-vivo* pharmacokinetic data was investigated. C_{max} was positively correlated to cumulative *in-vitro* drug release at T_{max}, however not in a significant manner. In general, for this type of dosage form and drug, no satisfactory IVIVC are observed. The model used for *in-vitro* drug release testing neglect some crucial aspects of physiological conditions and cannot replace biological systems.

2.2. Comparison of the properties of sustained release injectables formed *in-situ* with microspheres

Sustained release injectables formed *in-situ* and microspheres are both technologies intended for parenteral application, planned to achieve a sustained drug release. In both technologies, sustained effect is caused by biodegradable PLA/PLGA polymer matrix in which the active ingredient is embedded. Active ingredient release is controlled by a mixed process of drug diffusion and polymer matrix erosion. However, both technologies differ considerably in manufacturing procedures and in *in-vitro* release properties.

2.2.1. Manufacturing procedure

Manufacturing procedure for microspheres is much more elaborate as for sustained release injectables formed *in-situ*. A wide range of microencapsulation techniques have been developed to date. The selection of a specific technique is dependent on polymer type and on physico-chemical properties of the active ingredient. In the present study, an O/W emulsion solvent evaporation method was used as a simple and common preparation process for polylactid microspheres loaded with a lipophilic active ingredient [Conti et al., 1992]. However, this manufacturing procedure includes preparation of an O/W emulsion, followed by evaporation phase to remove the organic solvent phase in which the polymers are dissolved. A filtration is then needed to recover the solid microspheres and finally a drying step is required to remove solvent traces from the PLA/PLGA polymer particles. These different steps are time consuming and require the utilization of excess of organic solvents. Furthermore, the scaling up is a delicate procedure and a good batch to batch reproducibility is difficult to achieve, particularly with regard to parameters such as encapsulation efficiency, diameter of the particles, porosity and removal of residual solvent. For these considerations, it can be concluded that microspheres manufacturing procedures are very sensitive and challenging [Jalil et al., 1990; Conti et al., 1992; Cleland, 1998].

Sustained release injectables formed *in-situ* are prepared by using much more straightforward methods. Polymers are simply dissolved in the adequate amount of solvent and active ingredient is then dispersed or dissolved in this polymer solution. In the present study, the active ingredient NOA449851 was dissolved in the polymer solution. This manufacturing process though requires stirring equipments able to deal with the high viscosity of the polymer solutions. Filling process into the syringes needs also powerful pumps able to cope with viscous formulations.

2.2.2. *In-vitro* drug release

In-vitro drug release profiles of the different microspheres batches can be compared with the ones of related sustained release injectables formed *in-situ*. Formulations *"PLA-AI(1:1)", "85/15 PLGA-AI(1:1)"* and *"75/25 PLGA-AI(1:1)"* are related to the three microspheres batches having a weight polymer to drug ratio 1:1, formulations *"PLA-AI(5:1)"*, *"85/15 PLGA-AI(5:1)" and "75/25 PLGA-AI(5:1)"* to the three microspheres batches with the weight polymer to drug ratio 5:1 while formulations *"PLA-AI(100:1)"*, *"85/15 PLGA-AI(100:1)" and "75/25 PLGA-AI(100:1)"* to the three microspheres batches with the weight polymer to drug ratio 100:1. Compositions and the *in-vitro* dissolution profiles of sustained release injectables formed *in-situ* formulations are described in Chapter 3 whereas compositions and *in-vitro* dissolution profiles of microspheres are described in Chapter 7.

Microspheres are small in size (from about 10 µm to 100 µm in diameter) and therefore have large surface to volume ratios. The high surface area of the microspheres facilitate water penetration into the particles and at the same time allow a faster diffusion of the active ingredient in the medium. Whereas, implants arisen from the sustained release injectables formed *in-situ* generate a single bulk matrix with little surface to volume ratio.

Surprisingly, in every investigated case drug release from sustained release injectables formed *in-situ* was faster than from the related microparticles. *In-vitro* drug release from microspheres with weight polymer to drug ratio 1:1 was in a range between 3.47 % and 3.87 % of the total amount of active ingredient integrated after 40 days of dissolution test, whereby it was between 39.08 % and 42.64 % for the related sustained release injectables formed *in-situ* with same weight polymer to drug ratio. For formulations with weight polymer to drug ratio 5:1, active ingredient release after 40 days of dissolution test represented between 1.04 % and 2.23 % of the drug integrated in the microspheres and between 14.75 % and 16.39 % of the drug integrated in related sustained release injectables formed *in-situ*. Drug release from sustained release injectables formed *in-situ* with polymer to drug ratio 100:1 was influenced by the polymer type used and reached 76.54 % of the active ingredient for 75/25 PLGA based formulation, 25.06 % for 85/15 PLGA based formulations and 21.69 % for PLA based formulation. In contrast, the amount of active ingredient released form the related microspheres with weight polymer to drug ratio 100:1 during the 40 days of the dissolution test was not sufficient to be detected by HPLC method, independently of the polymer type used.

In order to understand the faster release from sustained release injectable formed *in-situ* than from microspheres, it should be realized that diffusion of active ingredient through the polymer matrix plays a major role in drug release process. The polymer matrix of the microspheres is already solidified before injection in the aqueous medium. Therefore it presents other permeability properties for the active ingredient as polymer matrix of implants formed *in-situ* which is not immediately solidified, but coagulates slowly *in-situ* as result of the diffusion of the organic solvent in the surrounding milieu. The drug release related to the solidification phase of the implant and therefore concomitant to the organic solvent diffusion is one of the major releasing property of the sustained release injectables formed *in-situ*. Active ingredient diffusion out of the implant during coagulation phase of the polymeric matrix is responsible for the initial burst effect.

These observations are in agreement with those from R.A. Jain [Jain et al., 2000] who compared *in-vitro* release of cytochrom C from PLGA microspheres and from sustained release injectables formed *in-situ,* based on PLGA and triacetin. *In-situ* formed implant also exhibited faster drug release than related microspheres. The authors attributed this phenomenon to the rigid and dense nature of the microspheres matrix.

Microspheres and sustained release injectables formed *in-situ* with high drug loads (polymer to drug ratios of 1:1 and 5:1) exhibit drug release characteristics independent of poly-glycolic acid to poly-lactic acid ratios of the PLA/PLGA copolymers used to form the drug delivery device matrix (Chapter 3, Figures 3.3 and 3.4 as well as Chapter 7, Figures 7.19 to 7.21). This confirms the hypothesis that the major releasing mechanism for both technologies, microparticles and sustained release injectables formed *in-situ,* is drug diffusion through polymer matrix following the Fickian's diffusion theory and not polymer matrix degradation. Therefore, the active ingredient concentration gradient influence release rate process. Actually, for both technologies, a bigger fraction of active ingredient was released when drug loading was higher.

Drug loading of sustained release injectables formed *in-situ* can change release rate in various ways. The drug release rate may be increased at high drug loadings because of formation of large and interconnected pores throughout the matrix [Shah et al., 1993; Eliaz et al., 2000 (1), (2); Eliaz et al., 1997; Chandrashekar et al., 1996]. In contrast to this, an increased drug/drug interaction within the polymer matrix can occur at high active ingredient loading, leading to a lower release rate [Yewey et al., 1997].

Raman and IR spectroscopy investigations of sustained release injectables formed *in-situ* and of microspheres were performed to detect interactions between active ingredient and polymer matrix. In all investigated formulations, independently on active ingredient loading and on polymer type used to form the matrix, no evidence of any type of interaction between both components was detected (Chapter 3, Section 3.4 and Chapter 7, Sections 3.3 and 3.4). Additionally, conformation of the active ingredient within the polymer matrix was investigated using Raman spectroscopy as well as X-ray diffraction. The active ingredient, when embedded in the PLA/PLGA polymer matrix, was found to be in the amorphous state, independently of drug loading and of polymer type. The lack of interaction between active ingredient and polymer matrix as well as the amorphous state of the drug within polymer matrix are parameters which both increase drug release rate.

With these considerations, the faster release of active ingredient from highly drug loaded sustained release injectables formed *in-situ* is in agreement with the expectations. In the present experiment, no drug/drug interaction or polymer/drug interaction seem to slow down the active ingredient release from the different formulations. Additionally, diffusion rate is enhanced by a higher concentration gradient for higher drug loaded formulations and by probable formation of pores within the polymer matrix.

As already mentioned, the active ingredient release patterns from the sustained release injectables formed *in-situ* with weight polymer to drug ratio 1:1 and 5:1 as well as from the microspheres were not affected by the polymer type. The different properties of the polymeric drug carrier are generally used to control the drug release. As widely reported in the literature [Middleton et al., 2000; Wang et al., 1997; Sinha et al., 1998], the different PLA/PLGA copolymers are characterized by various hydrolysis rate when placed in an aqueous medium. The highly lipophilic character of the active ingredient NOA449851 may have prevented penetration of the water molecules into the polymeric matrix. This would have resulted in a reduced rate of hydrolysis of the ester bounds of the polymer and consequently in a low erosion of the polymeric matrix. All these considerations confirm that the active ingredient release mechanism is primarily based on drug diffusion through the polymeric matrix. Diffusion as main release mechanism from sustained release injectables formed *in-situ* has been suggested various times in the related publications [Lambert et al., 1995; Dunn et al., 1996; Singh et al., 1997; McHugh et al., 1999; Graham et al., 1999; DesNoyer et al., 2001; Eliaz et al., 2002].

However, drug release was dependent on the polymer type in case of sustained released injectables formed *in-situ* with very low drug load. After 20 days of *in-vitro* dissolution testing, drug release from 75/25 PLGA based formulation began to increase, whereas active ingredient release from implants based on 85/15 PLGA and PLA polymers remained constantly low (Chapter 3, Figure 3.5). At this stage, drug release from 75/25 PLGA formulation was mainly determined by polymer erosion, as hydrolysis of these polymers is known to occur faster than for 85/15 PLGA and pure PLA polymers [Middleton et al., 2000; Wang et al., 1997]. The important decrease lipophilic active ingredient content in the formulation (polymer to drug ratio 100:1) may have facilitated water permeation into polymer matrix in comparison to formulation with much higher drug contents, as for example with the polymer to drug ratios 5:1 or 1:1.

No active ingredient was released during the 40 days of the dissolution test from the three microparticles batches with weight polymer to drug ratio 100:1, as illustrated in Chapter 7, Figure 7.19. This may be explained by both the low concentration gradient and the difficulty of water to permeate into the densely solidified microparticles. As already mentioned above, the drying step of the manufacturing of the microparticles may result in denser packing of the polymer matrix which results in slower diffusion properties of the microspheres polymer matrix.

3. Critical issues of sustained release injectables formed *in-situ* technology

Some critical issues pointed out during investigations of sustained release injectables formed *in-situ* are discussed in the next section. This includes inconvenient syringeability due to high viscosity of formulations, selection criteria of an adequate active ingredient as well as stability of polymers during storage of injectable formulations. Finally, some comments on marketability of this technology and on further investigations which may be necessary to meet requirements from regulatory authorities are discussed.

3.1. Viscosity and syringeability of formulations

The high viscosity of sustained release injectables formed *in-situ* turned out to be a critical issue. The application of formulation through a 16 G or a 18 G needle was well tolerated by the dogs but would probably not be accepted by customers and veterinarians. Pain during injection procedure suffered by the companion animals is a pet-owner compliance and acceptance issue.

Viscosity of formulations was improved by reducing polymer or the active ingredient content. The viscosity of formulation containing 17.00 % of the PLA polymer and 17.00 % of the active ingredient NOA449851 was found to be 49.0 Pa*s at shear rates of 1 s^{-1}, while it was found to be 12.3 Pa*s for the formulation containing a reduced amount (12.75 %) of the PLA polymer at the same measurement conditions. Viscosity of related placebo formulations were found to be 7.2 Pa*s and 2.3 Pa*s, respectively (Chapter 4, Table 4.2). As an alternative to lower the viscosity, amount of solvent and therefore of injection volume could be increased. Moreover, the use of larger injection volumes may also result in more pain at the injection site and therefore can only solve the problem to a limited extent. Changes in the composition of the formulation must be carefully balanced as the drug release properties and particularly the initial burst effect can dramatically change with slight modifications of formulation. This has been reported for the *in-vitro* dissolution tests with the reduction of the polymer amount from 17.00 % to 12.75 % (Chapter 3, Figure 3.6).

Use of co-solvents modified viscosity and syringeability of the sustained release injectables formed *in-situ*. Viscosity of formulation was increased with substitution of 10.00 % of the main solvent triacetin with co-solvent anhydrous glycerol, whereby it was lowered with glycerol formal and ethanol absolute as co-solvent. For shear rate of 1 s^{-1}, the viscosity of the

anhydrous glycerol containing formulation was 118 Pa*s, while it was 49.0 Pa*s for the formulation containing triacetin as sole solvent. The addition of the co-solvents glycerol formal and ethanol absolute decreased the viscosity of formulations from 49.0 Pa*s to 30.3 Pa*s and 5.5 Pa*s, respectively. Co-solvents can be used as release modifying agents, since initial burst was clearly influenced by the solvent mixture composition. As investigated *in-vitro* (Chapter 3, Figures 3.8 to 3.10) and *in-vivo* (Chapter 4, Figures 4.7, 4.9 and 4.10), it was found that addition of hydrophilic co-solvents to the triacetin composition of the formulation reduced the magnitude of initial burst. This positive impact of use of co-solvents is desired since an important initial burst can lead to systemic side effects and to shorter release of the drug. As ethanol absolute was the excipient which reduced the most considerably viscosity and simultaneously lowered the initial burst effect, it was selected as co-solvent for a candidate formulation for stability testing (Chapter 7).

To reduce efficiently the viscosity and therefore improve syringeability of formulation, an injectable containing simultaneously a reduced amount of PLA polymer as well as 10.00 % of the co-solvent ethanol absolute was prepared. The viscosity of 2.2 Pa*s was found to be lower than formulations containing only the co-solvent ethanol absolute (5.5 Pa*s) or only the reduced amount of polymer (12.3 Pa*s) as related in Chapter 4, Table 4.2. The *in-vitro* release profile as illustrated in Chapter 3, Figure 3.11 had a “b/a” factor of 129.7 and was therefore qualified as very bad. It showed an important initial burst representing a release of 31 % of the total active ingredient during the first 7 days, followed by a slow continued release of only 0.3 % of the total integrated drug substance per day. Surprisingly, *in-vivo* blood profile of this formulation showed a satisfactory release of the active ingredient. Maximal concentrations reached values of 61.2 ng/ml and 48.9 ng/ml, which are lower than the maximal concentrations of 92.7 ng/ml and 117.2 ng/ml obtained after the application of the corresponding formulation without the hydrophilic co-solvent ethanol absolute. It is probably disadvantageous to lower the active ingredient concentration in the formulation to improve the syringeability of the formulation, because of the consequently reduced efficacy and duration of the treatment.

3.2. Requirements for active ingredient

The therapeutic agents suitable for sustained release injectable drug delivery systems should need to be administered for long period of time, be highly potent and have compliance issues. In addition, the resulting controlled release formulation with the active ingredient in question should have an attractive benefit/cost ratio for the customer. In order to reduce the risk that a possible significant initial burst cause prohibitive side effects, the active ingredients should have broad therapeutic index. Finally, the active ingredient should be sufficiently stable at 37°C during the envisaged depot injection release period, to allow a guaranteed and reproducible release form the injected depot formulation.

The active ingredient selected for the present study, the anti-infective NOA449851, was dissolved in the polymer solution and showed good stability during storage of the injectable formulations at different temperatures (Chapter 7). It generated no toxic effect at the injection site as well as no systemic effects during the whole duration of the *in-vivo* trials (Chapter 4). In addition, its lipophilic character prevented a too fast diffusion from the lipophilic PLA polymer matrix.

However, the selected active ingredient was not an ideal candidate for such a technology. The high drug loading of 17.00 % was necessary to assure sufficient blood level for about six months. With a more potent active ingredient, its required content would decrease and consequently polymer concentration could be reduced, leading to a lower viscosity and a better syringeability of formulation. Therefore, it can be assumed that high potent and save active ingredients such as proteins, vaccines or chemotherapeutics would be more adequate for this technology.

3.3. Stability of PLA polymers

The use of biodegradable polymers alleviates the need for surgical removal of the implant after termination of the therapy, thereby increasing patient acceptance and compliance. Therefore, to reach biodegradation, chemical properties of polymers should allow hydrolysis of their ester-bonding at physiological conditions. During storage, the stability of these polymers, especially when dissolved in organic solvents, are consequently relatively poor. Degradation rate of the PLA polymers was investigated in Chapter 7 by measuring their apparent molecular weight during six months storage. It was found that hydrolysis process and therefore stability of the formulation was strongly influenced by the storage temperature. Polymers showed better stability at a storage temperature of 5°C in contrast to storage

conditions tested such as 25°C and 40°C. Decrease in molecular weight for PLA polymers was smaller than 20 % of the initial value during the six months storage at 5°C, while it reached about 50 % for storage temperature of 25°C and even 90 % for storage temperature of 40°C in the same period of time. Therefore, storage conditions at 5°C is indispensable for maintaining the molecular weight of the polymer relatively stable. Although these storage conditions are not optimal, especially for the veterinarian use, it should be realized that the use of more sophisticated release principles most of the time require more demanding storage conditions allowing the use of these products.

3.4. Marketability

Chronic oral administration of tablets to companion animals is particularly challenging and therefore there is a continuing need for alternative options like long acting injections and implants, with a desired duration of release varying from two weeks to two years [Ahmed et al., 2002]. One limitation of these newly developed drug delivery systems is that their development and sales cost to benefit ratio is too high which restricts their use over conventional dosage forms. Formulation and drug delivery considerations for companion animals are dictated by a set of circumstances such as ease of use to the pet-owner, pet compliance and dosing flexibility. Systemic safety and toleration of the animal are also important considerations along with owner safety and convenience. Therapy for companion animals is not usually as cost sensitive as for livestock.

To obtain approval from regulatory authorities, additional parameters must be tested. All of these factors can be severely detrimental to commercial success of the product. Parenteral delivery systems have to meet pharmacopoeial requirements with respect to sterility. The chemical instability of the polymeric matrix material limits the options for obtaining a guaranteed sterile product. Usual methods such as dry or moist heat sterilization methods cause an unacceptable degree of degradation and hydrolysis of the polymers. Toxicological problems due to residual amounts are encountered with the use of ethylene oxide. In the case of sustained release injectables formed *in-situ*, the best option for terminal sterilization is the use of gamma-irradiation. This method showed good efficacy as terminal sterilization method for PLA/PLGA polymer based drug delivery systems. However, γ-irradiation of saturated polyesters can result in simultaneous chain scission and crosslinking. As mentioned in the literature, there is an evidence of the decrease in weight average molecular weight of polymers after different dose of gamma-irradiation [Mohr et al., 1999; Montanari et al., 1998; Volland et al., 1994]. The active ingredient can also be sensitive to this sterilization

method. Therefore, sterilization conditions need to be carefully adjusted for the final dosage form.

As already extensively discussed, the high viscosity of the formulation leads to acceptance problems due to the required big needle diameter. Reduction of viscosity is limited by some factors including polymer concentration, active ingredient loading as well as maximal injectable volume. The use of a high pressure injector gun which permits the rapid subcutaneous application of such viscous formulations without demanding to much force to be applied by the veterinarian could eventually solve this problem.

Finally, one of the most crucial properties to judge whether a depot injectable is useful is the degree of obtained efficacy of the treatment. In the present study, pharmacokinetic parameters such as blood values were measured, but no efficacy tests were performed with artificial infection of the animals with the target parasite.

3. Conclusion

New active ingredients are continuously being developed. By utilizing new form of sustained release drug delivery systems, it will be possible to deliver these drugs at constant rates over a prolonged period of time. Sustained release injectables formed *in-situ* based on PLA/PLGA polymers seem to be a suitable approach for subcutaneous long term release of the new lipophilic anti-infective NOA449851. This technology fulfills, for this particular compound, some basic requirements such as a good tolerability, controlled release of the active ingredient over a long period of time as well as an acceptable stability of the formulation during storage for several months at low temperature conditions. The release properties of the active ingredient could be modified by changing composition of formulation and possible detrimental burst effects could be suppressed by careful selection of the formulation co-solvents. It is always important for further development of formulation to match its composition with requirements of the market and authorities. It is to be expected that in the future, development of new implantable systems will, increasingly, help reducing the cost for drug therapy, and potentiate medical treatments and, simultaneously enhance patient compliance.

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WORK EXPERIENCE

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