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## ColoPulse tablets in inflammatory bowel disease

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Development and potential application of an  
oral ColoPulse infliximab tablet with colon  
specific release: a feasibility study

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## Abstract

The monoclonal antibody infliximab is one of the cornerstones in the treatment of Crohn's disease. Local delivery of infliximab would be an alternative to overcome the inherent disadvantages of intravenous therapy. For this purpose 5 mg infliximab tablets were developed. To stabilize the antibody during production and storage it was incorporated in a sugar glass containing the oligosaccharide inulin. To obtain colon-specific release a ColoPulse coating was applied. The tablets were stored for 16 months under different conditions based on ICH climatic zone I:

Condition 1: 25°C/60% RH closed vial

Condition 2: 25°C/60% RH open vial

Condition 3: 40°C/75% RH closed vial

With a panel of tests (i.e. HP-SEC, UV, CD) a stability indicating profile was obtained. Infliximab tablets were stable for up to four months when stored at temperatures varying from 25 - 40 °C. Tablets stored under condition 1 were most stable and displayed 16 months after production still a biological activity of 83% compared to a freshly prepared infliximab solution. This study is a first step in the development of a novel strategy in the treatment of patients with Crohn's disease.

## 1. Introduction

Crohn's disease is an autoimmune disorder of the gastrointestinal tract with huge impact on quality of life. The entire gastrointestinal tract can be affected, but in most cases inflammation is localized in the terminal ileum and the colon [1]. Currently the treatment of Crohn's disease is mainly symptomatic and aimed at the induction of remission and prevention of flare-ups. Pharmacotherapeutic treatment depends on patient characteristics and severity of disease. Several drugs are available to treat different stages of the disease, including 5-aminosalicylates, corticosteroids, methotrexate and thiopurines. Infliximab is considered a cornerstone in the treatment of moderate to severe Crohn's Disease, achieving rapid symptom relieve and maintaining remission [1,2].

Infliximab, a chimeric monoclonal antibody, was approved in 1998 by the Food and Drug Administration (FDA) for the treatment of Crohn's disease. It is now also used in other inflammatory diseases. The antibody binds to transmembrane and soluble tumor necrosis factor alpha (TNF-alpha) and exhibits its effect in Crohn's disease mostly by causing apoptosis of TNF-alpha expressing, activated T-lymphocytes and by neutralizing TNF-alpha [3,4].

At the start of therapy infliximab is administered by intravenous infusion at a dose of 5 mg/kg in the weeks 0, 2 and 6, followed by an infusion of the same dose every eight weeks. Although the antibody has proven to be effective in the treatment of Crohn's disease, it has several disadvantages when administered intravenously. Systemic exposure to infliximab is known to give rise to anti-infliximab antibodies resulting in infusion reactions, increased clearance and loss of response in a considerable number of patients. Furthermore, intravenous administration of infliximab is associated with serious adverse events, for example infectious complications, that can occur when the immune system of the patient is compromised. Also acute infusion reactions, hypersensitivity and anaphylactic shock have been reported [3]. Last but not least, regular intravenous therapy is perceived as a serious burden to the patient compared to chronic oral therapy. Because the mentioned clinical disadvantages are related to systemic exposure they may be overcome by a site-specific administration of (a lower dose of) infliximab. Small scale studies have shown a good clinical effect after local injections of infliximab [5-7].

As substitute for local administration by injections, the aim of this study is to develop an infliximab tablet with local release at the site of inflammation in patients with ileocolonic Crohn's disease. This aim is supported by the recent review of Moroz et al. [8]. They concluded that oral delivery to gastrointestinal

targets is currently more promising than systemic delivery because of the accessibility and the lack of intestinal permeability enhancement. They also stated that in the treatment of inflammatory bowel diseases capturing TNF-alpha in the intestinal lumen is a promising alternative to systemic treatment with anti-TNF-alpha antibodies (e.g. adalimumab, infliximab, and certolizumab pegol) with regard to systemic immunosuppression induced by these antibodies. In addition, oral administration is more patient friendly than the parenteral route as it is needle-free. The concept of local delivery of infliximab is also supported by the results of the Atlas study as described by Yarur et al. [9]. The results of this study suggest that local tissue inflammation characterized by high levels of TNF serves as a sink for anti-TNF. The authors stipulated that patients with high serum anti-TNF levels have active disease because tissue levels of anti-TNF are insufficient to neutralize local TNF production.

From several studies it appears that the colon is a suitable delivery site for proteinaceous drugs due to limited proteolytic activity (compared to higher parts of the gastrointestinal tract) combined with a relatively long residence time [10,11]. However, it remains a challenge to deliver a proteinaceous drug undamaged to the colon via oral administration. In the literature different strategies for colon targeting have been described. They include pH-responsive systems, time-based systems and systems triggered by the colonic flora, as well as combinations of such systems [12]. The ColoPulse technology is an example of a pH-responsive system which in several human studies was shown to deliver a drug site-specific into the ileocolonic region.

The ColoPulse system consists of a coating that dissolves at  $\text{pH} > 7.0$ , enabling drug release in the ileocolonic region. The release from the ColoPulse system is faster and more pulsatile than from other pH responsive systems because a superdisintegrant is incorporated in the coating [13]. In an *in-vitro* study release from several commercially available mesalazine drug products with a pH sensitive coating was tested in a modified dissolution test and *in vitro* colon selectivity was found to be suboptimal [14]. All formulations showed release of a substantial part of the active substance in the simulated stomach and jejunum. Using the same dissolution test for ColoPulse based dosage forms a different *in vitro* release profile was found with mainly release in the simulated jejunum / colon [15].

ColoPulse tablets containing  $^{13}\text{C}$ -urea as a marker substance revealed no relevant difference in site-specific delivery and release kinetics between healthy volunteers and patients with Crohn's disease [16]. To gain insight in the influence of food and time of food intake on the release from a ColoPulse tablet a study was performed in healthy volunteers and in patients with Crohn's

disease in remission. There was no relevant influence of food and time of food intake on the release characteristics and bioavailability in both healthy volunteers and in Crohn's patients when a standardized breakfast 3 h after administration of a ColoPulse tablet was compared to a non-standardized breakfast after 1 h.

The results from another study in which the relationship between *in vivo* gastrointestinal pH measurements and *in vivo* release from a ColoPulse tablet was studied in healthy volunteers confirm that release from a ColoPulse tablet indeed occurs in the distal ileum and colon and after pH 7.0 is reached [17].

This leads to the conclusion that the characteristics of a ColoPulse tablet correspond with the described aim of developing a modified release tablet for local treatment with infliximab in patients with Crohn's disease, because release from a ColoPulse tablet occurs at the ileocolonic region and without the use of permeation enhancers.

Another major challenge in the development of an infliximab tablet is to maintain the protein's stability during manufacturing and shelf life. To prevent loss of activity, the protein can be stabilized by incorporating it into a sugar glass. This can be achieved by freeze-drying of a solution in which both the protein and a suitable sugar are dissolved [18,19].

Combining the incorporation of infliximab into a sugar glass matrix with subsequent formulation of a ColoPulse tablet is considered an alternative to overcome the problems and drawbacks related to intravenously administered infliximab. Our hypothesis is that orally administered infliximab could be a novel strategy in the treatment of Crohn's disease. This would be a significant step forward in therapy. However, until now no data about formulation and stability of such a dosage form are available. In this paper we describe a suitable formulation for infliximab tablets with stability data of more than one year after production. This will enable us to study the effect of local treatment with ColoPulse infliximab tablets in Crohn's patients in the near future.

## **2. Materials and methods**

### **2.1. Materials ColoPulse infliximab tablets**

Polyethylene glycol 6000, caffeine, colloidal anhydrous silica, sodium stearyl fumarate, talc (BUFA, the Netherlands), microcrystalline cellulose (Avicel PH102, FMC Biopolymer, USA), croscarmellose sodium (Ac-di-sol, FMC Biopolymer, USA), methacrylic acid-methyl methacrylate copolymer 1:2 (Eudragit S100, Röhm, Germany), were obtained via a certified wholesaler

(Spruyt-Hillen, the Netherlands). Inulin 4.0 kD was obtained from Sensus (the Netherlands). Ethanol 96% and water for injections were obtained from Fresenius Kabi (Germany). Infliximab (Remicade®) vials 100 mg were obtained from MSD (the Netherlands). All ingredients were of pharmacopoeial grade (Ph Eur or USP).

## **2.2. Composition and production of ColoPulse infliximab tablets**

### **2.2.1. Preparation of the sugar glass**

A sugar glass was prepared by reconstitution of vials Remicade® containing 100 mg infliximab, sucrose, polysorbate 80, dibasic and monobasic sodium phosphate with 10 ml of a 17.5 wt% inulin solution in water. The infliximab-inulin solution was then transferred into vials of 4 ml each. Subsequently the vials were placed into liquid nitrogen to freeze the solution and the vials were placed in a Christ model Alpha 2–4 freeze-dryer (Salm and Kipp, the Netherlands). Primary drying was performed for 24 h at a shelf temperature of -35°C and pressure of 0.220 mbar. Thereafter, secondary drying was done for 24 h at a shelf temperature of 20°C and a pressure of 0.05 mbar. After freeze-drying samples were stored at 20°C/45% relative humidity (RH) for at least 48 h until further use. The glass transition temperature ( $T_g$ ) as determined using a DCS 2920 (TA instruments, Belgium) was 129.7°C. The water content as determined with a Karl Fischer titration using a 720 KFS Titrino (Metrohm, Switzerland) was 9.8%.

### **2.2.2. Production of 5 mg infliximab tablets**

To obtain a powder with sufficient flowability the slugging technique was used to perform dry granulation. Tablets with a diameter of 5 cm were made using an ESH hydraulic press (Hydro Mooi, the Netherlands) with a compaction force of 10 kN and a compaction speed of 2.5 kN/s after which the tablets were passed through a 0.8 mm sieve.

Two batches of infliximab 5 mg tablets were produced (batch A 180 tablets and B 45 tablets). The quantitative composition of batches A and B is shown in table 1. The amount of sugar glass was corrected for the water content in the infliximab sugar glass. Batch A also contained 25 mg caffeine for quality control purposes and for dissolution testing. The sugar glass was mixed in a turbula mixer with the excipients and subsequently the powder was compacted with a Korsch eccentric press (Berlin, Germany) to yield biconvex tablets with a diameter of 9 mm and a weight of 350 mg. The instrument settings were adjusted in such a way that tablets with a crushing strength of 150-200 N were

obtained. The tablets were analyzed according to the European Pharmacopoeia 8<sup>th</sup> edition. The results of the quality control tests are shown in table 2.

**Table 1:** Composition of 5 mg infliximab tablet cores.

Substance	Function	Batch A	Batch B
Infliximab sugar glass	Active substance	131 mg	131 mg
Caffeine	Marker substance	25 mg	0 mg
Microcrystalline cellulose	Excipient	175 mg	200 mg
Croscarmellose sodium	Excipient	14 mg	14 mg
Colloidal anhydrous silica	Excipient	1.8 mg	1.8 mg
Sodium stearyl fumarate	Excipient	3.5 mg	3.5 mg
Total weight		350 mg	350 mg
Number of tablets		180	45

**Table 2:** Quality control data of 5 mg infliximab ColoPulse tablets presented as mean (standard deviation) where applicable.

Parameter	Specification	Batch A (with caffeine)	Batch B (without caffeine)
<i>Infliximab 5 mg cores (uncoated)</i>			
Yield	n.a. <sup>a</sup>	147 tablets	42 tablets
Appearance	White biconvex tablet	Complies	Complies
Crushing strength	150-200 N	168 N	162 N
Friability	< 1% (Ph Eur)	0.109%	- <sup>b</sup>
Disintegration (cores)	All < 15 min (Ph Eur)	All < 15 min	- <sup>b</sup>
Uniformity of dosage units (caffeine, n = 10)	AV < 15 (Ph Eur)	13.9	- <sup>b</sup>
Content (caffeine)	90-110%	96.7% (4.9)	- <sup>b</sup>
Amount infliximab (mg) able to bind to TNF-alpha	4.5-5.5 mg	5.5 mg (0.4)	- <sup>b</sup>
Tablet weight (mg)	350 mg	348.1 mg (3.5)	346.4 mg (3.3)
<i>Infliximab 5 mg ColoPulse tablets (coated)</i>			
Coat thickness (mg/cm <sup>2</sup> )	13-17 mg/cm <sup>2</sup>	9.5 mg/cm <sup>2</sup>	10.5 mg/cm <sup>2</sup>
Lag time (n = 6)	> 240 min	215.2 min (8.8)	- <sup>c</sup>
Pulse time (n = 6)	< 60 min	30.5 min (10.7)	- <sup>c</sup>
Release at t <sub>360 min</sub> (n = 6)	> 80%	94.5% (11.9)	- <sup>c</sup>

<sup>a</sup> n.a., not applicable

<sup>b</sup> Not determined due to small batch size

<sup>c</sup> Not possible due to absence of caffeine



### 2.2.3. Coating of infliximab tablets

A coating was applied on 75% of the tablets of batch A and all tablets of batch B using the ColoPulse technology as described before [15]. In this study tablets were coated in a Strea 1 fluidized bed coater (Aeromatic, USA). Coating time was 50 minutes. The coating consisted of a mixture of PEG 6000 : Eudragit S100 : Ac-di-sol : Talc in a ratio of 1:7:3:2 (w/w/w/w). The solvent used was ethanol 96%. Coating thickness was determined and expressed as the amount of Eudragit S100 applied per cm<sup>2</sup> using the following formula:

$$\text{Coating thickness} = \frac{\text{weight coated tablet} - \text{weight uncoated tablet}}{\text{surface uncoated tablet}} \times \text{fraction eudragit S100}$$

The coating thickness applied was 9.7 mg/cm<sup>2</sup> for batch A and 10.5 mg/cm<sup>2</sup> for batch B.

## 2.3. Stability study

### 2.3.1. Storage conditions

The tablets were stored under different conditions, based on the ICH guidelines, climatic zone I [20]:

- Condition 1: 25°C/60% RH closed vial
- Condition 2: 25°C/60% RH open vial
- Condition 3: 40°C/75% RH closed vial

The tablets were individually packed in a closed or open glass container. When applicable vials were hermetically closed with a screw cap. The above-mentioned storage conditions were created in an exsiccator. A saturated solution of potassium bromide was used to create an atmosphere of 60% RH and the exsiccator was then placed in a cabinet of 25°C during the study period of 16 months. An RH of 75% was reached using an exsiccator with a saturated solution of ammonium chloride; the exsiccator was placed in a cabinet of 40°C during the study period of 16 months.

### 2.3.2. Stability indicating profile

For large proteins like infliximab (150 kD), there is not one single analytical method that can be used to assess the different characteristics and the stability of the protein. Therefore a stability indicating profile has to be established guaranteeing that potential changes in identity, purity and potency of the protein will be detected [21]. To achieve this the use of a panel of different analytical methods is necessary. The evaluation of the combined results will give

information about the characteristics and stability of the protein. The tests performed in the stability study with infliximab tablets are shown in table 3. The tablets were analyzed at t = 0, 1, 2, 3, 4, 5 and 16 months after production according to the schedule in table 3. Tablets at t = 0 were uncoated except for the dissolution test and Circular Dichroism (CD) analysis. At all other time points the tablets were covered with the ColoPulse coating. The analytical methods used are described shortly in 2.3.3 to 2.3.9. In all tests phosphate buffered saline (PBS) pH 7.4 containing 0.45% sodium chloride, 15 mM monobasic sodium phosphate dihydrate, 53 mM dibasic sodium phosphate dodecahydrate and water for injection was used for dilutions. Solutions were filtrated as described in the individual method sections to remove insoluble parts of the coating.

**Table 3:** Test schedule stability study infliximab 5 mg ColoPulse tablets. Tests are marked with X and each test was performed for all storage conditions with n = 3 unless otherwise specified:

Condition 1: 25°C/60% relative humidity closed vial

Condition 2: 25°C/60% relative humidity open vial

Condition 3: 40°C/75% relative humidity closed vial

Test	Aim	t = 0 month	t = 1 month	t = 2 months	t = 3 months	t = 4 months	t = 5 months	t = 16 months
ELISA	Content	X <sup>a</sup>		X		X		X
SDS-PAGE	Molecular mass/degradation products	X <sup>a</sup>		X		X		X
Bio-assay	Biological activity				X			X
Dissolution	Dissolution profile	X <sup>f</sup>	X	X	X			X <sup>b</sup>
Circular Dichroism	Secondary and tertiary structure	X		X <sup>c</sup>	X	X		X <sup>d</sup>
UV	Aggregation	X <sup>a</sup>				X		X
HP-SEC	Aggregation				X <sup>e</sup>		X <sup>e</sup>	X

<sup>a</sup> uncoated

<sup>b</sup> only for storage condition 1 and 3

<sup>c</sup> only for storage condition 1

<sup>d</sup> n = 2 for storage condition 3 Far UV

<sup>e</sup> n = 1 for each storage condition

<sup>f</sup> n = 6

### **2.3.3. ELISA**

An ELISA was used to gain more insight in content changes of the amount of infliximab able to bind TNF-alpha in the ColoPulse tablets. Tablets of batch A were dissolved in 5.0 ml PBS to yield a concentration of 1.0 mg/ml ( $n = 3$  for each storage condition) followed by filtration through a 0.22  $\mu\text{m}$  Millex® GP filter. As control a fresh infliximab solution was prepared using the same method. Solutions were further diluted with albumin 200 mg/ml (Sanquin, the Netherlands) to yield a final infliximab concentration of 5  $\mu\text{g}/\text{ml}$  in polypropylene vials. Controls of Remicade® with final concentrations of 1.0, 2.5, 5.0 and 10.0  $\mu\text{g}/\text{ml}$  ( $n = 5$ ) in PBS, prepared as described above, were analyzed to validate the method of sample preparation and were included in every run. Accuracy and the coefficient of variation were calculated. The ELISA was performed by Sanquin, the Netherlands according to a protocol described by Vande Castele et al. [22].

### **2.3.4. SDS-PAGE**

The molecular mass of the different subunits and possible soluble aggregates or smaller sized degradation products of infliximab in the tablets were monitored using SDS-PAGE. SDS PAGE separates proteins according to their molecular weights. Possible (soluble) aggregates present in the tablet can thus be detected as well as smaller size degradation products. Coated tablets of batch A were dissolved in 10.0 ml PBS ( $n = 3$  for each storage condition) followed by filtration through a 0.22  $\mu\text{m}$  Millex® GP filter. This procedure was also applied to prepare a fresh infliximab solution from Remicade®. SDS-PAGE was performed by using a vertical Bio-Rad Mini-Protean 3 gel electrophoresis system (Bio-Rad, USA) under reducing (dithiothreitol, Invitrogen, The Netherlands) and non-reducing conditions according to the manufacturer's instructions using a 10% gel. The gel was loaded with appropriate volumes containing 10  $\mu\text{g}$  infliximab and 7.5  $\mu\text{l}$  of a control (Precision Plus Protein Standard all blue, Bio-Rad, USA). After running the gel was colored with Coomassie Brilliant Blue staining solution (Bio-Rad, USA) according to the manufacturer's instructions.

### **2.3.5. Bioassay**

A bioassay was used to determine the TNF-alpha neutralizing activity of infliximab. Coated tablets of batch A were dissolved in 5.0 ml PBS to yield a concentration of 1.0 mg/ml followed by filtration through a 0.22  $\mu\text{m}$  Millex® GP filter ( $n = 3$ ). This procedure was also applied to prepare a fresh infliximab solution from Remicade®. PBS was used as negative control. The bioactivity of

infiximab tablets was determined with a TNF-alpha sensor assay using HeLa 8D8 cells that express Green Fluorescence Protein (GFP) under control of an NF- $\kappa$ B response element. GFP expression was quantified by flow cytometry [23]. In this bioassay TNF-alpha stimulates the expression of GFP and expression of GFP is inhibited by infiximab by neutralisation of TNF-alpha. The GFP expression of cells incubated with PBS was set at 100% (TNF-alpha inhibition 0%) and the GFP expression of cells incubated with infiximab solution was set at 0% (TNF-alpha inhibition 100%).

### **2.3.6. CD Spectroscopy**

The secondary and tertiary structure of infiximab was analyzed by far-UV and near-UV CD, respectively [24]. Coated tablets of batch B (without caffeine) were dissolved in 5.0 ml PBS to a concentration of 1.0 mg/ml followed by filtration through a 0.22  $\mu$ m Millex® GP filter (n = 3 for each storage condition). This solution was used to record the near-UV spectrum. 100  $\mu$ l of the solution was further diluted with PBS to a concentration of 0.025 mg/ml for recording of the far-UV spectrum. The same procedure was carried out to prepare a fresh infiximab solution in PBS.

The samples were placed in a 10 mm quartz cuvet and the UV-spectra were recorded using a Jasco-J-850 CD spectrometer (Jasco, Italy). The UV spectra were collected in a continuous scanning method for the range 205-250 nm for the far-UV and 250-350 nm for the near-UV at a scanning speed of 50 nm/min, a response time of 2 sec, a bandwidth of 2 nm and an accumulation of 5 scans. The spectra were background corrected for the solvent, smoothed with Savitzky-Golay using a convolution width of 9 and converted to specific ellipticity.

### **2.3.7. UV Spectroscopy**

To gain insight in the degree of insoluble aggregates of infiximab in the tablets, the aggregation index was determined using UV spectroscopy. Coated tablets of batch B were dissolved in 5 ml PBS to yield a concentration of 1.0 mg/ml followed by filtration through a 0.22  $\mu$ m Millex® GP filter (n = 3 for each storage condition) and were measured on the same day (t = 0, 4 and 16 months) or the next day (t = 1 month). A freshly prepared infiximab solution of 1.0 mg/ml in PBS made from Remicade® was used as a control.

UV spectroscopy was performed with a Unicam UV 500 spectrophotometer (Thermo Spectronic, United Kingdom). Samples were measured in a quartz cuvet with path length of 10 mm and UV absorbance was measured at 280 and 340 nm. The absorbance was not corrected for the absorbance of Eudragit S100 at these wavelengths, because this test was intended to follow the development

of soluble aggregates over time and to compare results between the different storage conditions.

The aggregation index (AI) was calculated from the UV spectra using the following formula, which was described in the literature [25]:

$$AI = \frac{\text{Absorbance}_{340 \text{ nm}}}{(\text{Absorbance}_{280 \text{ nm}} - \text{Absorbance}_{340 \text{ nm}})} * 100$$

### 2.3.8. High Performance Size Exclusion Chromatography

High Performance Size Exclusion Chromatography (HP-SEC) was used to determine the degree of soluble aggregates of infliximab in the tablets [26]. HP-SEC was performed on a LaChrom Elite HPLC system with UV detection (Hitachi, US). A Superdex 200 10/300 GL column was used in combination with an eluent containing 0.02M sodium dihydrogen phosphate dihydrate and 0.02M sodium hydrogen phosphate dihydrate in sodium chloride 0.9%, pH 7.0. Coated tablets of batch B were dissolved in 5 ml PBS to yield a concentration of 1.0 mg/ml followed by filtration through a 0.22  $\mu\text{m}$  Millex® GP filter ( $n = 3$  for each storage condition). An infliximab solution of 1.0 mg/ml in PBS made from Remicade® was used as a control. 20  $\mu\text{l}$  were injected onto the column and separation was performed at a flow rate of 0.7 ml/min. The absorption was measured at 220 and 280 nm.

### 2.3.9. Dissolution test

The pulsatile release properties of a ColoPulse tablet are reflected by the lag time and the pulse time. Caffeine was used as a marker substance to confirm that ColoPulse infliximab tablets met the specified release specifications and, for example, did not release active substance at a pH < 7.0. The lag time is the time point at which the tablets start to release the active substance and was defined as the time at which 5% of caffeine was released. The pulse time reflects the pulsatile release characteristics and was defined as the period between the lag time and the time at which 70% of caffeine was released. These parameters were established in a dissolution test with a total duration of 360 minutes during which the pH was varied in time to simulate the different stages of the gastrointestinal tract as described before. This dissolution test known as Gastro-Intestinal Simulation System (GISS) was described before by Schellekens et al. [14].

The dissolution profile of the coated tablets of batch A ( $n = 3$  for each storage condition,  $n = 6$  for  $t = 0$ ) was determined using the GISS. The test was

performed as described previously [14]. In short, the different parts and residence times of the gastrointestinal tract were simulated by varying the pH, the volume and the osmolality of the dissolution medium in four different phases. A summary of the specifications of this dissolution test is shown in table 4.

The release of caffeine from the tablets was measured with UV spectroscopy at a wavelength of 273 nm. At the end of the dissolution test a visual check on disintegration of the tablet was performed.

**Table 4:** Specifications of the dissolution test (GISS).

Phase	Gastrointestinal Segment	Volume (ml)	Residence time (h)	pH	Osmolality (mosmol/kg)
I	Stomach	500	2.0	1.2 ± 0.10	150 ± 25
II	Jejunum	629	2.0	6.8 ± 0.20	250 ± 50
III	Ileum (distal)	940	0.5	7.5 ± 0.25	250 ± 50
IV	Colon (proximal)	1000	1.5	6.0 ± 0.25	250 ± 60

## 2.4. Statistical procedures

Where applicable the results were evaluated by descriptive statistics with SPSS version 22. The center was characterized by the mean and the dispersion by the standard deviation (SD), CV and range. A paired samples t-test and an independent samples t-test (two tailed,  $\alpha = 0.05$ ) were used to compare the results between different time-points and different storage conditions, respectively. Results were considered significant when  $p < 0.05$ .

## 3. Results

Infliximab 5 mg ColoPulse tablets met all current quality control criteria at  $t = 0$  as shown in table 2, except for coating thickness and the related lag time. Despite the coating thickness being lower than expected (which affects mainly the lag time), the stability study was performed, because a reduced thickness of this type of coating is unlikely to influence protein stability in the tablet core. The results of all different tests, together describing the characteristics and stability indicating profile, are described below. A summary of the results at  $t = 16$  months can be found in table 5. The appearance of the tablets showed no difference over time or between different storage conditions.

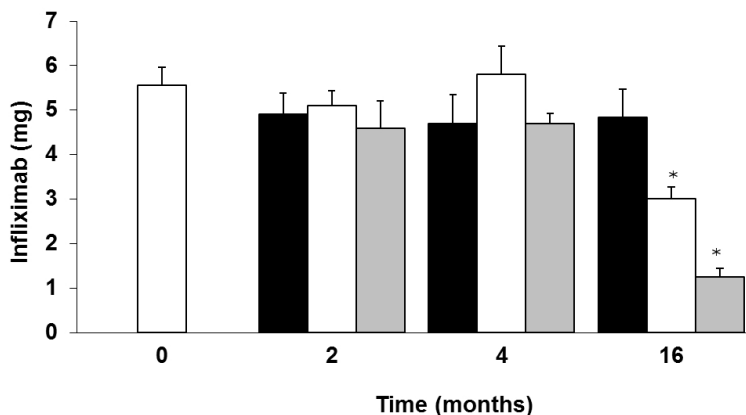
**Table 5:** Summary of results at t = 16 months presented as mean (standard deviation).  
N of each test can be found in table 3.

Test	Aim	Requirement	Result condition 1	Result condition 2	Result condition 3
ELISA	Content	≥ 4.5 mg	4.8 mg (0.64)	3.0 mg (0.26)	1.2 mg (0.21)
SDS-PAGE	Molecular mass/ degradation products	No difference compared to freshly prepared infliximab solution	No difference	No difference	No difference
Bio-assay	Biological activity	> 80%	83.4% (17.0)	72.9% (12.6)	12.2% (7.9)
Dissolution	Dissolution profile	Lag time > 240 min Pulse time < 60 min Release at t <sub>360 min</sub> > 80%	246 min (8.3) 10.0 min (5.0) 102% (5.0)	Not available	255 min (4.0) 9.3 min (3.5) 107% (3.5)
Circular Dichroism	Secondary and tertiary structure	No difference compared to freshly prepared infliximab solution	No difference	Differences observed	Differences observed
UV	Aggregation	Not applicable	17.5 (4.7)	25.0 (9.4)	46.6 (11.2)
HP-SEC	Aggregation	No aggregation/degradation	No aggregation/degradation	Aggregation	Aggregation + degradation

### 3.1. ELISA

The suitability of the sample preparation for ELISA was tested with controls made from Remicade® with final infliximab concentrations of 1.0, 2.5, 5.0 and 10.0 µg/ml. The mean accuracy of the method was -0.6% (range -6.1 - 6.0%) and the mean CV was 18.3% (range 16.1 - 22.0%) depending on the concentration. The sample preparation method was judged suitable for determination of the content of infliximab 5 mg ColoPulse tablets.

The amount of infliximab able to bind to TNF-alpha of 5 mg infliximab tablets stored under different storage conditions at t = 0, 2, 4 and 16 months is shown in figure 1. At t = 0 the mean amount was 5.5 mg (n = 3, SD 0.42). After 2 and 4 months no difference was found between the different storage conditions and all measurements, except for condition 2 at t = 4 months (5.8 mg), were within the specifications of 4.5 - 5.5 mg.



**Figure 1:** Mean amount of infliximab able to bind to TNF-alpha and standard deviation of infliximab 5 mg ColoPulse tablets (n = 3) at different storage conditions. Analysis at t = 0 was performed with uncoated tablets.

\* indicates a statistically significant difference ( $p < 0.05$ ) compared to t = 0

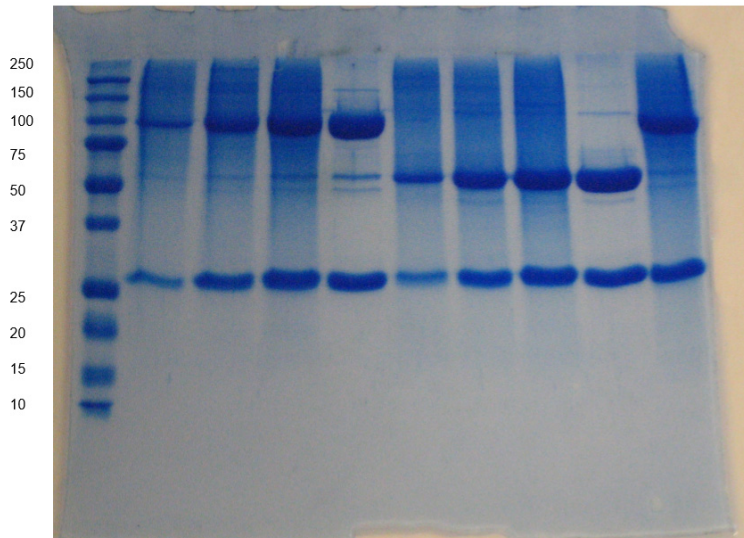
- condition 1: 25°C/60% RH closed vial
- condition 2: 25°C/60% RH open vial
- condition 3: 40°C/75% RH closed vial

The amount of infliximab able to bind to TNF-alpha at t = 16 months under storage conditions 2 and 3 decreased to a mean value of 3.0 mg and 1.2 mg, respectively. The mean amount at t = 16 months under condition 1 closed vial was 4.8 mg (n = 3, SD 0.66, 87.3% of T = 0). The results did not differ significantly between t = 0 and t = 16 months for tablets stored at this condition ( $p = 0.187$ , paired samples t-test).

### 3.2. SDS-PAGE

In figure 2 the result of a representative gel (1 out of 3) at t = 16 months is shown. Under non-reducing conditions two fragments with a molecular weight of approximately 100 and 25 kD were found. Also two lightly colored bands corresponding to protein fragments of about 55 and 50 kD were found. Under reducing conditions two fragments were found of approximately 50 and 25 kD. Also a lightly colored band corresponding to a protein fragment of about 55 kD was found. There was no difference between the position of fragments at the different time points (t = 0, 2, 4 and 16 months) and the position of the fragments was identical to that of the fragments obtained using an infliximab control solution.

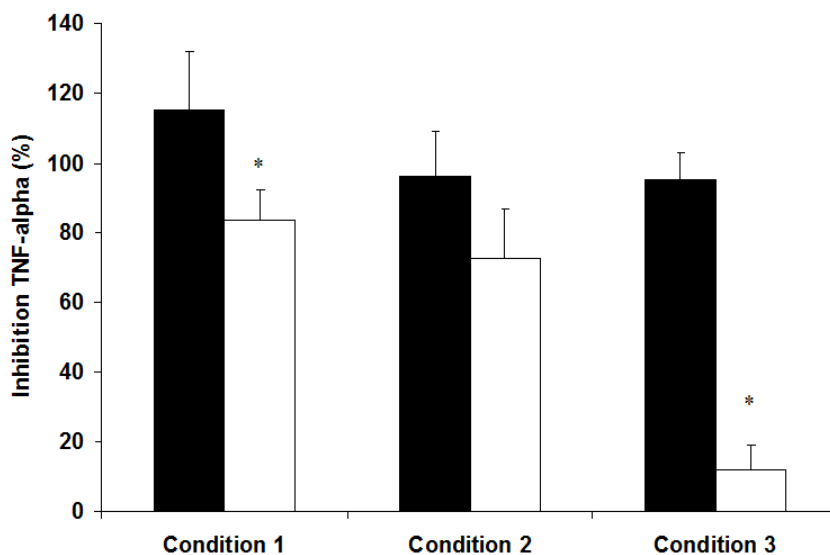




**Figure 2:** SDS-PAGE at t = 16 months. Lanes from left to right: ladder/non-reducing conditions (tablets storage condition 1/2/3/infliximab solution) / reducing conditions (tablets storage condition 1/2/3/infliximab solution) / extra: non-reducing tablet condition 3

### 3.3. Bioassay

In figure 3 a summary of the bioassay results is presented. At t = 16 months storage under conditions 1 - 3 shows a mean of 83.5, 72.9 and 12.2% TNF-alpha inhibition, respectively, compared to a fresh infliximab control solution. At t = 3 months this was 115.5, 96.3 and 95.2%, respectively. There was a significant difference in TNF-alpha inhibition between the freshly prepared infliximab solution and infliximab incorporated in tablets stored under condition 1 and 3 when results at t = 3 months and 16 months were compared ( $p = 0.045$  and  $p = 0.001$ , respectively, paired samples t-test).

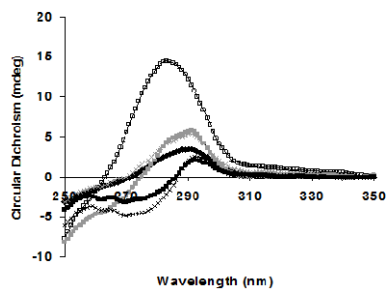
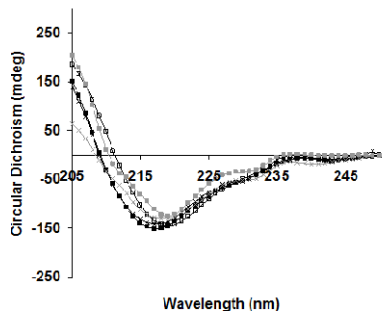


**Figure 3:** Bioassay: Mean TNF-alpha inhibition after 3 months (■) and 16 months (□) storage at different conditions (n = 3). TNF-alpha inhibition of a freshly prepared 1 mg/ml infliximab solution was set at 100%. \* indicates a statistically significant difference

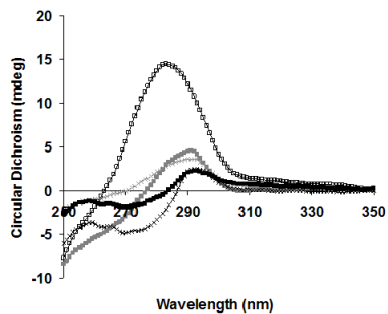
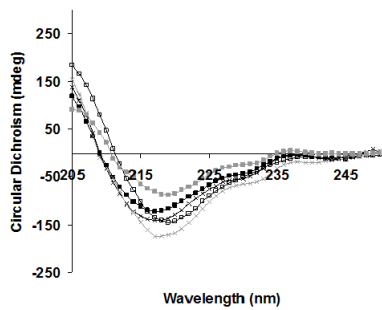
### 3.4. CD spectroscopy

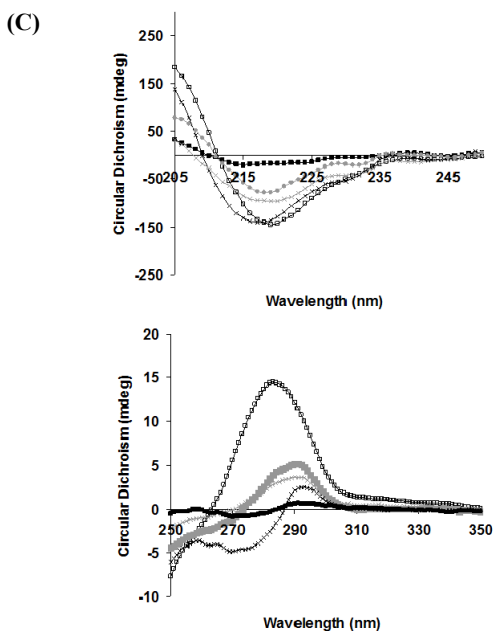
The far-UV and near-UV CD spectra are shown in figure 5 for all storage conditions and all time points. The shape of the far-UV CD spectra, representing the secondary structure of a protein, shows little change over time during storage under conditions 1 and 2. During storage under condition 3, however, substantial changes are visible. The shape of the near-UV CD spectra, representing the tertiary structure of a protein, shows little difference between t = 2 (condition 1 only), 3 and 4 months for all storage conditions. However, for t = 16 months differences were observed, mostly for storage condition 2 and 3.

(A)



(B)





**Figure 5:** Far and near UV CD spectra of infliximab 5 mg tablets (mean of  $n = 3$ ) at different storage conditions and time points

□  $t = 0$  (uncoated) / ♦  $t = 2$  months (only condition 1) / X  $t = 3$  months / ■  $t = 4$  months / ▀  $t = 16$  months / X Infliximab standard (spectra from analysis  $t = 16$  months)

5A: condition 1: 25°C/60% RH closed vial

5B: condition 2: 25°C/60% RH open vial

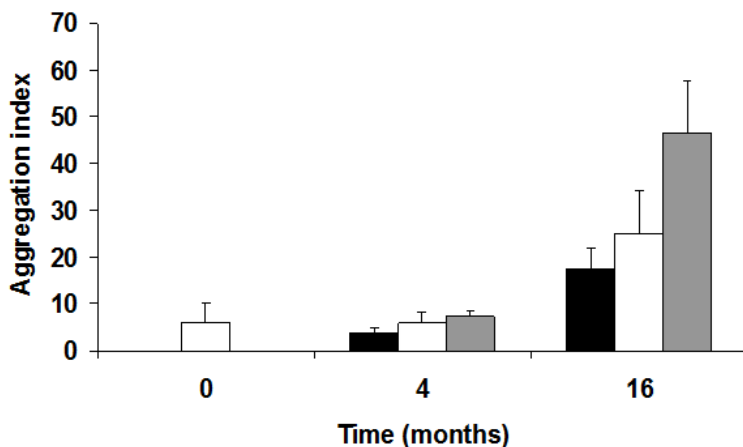
5C: condition 3: 40°C/75% RH closed vial

### 3.5. UV Spectroscopy

Using UV spectroscopy the aggregation index of infliximab in the tablets was calculated from the absorption at 280 and 340 nm. The results of these measurements are presented in figure 6. At  $t = 16$  months the aggregation index increased to a mean value of 17.5, 25.0 and 46.7 for storage conditions 1, 2 and 3, respectively. For all conditions the aggregation index increased during storage. At both time points the aggregation index of condition 1 was lower than that of condition 2 and condition 2 showed a lower aggregation index than condition 3.

This could also be observed when condition 2 and 3 were compared.

Over time absorption at 280 nm decreased and absorption at 340 nm increased (data not shown). This confirms that the measured aggregates could be attributed to infliximab and not to other solid substances like inulin which is not likely to degrade.



**Figure 6:** Mean aggregation index and standard deviation of infliximab 5 mg tablets (n = 3) at different storage conditions.

- condition 1: 25°C/60% RH closed vial
- condition 2: 25°C/60% RH open vial (t = 0: core)
- condition 3: 40°C/75% RH closed vial

### 3.6. High Performance Size Exclusion

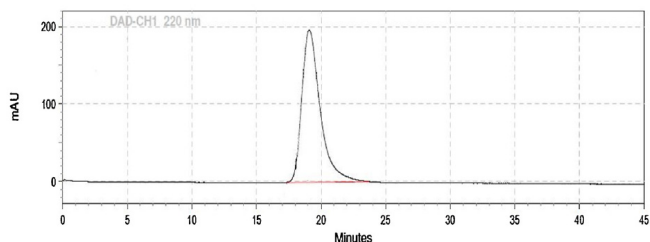
Additional information about soluble aggregates of infliximab was obtained using HP-SEC at t = 3, 5 and 16 months of storage. At t = 3 and 5 months the chromatograms of the samples stored under condition 1, 2 and 3 were comparable to each other (data not shown). In figure 7 representative results of t = 16 months are shown for all storage conditions. The infliximab peak has a retention time of approximately 19 minutes.

For condition 1 some small extra peaks could be observed at all time points when results were compared with a freshly prepared infliximab solution as measured at 220 nm and at 280 nm. Further experiments (data not shown) pointed out that these peaks could be attributed to the Eudragit of the ColoPulse coating. Eudragit showed at 220 nm a small peak between 11 and 14 minutes. At 280 nm absorption of Eudragit could be seen at approximately 11 and 25 minutes.

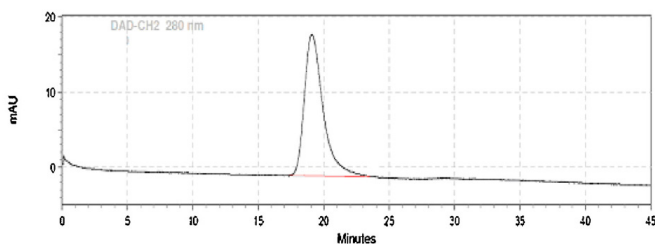
Tablets stored at condition 2,  $t = 16$  months showed several extra peaks (compared to condition 1) before the infliximab peak at 280 nm. These could possibly be attributed to the formation of soluble aggregates.

The chromatogram of the samples stored under condition 3 for 16 months showed several differences when compared with the freshly prepared infliximab solution. The large peaks before and after the protein peak, indicate substantial aggregation and degradation, respectively.

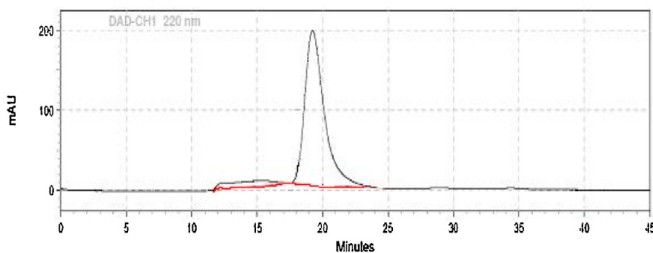
**(A) 220 nm**



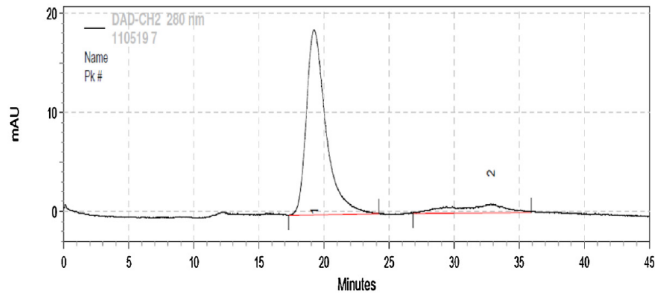
**280 nm**



**(B) 220 nm**

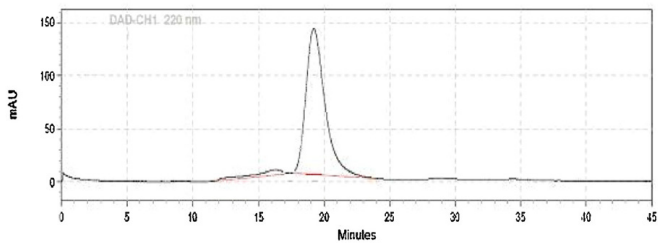


**280 nm**

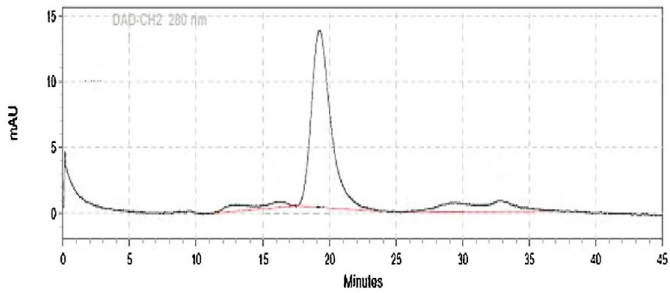


(C)

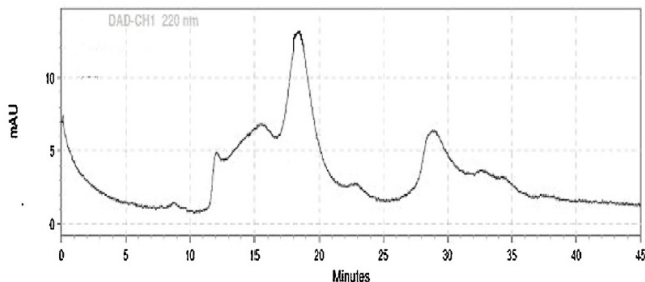
**220 nm**



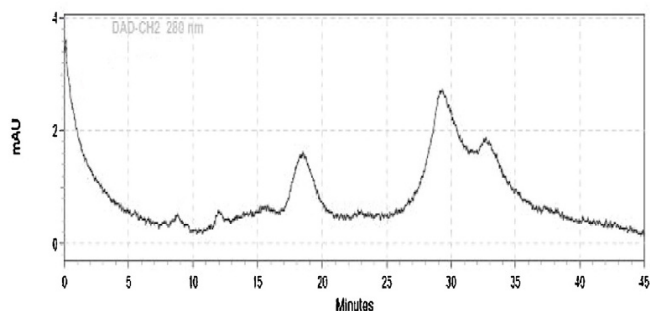
**280 nm**



(D) 220 nm



280 nm



**Figure 7:** HP-SEC representative chromatograms at  $t = 16$  months for an infliximab solution (A) and for all storage 1 and 3 of infliximab 5 mg tablets (B-D).

Measurements were performed at a wavelength of 220 en 280 nm.

7A: infliximab solution

7B: condition 1: 25°C/60% RH closed vial

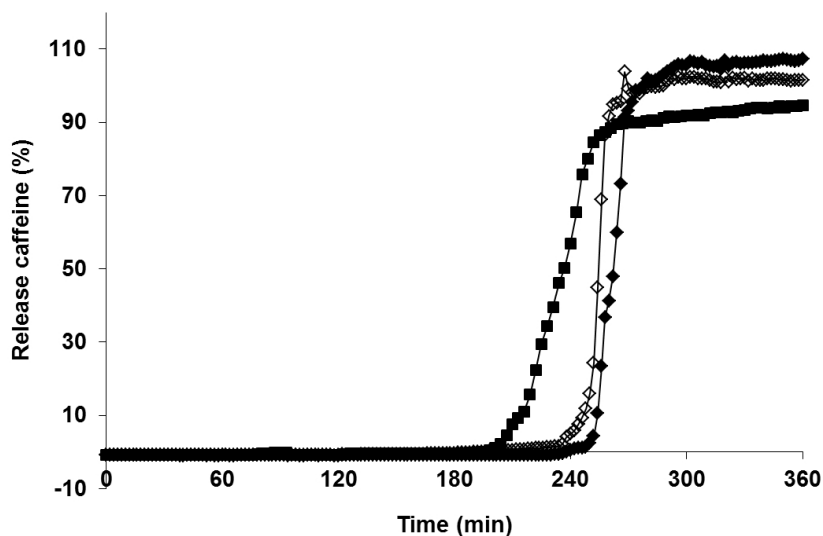
7C: condition 2: 25°C/60% RH open vial

7D: condition 3: 40°C/75% RH closed vial

### 3.7. Dissolution

The dissolution profile after production of the ColoPulse tablets and at  $t = 16$  months is shown in figure 4. All tablets met the criteria for release and pulse time at all time points. The mean lag time at  $t = 0$  was 215 min (range 201-224 min) which did not meet the specification of  $> 240$  min. This was also observed at time points  $t = 2$  and 3 months (range 215-263 min). At  $t = 16$  months mean lag time (both storage conditions combined) was 250.7 min (range 237-259 min). All tablets had disintegrated at  $t = 360$  minutes as visually determined.





**Figure 4:** Dissolution profiles of ColoPulse infliximab tablets at  $t = 0$  (■) and  $t = 16$  months (◆, condition 1 and ◇ condition 3). The graph shows the mean of  $n = 6$  ( $t = 0$ ) and  $n = 3$  ( $t = 16$  months) tablets.

## 4. Discussion

To the best of our knowledge, this is the first study in which the feasibility to formulate an oral infliximab tablet has been explored. Infliximab 5 mg tablets stored under ICH condition I showed no relevant loss of content, potency and change in structure up to 4 months after production. At  $t = 16$  months major differences were observed for storage under accelerated (extreme) conditions ( $40^{\circ}\text{C}/75\% \text{RH}$ ). However, after 16 months infliximab from a ColoPulse tablet stored under normal conditions ( $25^{\circ}\text{C}/60\% \text{RH}$ , closed vial) still displayed a mean biological activity of 83% compared to a fresh infliximab solution.

### 4.1. Selection of infliximab dosage

In the literature no information is available yet about the optimal dosage of orally administered infliximab for topical therapy in patients with Crohn's disease in the ileocolonic region. In most publications the dosage described for local injections varies between 10 – 40 mg [5-7]. This corresponds on average with 2.5 – 10.0% of the intravenous dosage for a patient of 80 kg, however, the frequency of local administration of infliximab every 2 – 6 weeks [5–7] is higher than intravenous administration which is given once every eight weeks.

The colon is a suitable absorption site for protein and peptide drugs due to the absence of digestive enzymes and a relatively low proteolytic activity besides providing a longer residence time than in the small intestine. However, intestinal transport and diffusion of protein drugs is difficult to predict [10]. But the fact that intravenously administered infliximab exhibits its effects in the lamina propria (part of the mucosa) [27] suggests that infliximab is able to migrate across the intestinal epithelium.

Our hypothesis is that a daily (low) oral dose of infliximab will be as effective as intravenously administered infliximab 5 mg/kg every 8 weeks, because of the continuous exposure of the intestinal mucosa to infliximab. Furthermore the clinical effect of infliximab is also related to trough levels [28] and with our proposed design continuous low (local) trough levels can be reached during therapy. Based on the assumption that local bioavailability will be around 75% due to enzymatic degradation, degradation by the colon flora and loss with feces, a daily dosage range of 10 – 20 mg infliximab was chosen for our planned study. Considering this, tablets with 5 mg infliximab were selected because of being a suitable amount per dosage unit, also in view of envisaged need of dosing flexibility.

## **4.2. Tablet formulation and quality control**

Infliximab tablets were compounded using the commercial product Remicade®. The formulation of Remicade® is mainly based on sucrose and the appearance of the freeze dried powder can best be described as a porous cake. This formulation as such is not suitable for the production of tablets due to the poor flowability of the powder. Furthermore the protein should be further stabilized to make long-term storage of the tablets possible, preferable at room temperature.

Flowability was improved using dry granulation of Remicade® followed by sieving. To stabilize a protein and to protect it from degradation during tablet production and storage it could be formulated into a sugar glass. In a sugar glass the mobility of molecules is strongly reduced when the environmental temperature is considerably below the glass transition temperature ( $T_g$ ), which enhances stability of the protein [29]. Therefore a sugar glass with a high  $T_g$  is preferable for production of ColoPulse infliximab tablets. Sucrose as present in Remicade® has a relatively low glass transition temperature ( $T_g$ ) of 60°C [30] when anhydrous and, since water acts as a plasticizer, this will be much lower when it contains some residual or absorbed moisture. This makes a formulation with sucrose considerably less suitable for formulation of infliximab tablets. A low  $T_g$  may lead to problems during tablet production because the RH of the environment should be low which may lead to static charging of the powder

resulting in poor flowability. In addition, a low  $T_g$  may result in compaction induced crystallization [31] which reverses the protective effect of the sugar glass. Several publications describe the oligosaccharide inulin as promising to stabilize proteins due to its high glass transition temperature amongst other physicochemical characteristics [30,32]. For this reason infliximab was incorporated in inulin resulting in a sugar glass with a  $T_g$  of approximately 130°C.

The quality control results of the tablet cores of batch A (formulation with caffeine) met all criteria as described before. Due to the explorative character of this study not enough material was available to perform a comparable control of batch B (formulation without caffeine). This formulation was used for various UV-based analytical procedures (UV, CD, HP-SEC) as caffeine, which shows UV absorption, would have disturbed these measurements. The aim of these tests was mainly to detect and follow protein aggregation during storage. Based on the assumption that the tablet mixture was homogeneous and the fact that the theoretical weight complied with the criteria, batch B was considered suitable for the mentioned aim.

### **4.3. Influence of relative humidity and temperature**

There are only few publications available about the short term stability of infliximab after reconstitution. No loss of biological activity occurred during a study period of two weeks at 4°C for a 400 mg/L solution in sodium chloride 0.9% [33]. In another study the immunoreactivity of infliximab at two different concentrations (50 and 0.069 µg/L) in 0.9% sodium chloride remained stable over the six-week study period at 4°C [34]. Data on long term storage or storage at room temperature are not available at all, but it is obvious that at higher temperatures infliximab will be less stable [35].

In our study tablets were stored for 16 months at 25°C, which is a convenient and normal storage condition when products are used in patient care. The temperature of 40°C was chosen as a challenging storage condition, but this condition does not reflect daily practice. The results obtained under this condition could be used to gain more insight in stability when tablets are for a short period stored under stress conditions, for example during transport.

The results of the different tests show that incorporation into an inulin sugar glass matrix prevents infliximab from degradation up to four months of storage under all storage conditions, which is already relatively long. At the next time point  $t = 16$  months, samples showed clear degradation of the antibody when stored at 40°C/75% RH but less for storage at 25°C/60% RH in a closed vial. A possible explanation for the degradation despite the high  $T_g$  of 130°C is that the

$T_g$  was lowered by the relatively high RH at 40°C resulting in crystallization of inulin and subsequent degradation of infliximab. The  $T_g$  and appearance of the tablet core should be closely monitored in the further development of this tablet.

Comparing the test results of condition 1 and 2 (closed and open vial, respectively) an improved stability was found when the tablets were stored in a closed vial. This emphasizes that the packaging and instructions for storage are extremely important for protein formulations and should also be considered carefully in the further development of this tablet formulation.

#### **4.4. Summary of stability indicating profile**

A panel of different methods of analysis was used to study the stability of 5 mg infliximab ColoPulse tablets during production and long-term storage. Combining the results of the different analysis for each storage condition leads to the conclusion that tablets stored under condition 1 showed the least differences between the time points, while tablets stored under condition 3 showed the most. This was expected because storage at high temperature and high humidity is a very challenging condition for an antibody, even when stabilized in a sugar glass. The observed pattern could be found for each test.

### **5. Conclusion**

Based on the presented results it can be concluded that formulation of 5 mg ColoPulse infliximab tablets is technically feasible. Incorporation of infliximab in a sugar glass matrix based on inulin resulted in a tablet that was stable for up to four months when stored at temperatures varying from 25 - 40 °C. 16 months after production tablets stored at 25°C in a closed vial still displayed a mean biological activity of 83% compared to a fresh infliximab solution. To obtain a stable oral formulation of infliximab suitable for use in clinical studies, packaging and refrigerated storage will be investigated in a follow-up study. However, the currently obtained results are a first step in the further development of a safe, effective and more patient friendly dosage form of infliximab for the treatment of patients with Crohn's disease.

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