Analyzing Drug Load and Release from Pharmaceutical Nanocarriers for the Treatment of Inflammatory Bowel Diseases

DISSERTATION

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„The greatest challenge to any thinker is stating the problem

in a way that will allow a solution.“

Bertrand Russell

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1 SUMMARY / ZUSAMMENFASSUNG

1.1 Summary

In the context of new strategy development for the treatment of inflammatory bowel diseases, especially Crohn's disease and ulcerative colitis, nanoparticular systems are considered as promising alternatives to the current treatments. Indeed, because of the accumulation of supramolecular objects in the epithelium of inflammatory bowel, a passive targeting could be achieved using drug carriers with the aim of increasing the efficacy and the safety for the patient.

The objective of the present dissertation is to establish appropriate analytical methods to characterize nanocarriers and secondarily microcarriers. Two different types of formulations, namely the lipid nanoparticles (Lipidot®) and the poly(lactic-co-glycolic acid) (PLGA) micro- and nanoparticles were investigated. Studies were performed using budesonide and cyclosporine A as active pharmaceutical ingredients (API). *In vitro* drug release testing methods based on the derivative spectrophotometry using a fiber optic system were successfully developed for PLGA drug carriers whereas for the first time a solid phase extraction method for nanoparticles was designed and implemented to evaluate the carrying performances of the Lipidot®. Furthermore, the concept of automation was strongly considered during the selection step of analytical techniques with the following outlook: speed up the commercialization of nanopharmaceuticals.

1.2 Zusammenfassung

Im Rahmen der Entwicklung von neuen Strategien für die Behandlung von entzündlichen Darmerkrankungen, hauptsächlich Morbus Crohn und Colitis ulcerosa, werden Nanopartikularsysteme als vielversprechende Alternativen zu aktuellen Behandlungen betrachtet. Tatsächlich könnte, aufgrund einer Akkumulation von supramolekularen Partikeln im Epithelium von entzündlichen Geweben, ein „passive targeting“ mit Nanoträgern erreicht werden und somit zu einer erhöhten Wirksamkeit und größeren Sicherheit für den Patienten führen.

Die vorliegende Dissertation beabsichtigt geeignete analytische Methoden zu entwickeln, um nanopartikulare/mikropartikulare Träger zu charakterisieren. Zwei verschiedene Formulierungen für den Transport von Wirkstoffen, nämlich die Lipid-Nanopartikel (Lipidot®) und die Poly(lactid-co-Glycolid) (PLGA) Mikro- und Nanopartikel wurden untersucht. Für die Experimente wurden Budesonid und Cyclosporine A ausgewählt. *In vitro* Freisetzungsmethoden wurden für PLGA-Wirkstoffträger mit einer Kombination aus Ableitungsspektrophotometrie und einem Fiberoptic Freisetzungssystem erfolgreich entwickelt. Außerdem, wurde erstmalig eine Festphasenextraktionsmethode für die Evaluierung der Beladung von Lipidot® für verkapselte Wirkstoffe entwickelt und angewendet.

Darüber hinaus wurde besonderer Wert auf das Konzept der Automatisierung während der Methodenentwicklung gelegt, um die Kommerzialisierung von Nanopharmazeutika zu beschleunigen.

2 INTRODUCTION

2.1 Background and Significance

2.1.1 Inflammatory bowel diseases and conventional therapies

The inflammatory bowel disease (IBD) actually consists of two major diseases of the digestive tract. However, these diseases are so pathologically close that they may not always be distinguished by the pathologist [1]. At the beginning of the 21st century, 1.4 million patients in the USA and 2.2 million in the Europe suffered from IBD [2]. The most frequent one, Crohn's disease (CD), principally affects the ileum and colon and less frequently other locations in the gastrointestinal tract (mouth, esophagus, stomach or anus), whereas Ulcerative Colitis (UC) is more restricted to the rectum, though whole the colon may be involved [3]. The inflamed locations are continuous for UC but discontinuous for CD and histologically, the inflammation is often transmural for CD, while it is restricted to the mucosa for UC. Both are relapsing inflammatory chronic diseases. Extraintestinal manifestations may be observed (25 % of patients), such as for example arthritis, tendinitis, erythema nodosum, pancreatitis, myocarditis,… [4]. In the extreme case of the fulminant ulcerative colitis, frequent bloody stools going with bleeding and anemia can necessitate blood transfusions. This form of the disease can be accompanied by colonic dilatation, megacolon, abdominal tenderness, weight loss and high fever. The CD as well presents mild to fulminant forms and can be "fistulizing" [4]. IBD typically appears at the ages from 10 to 30 year old or later at around 60 year old [1]. Concerning the etiologies, many scientific studies were performed to identify the causes of the disease but the state of the knowledge is unfortunately still very lacking. To date, the main hypothesis relies on an auto-immune response in the mucosal tissue due to environmental and genetic factors [3]. Furthermore, even though the diagnostic tools improved over the last decades, the incidence of the disease increased and spread world-wide [5]. Concerning the current conventional treatments for IBD, 5-aminosalicylates like mesalazine (first-line therapy for UC), antibiotics like metronidazole, corticosteroids like prednisone or budesonide (BUD) and immunosuppressive agents like azathioprine or

cyclosporine A (CSA) are used for both the inducing and the maintaining of remission phases [6]. For the refractory and fulminate forms, especially if no mucosal healing is reached, surgery is generally required [7]. For CD, 80 % of patients need an abdominal surgery and 10 % a permanent stoma, whereas for UC, 10 % to 30 % required a colectomy [8]. Moreover, the conventional treatments are often not efficient enough and accompanied by adverse side effects. The corticosteroids used for short-term strategies often lead to mild side effects and more rarely to strong side effects like psychosis. The long-term treatments however induced stronger and irreversible adverse side effects (exogenous hypercortisolism) [9]. Immunosuppressive agents increase the risk of malignancy [10], as well as opportunistic infections. 5-aminosalicylates have been associated to hematological side effects [11].

More recently, biological therapies were developed for IBD to restore the balance between the pro- and the anti-inflammatory signals. These treatments can be based for instance on the blockage of the tumor necrosis factor using the following monoclonal antibodies (mAb): infliximab (Remicade®), adalimumab (Humira®), certolizumab pegol (Cimzia®) or golimumab (Simponi®). The natalizumab (Tysabri®), approved by the FDA in 2004, inhibits the integrines to prevent the leucocytes adhesion at the digestive tractus. However, a serious adverse side effect namely the progressive multifocal leukoencephalopathy limits the use of this treatment. More recently in 2013, another mAb based on the same mechanism but without that adverse side effect was approved for IBD: the vedolizumab (Entyvio®). Another pharmacological alternative achieved by the ustekinumab (Stelara®) involves the blockage of the interleukins 12 and 23 required for the modulation of the immune system. Lastly, though recently developed, monoclonal antibodies therapies represent a very potent strategy for inflammatory bowel diseases [12].

To sum up, IBD can present severe forms and the etiologies are still poorly known. The current pharmacological treatments are often not efficient enough and lead to adverse side effects. This dissertation is actually to be placed in the context of new drug delivery strategies for inflammatory bowel diseases.

2.1.2 New therapy strategies for inflammatory bowel disease

C. Lautenschläger et al. [13] recently reviewed several alternatives to the current drug delivery strategies for IBD:

- Designing inactive prodrugs that release *in vivo* active drugs
- Embedding drugs into polysaccharide matrices
- Controlled released drug delivery systems (Pressure-controlled and Osmotic-controlled Release Oral delivery Systems (OROS))
- Self Micro-Emulsifying Drug Delivery Systems (SMEDDS) to increase the bioavailability
- Drug targeting using cell-based drug delivery systems (bacterial and eukaryote cells) or synthetic drug carriers (e.g. liposomes, micro- or nanoparticles)

Drug targeting can be considered of a great relevancy for IBD considering the properties of the inflamed lining of the intestine. Indeed, for an experimental rat model colitis, an accumulation of entrapped drug in the inflamed regions was observed and depended of the carrier size [14]. Three pathways were actually described for the gastrointestinal uptake of nanoparticles: an uptake by a paracellular pathway or via the jejunal cells lining the intestinal mucosa (intracellular uptake) or else via the M-cells and Peyer's patches [15]. In addition, an effect comparable to the so-called enhanced and permeation effect (EPR) is likely to occur in the inflamed locations of the gut. Initially described for tumor tissues, this effect is supposed to be caused by abnormal gaps between endothelial cells of the blood vessels of tumors [16]. The EPR-effect was thoroughly highlighted among the scientific publications during the last two decades but its relevance is more and more discussed nowadays [17, 18]. Even if the mechanisms of the accumulation of entrapped drug within the inflamed mucosal areas are not clearly identified, the concept of drug targeting using micro- or nanocarriers is still promising for IBD, as it was shown for example in the case of Polyethylene glycol (PEG)-functionalized poly(lactic-co-glycolic acid) (PLGA)-microparticles [19].

This strategy was selected in the setting of this dissertation. The selected drug carriers were lipid nanoparticles (Lipidot®) and polymeric particulate systems, namely the PLGA-microparticles and PLGA-nanoparticles. The carried APIs were two model drugs for the treatment of IBD: budesonide and cyclosporine A. In the case of budesonide (see Figure 1), a well-absorbed corticosteroid, the systemic bioavailability remains low because of a high first-pass hepatic metabolism. In addition, the risk of side effects is decreased with budesonide in comparison to prednisolone [20]. Moreover, opportunistic infections can be observed [4].

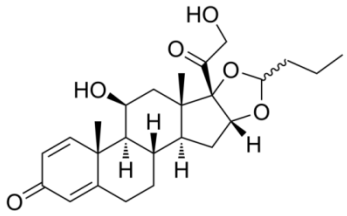

Figure 1: Budesonide

Concerning cyclosporine A, a molecule naturally produced by the fungus Tolypocladium inflatum, the chemical structure consists of a cyclic polypeptide of eleven amino acids (see Figure 2). Because of its immunosuppressant pharmacological activity, cyclosporine A can be a cause of opportunistic infections for the patient. Moreover, nephrotoxicity, hypertension, headache, gingival hyperplasia, hyperkalaemia, paresthesias, and tremors can be observed [21]. Nevertheless, this API has a strong efficacity, in particular for severe UC [22].

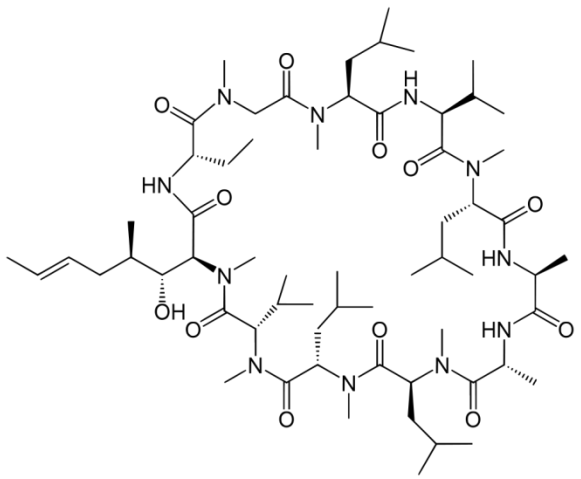

Figure 2: Cyclosporine A

2.1.3 Chemical characterization and performance tests for micro- and nanoparticulate drug carriers

To achieve drug targeting, the following three conditions should be fulfilled for drug carriers:

1. The initial free API amount is negligible compared to the entrapped API amount.
2. The API is maintained entrapped in the carrier until the target is reached.
3. The carrier releases the API at the target: at or in the tissue or in the cell.

In this setting, the repartition of the API between the different compartments of the formulation should be quantitatively known after manufacturing and naturally should not significantly differ at the time of the administration (product stability). Consequently, performant analytical tools with reliable physical or mathematical separation methods are required to demonstrate the physical and chemical suitability of the formulation.

Furthermore, the destiny of the API after administration requires monitoring. For this purpose, *in vitro* drug release testing (DRT) studies are generally performed in the case of conventional dosage forms. These tests are even considered as surrogate to clinical trials [23]. However, in the case of drug carriers, considering the three previously mentioned conditions, the concept of *in vitro* DRT appears completely different or even inappropriate as such. Indeed, though a sustained drug release at the targeted site is the main objective, the carrier should be on the contrary the most stable possible during the trip until the target so that a drug release does not prematurely occur. Actually, a concept for *in vitro* drug-load stability testing is currently missing but may be much more relevant for delivery strategies based on targeting than *in vitro* DRT only. Indeed, to re-create the mechanical and physicochemical environments crossed by the carrier until the target would be informational enough to optimize the formulation before further *in vivo* investigations. Unfortunately, no stress tests for micro- or nanoparticulate drug carriers have been noticed in the scientific literature precisely for the assessment of the drug-load stability of the system. However, the chemical and thermical stress tests usually carried out for the stability of conventional

formulations can be adapted to the nano-/microcarriers. Besides, biorelevant media can be used to reproduce the stress conditions of the route of application until the target.

Nowadays, many characterization tools could already be conceivable for the separation of API from particles or for *in vitro* drug release tests of drug carriers. Still, few of these techniques have intensively been investigated for drug carriers. The following parts review the state of the art for these techniques.

2.1.3.1 Separation methods: State of the art for micro- and nanoparticles

The quantification of free residual API still present after the manufacturing of micro- or nanoparticles or after release over an *in vitro* DRT often requires separations methods. A short overview of the relevant separation techniques is given below:

The dialysis-based methods are strongly represented among these techniques. Dialysis is often used as a purification technique to remove free residual API or excess of excipients after manufacturing. The technique generally involves a dialysis tube filled with the formulation and placed in a large volume of aqueous medium. The medium is maintained under stirring from half a day to several days and may be refreshed at interval times to maintain the concentration gradient as strong as possible [24, 25] (see Figure 3). Such dialysis separation methods are often implemented to calculate the loading efficiency of the manufacturing process [26]. Nevertheless, a significant limitation of the technique is the time-consuming permeation of the API through the membrane and the subsequent drug release that could occur during the process. In addition, a significant amount of nanoparticles has been documented to be lost by accumulation at the membrane [27]. Dialysis methods used for *in vitro* drug performance tests are detailled in paragraph 2.1.3.2.

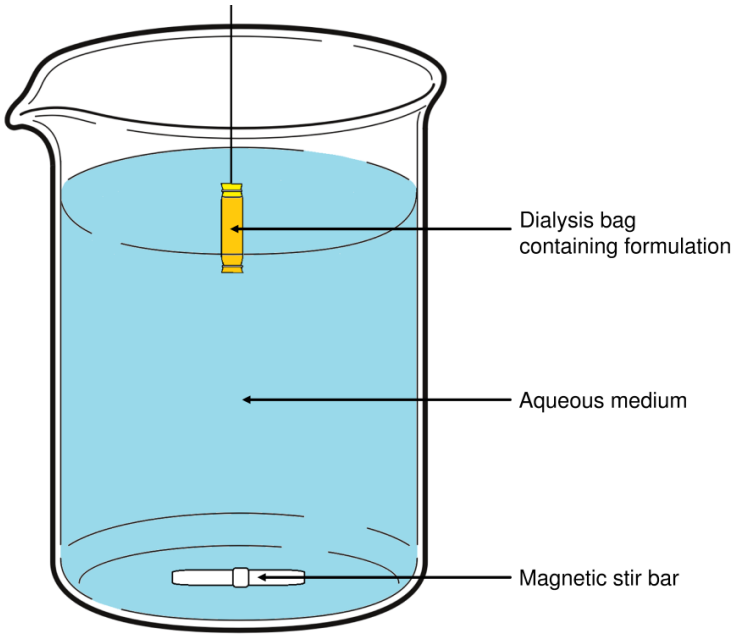

Figure 3: Basic configuration for the dialysis separation techniques

Further, a Spin Centrifugation–Dialysis (SCD) method was developed for the purification of nanoparticles. This preparative method consisted of a centrifugation step (1,000 g for 12 h) and a dialysis step (4-6 h). Although it would remain time consuming for an analytical method, the technique is inexpensive, easy, automatable and presented equivalent results to the reference technique of the study, namely the Tangential Flow Filtration (TFF), presented below [28].

Tangential Flow Filtration (TFF) or Cross-Flow Filtration (CFF) is a technique where the flow of a liquid containing the particles is parallel to the filter. The API to be separated from the particles permeates through the filter. CFF is usually used in R&D for the purification of nanoparticles or microparticles. The main advantage over dead-end filtration techniques is the decreased risk of clogging at the filter (see Figure 4). An electrostatic field can even be applied to optimize the filtration [29]. However, the deformability property of semi-solid nanoparticles is a limitation for the use of CFF. Lipid nanocapsules with initial size were reported to be detected in CFF filtrates for membranes with MWCO smaller than the particle diameter [27].

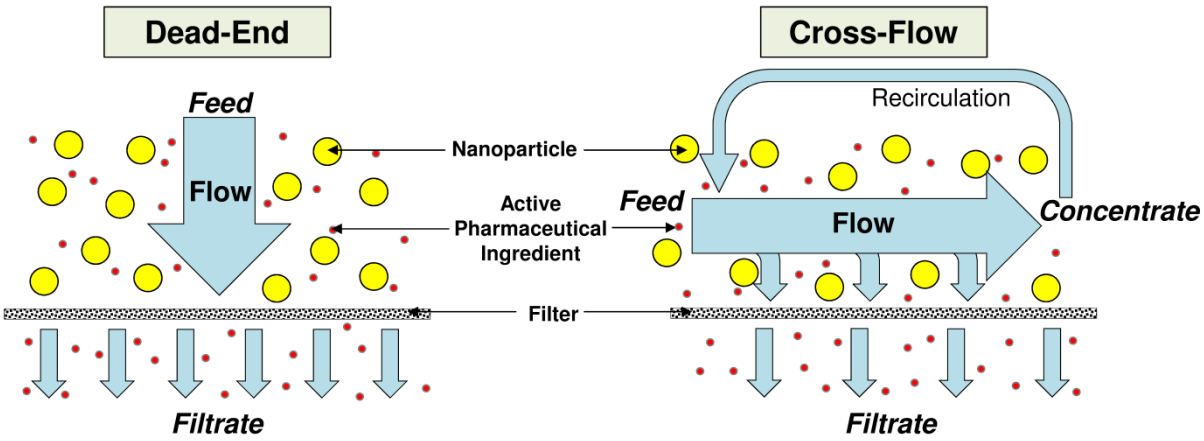

Figure 4: **Dead-End Filtration (left) versus Cross-Flow Filtration techniques (right)**

Giddings introduced in 1966 the concept of Field-Flow Fractionation (FFF) [30] which consists a separation of particles by differential migration. A field is applied perpendicularly to a narrow tube containing the pumped fluid with particles [31]. The tangential flow of the fluid is laminar and faster in the center of the tube. The active pharmaceutical ingredients, then the smallest particles, are eluted before the largest particles carried by the field to the so-called “accumulation wall” (see Figure 5).

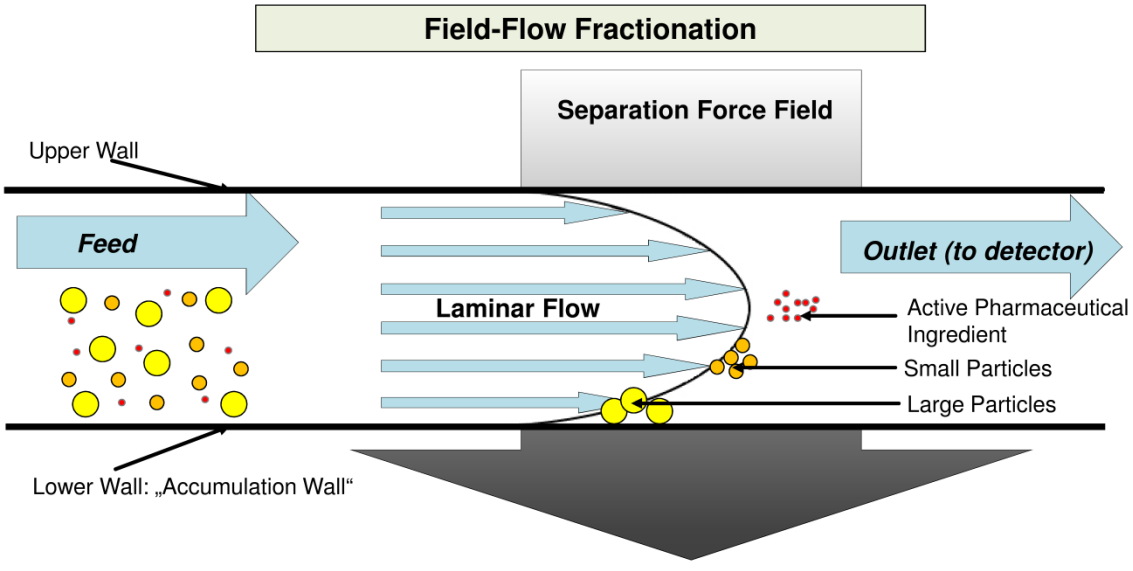

Figure 5: **Principle of the Field-Flow Fractionation (FFF) techniques**

The most common FFF techniques are the Symmetric and Asymmetric Flow FFF. In the case of Symmetric Flow Field-Flow Fractionation (SF4), the perpendicular field consists of a fluid flow crossing the tube, entering through an upper semi-permeable membrane and exiting through a lower semi-permeable membrane [32] (see Figure 6).

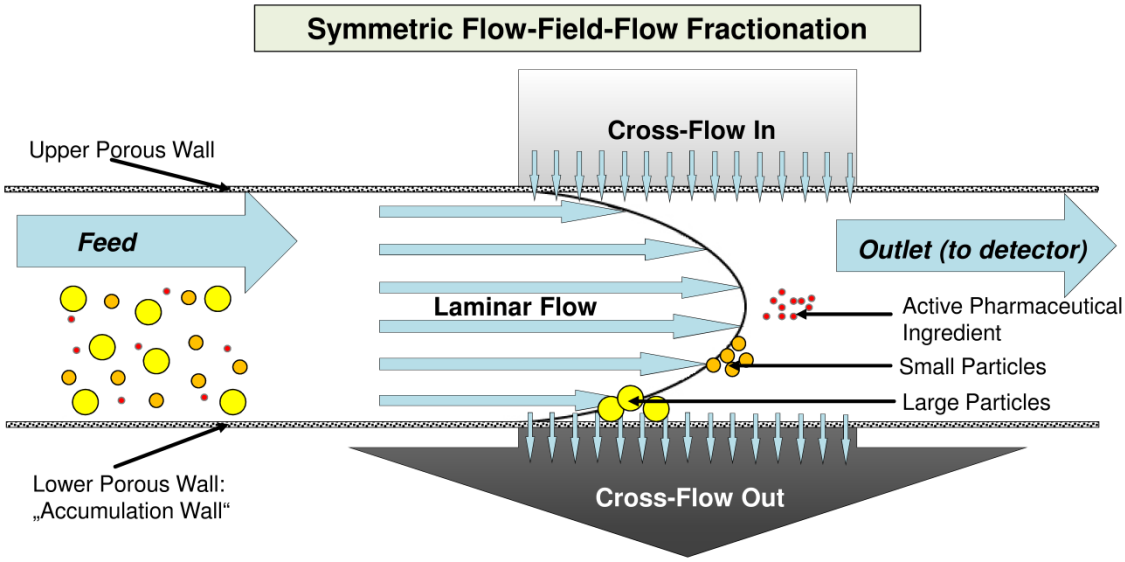

Figure 6: Principle of the Symmetric Flow-Field-Flow Fractionation (SF4) technique

For Asymmetric Flow FFF (AF4), the perpendicular field is caused by the tangential fluid itself exiting through a single bottom semi-permeable membrane [33] (see Figure 7).

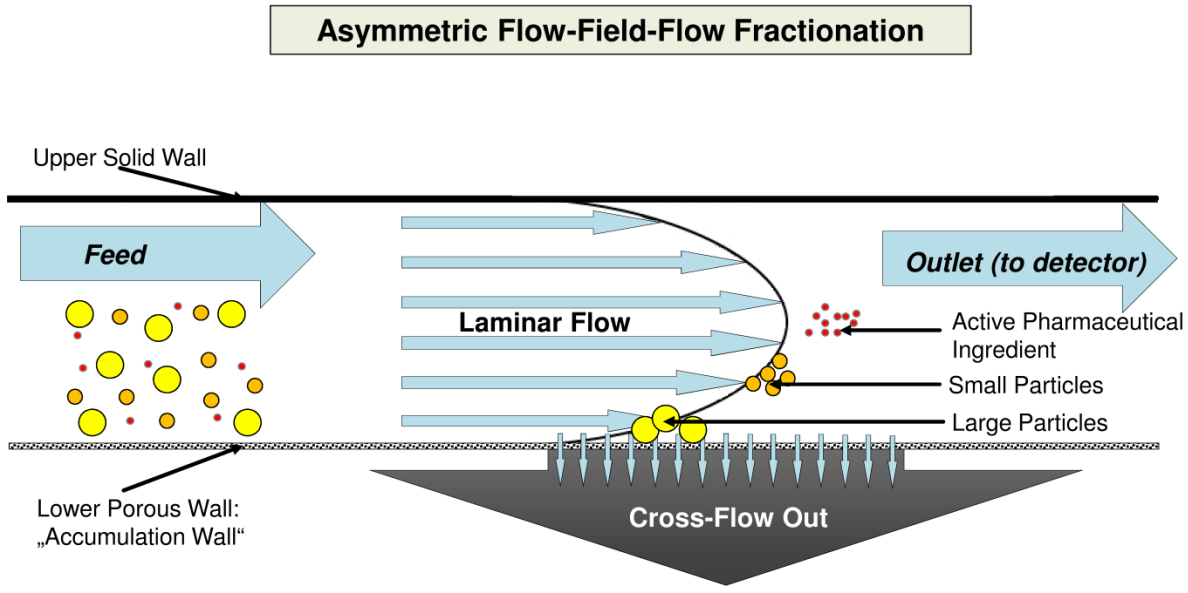

Figure 7: Principle of the Assymmetric Flow-Field-Flow Fractionation (AF4) technique

Actually, a plethora of FFF techniques are available depending on the separation force field: Sedimentation FFF (SF3), Magnetic FFF (MgFFF), Medium Temperatur Asymmetric Flow-FFF (MT AF4), Hollow-Fiber Flow FFF (HF5), Dielectrophoretic FFF (DEP-FFF), Split Flow Thin Cell Fractionation (SPLITT), Thermal FFF (ThFFF)... The latter is an example of FFF where a perpendicular temperature gradient is achieved warming up the upper wall and cooling down the bottom wall (see Figure 8).

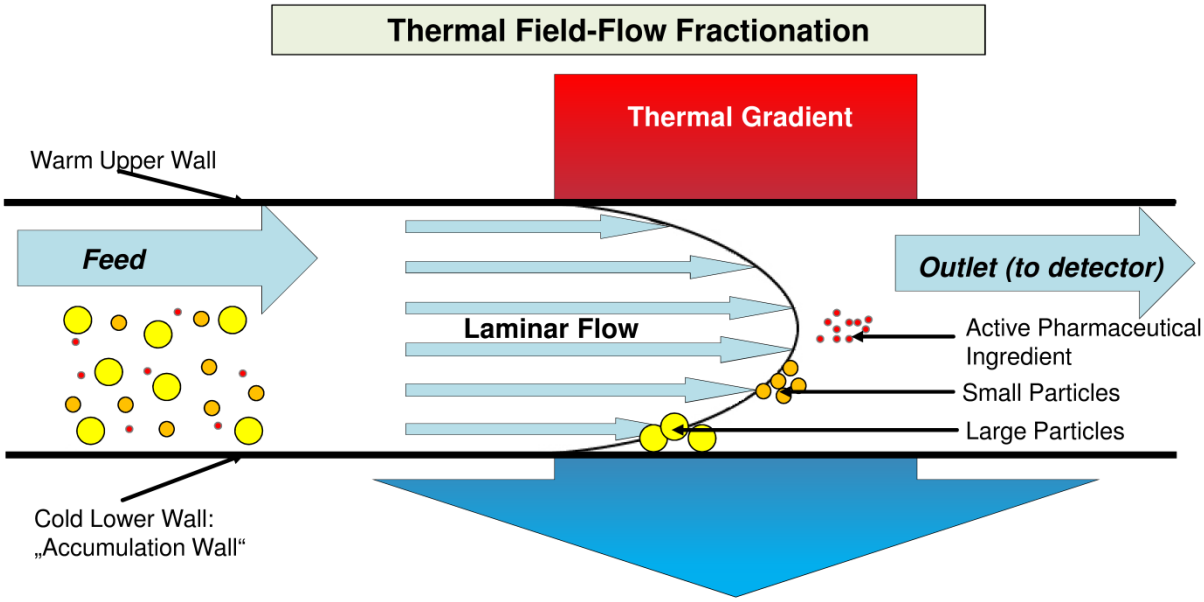

Figure 8: Principle of the Thermal Field-Flow Fractionation (ThFFF) technique

A coupling of FFF to a physical detection technique can be achieved with many physical detectors. Although field flow fractionation offers decisive advantages for industry (automation, robustness…), very few methods are described in the field of the separation of free drug from nanoparticles. The technique is usually rather used for the separation of particles or polymers one to another. Nevertheless, the determination of the encapsulation efficiency of APIs has been already performed for liposomes using AF4 [34]. In addition, a method based on AF4 was successfully implemented not to monitor the *in vitro* drug release but the nanoparticle release from tablet dosage form [35].

As for FFF, Analytical Ultracentrifugation (ANUC or AU) can be used to physically characterize particles, polymers or macromolecules (size, distribution, molecular mass) [36]. The principle of the separation is based on the sedimentation velocity after centrifugation at a very high rotation speed (e.g. 100,000 rpm or 1,000,000 g). Usually, for chemical characterization, ultracentrifugation is used to precipitate particles and to quantify the free residual drug in the supernatant or the entrapped drug released after particle disintegration [37].

Concerning the techniques of filtration, the samples are generally eluted under low pressure through 0.1 to 1 µm-sized pore filters (microfiltration, MF) or 0.01 to 0.1 µm-sized pore filters (ultrafiltration, UF). The molecules (API), smaller than the pores, are eluted, whereas the particles, larger than the pores, are retained on the filter (see Figure 9). Though the simplicity of the technique, ultrafiltration presents disadvantages, especially the formation of cakes obstructing the filters [38, 39]. Moreover, the shape of soft nanoparticles may deform, as previously mentioned for CFF [27], and thus, nanoparticles or fragments may be partially co-eluted in the stream with the API.

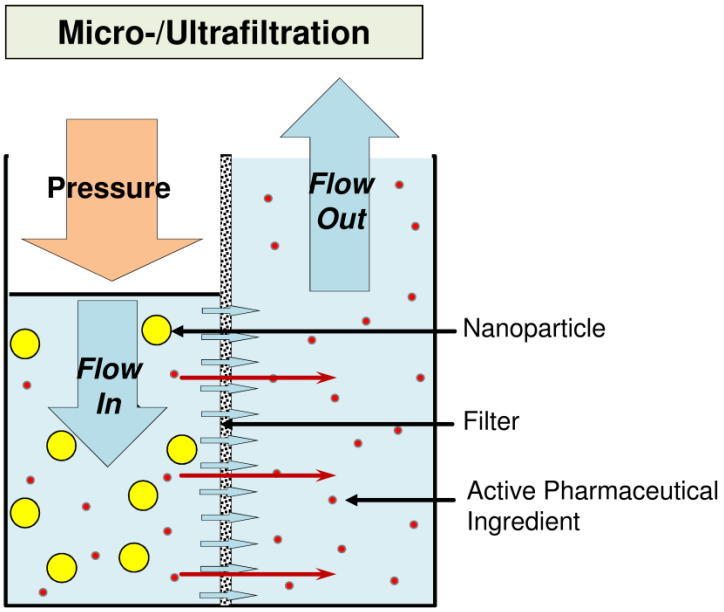

Figure 9: Principle of the Ultra- and Microfiltration techniques

Capillary Electrophoresis (CE) is a family of separation techniques based on the different electrophoretic motilities of electrically charged analytes subjected to an electro-osmotic flow (EOF) through a very narrow capillary (see Figure 10). The inner walls of the capillaries are often covered with ionizable silanol groups whose charge mainly depends on the pH of the buffer solution. Negatively charged silanoate groups tightly retain cations from the buffer forming a positively charged fixed layer. An outer mobile layer consisting of solvated cations can be set in motion when an electrical potential difference between the two ends of the capillary is applied. The generated flow of buffered solution corresponds to the electro-osmotic flow (EOF).

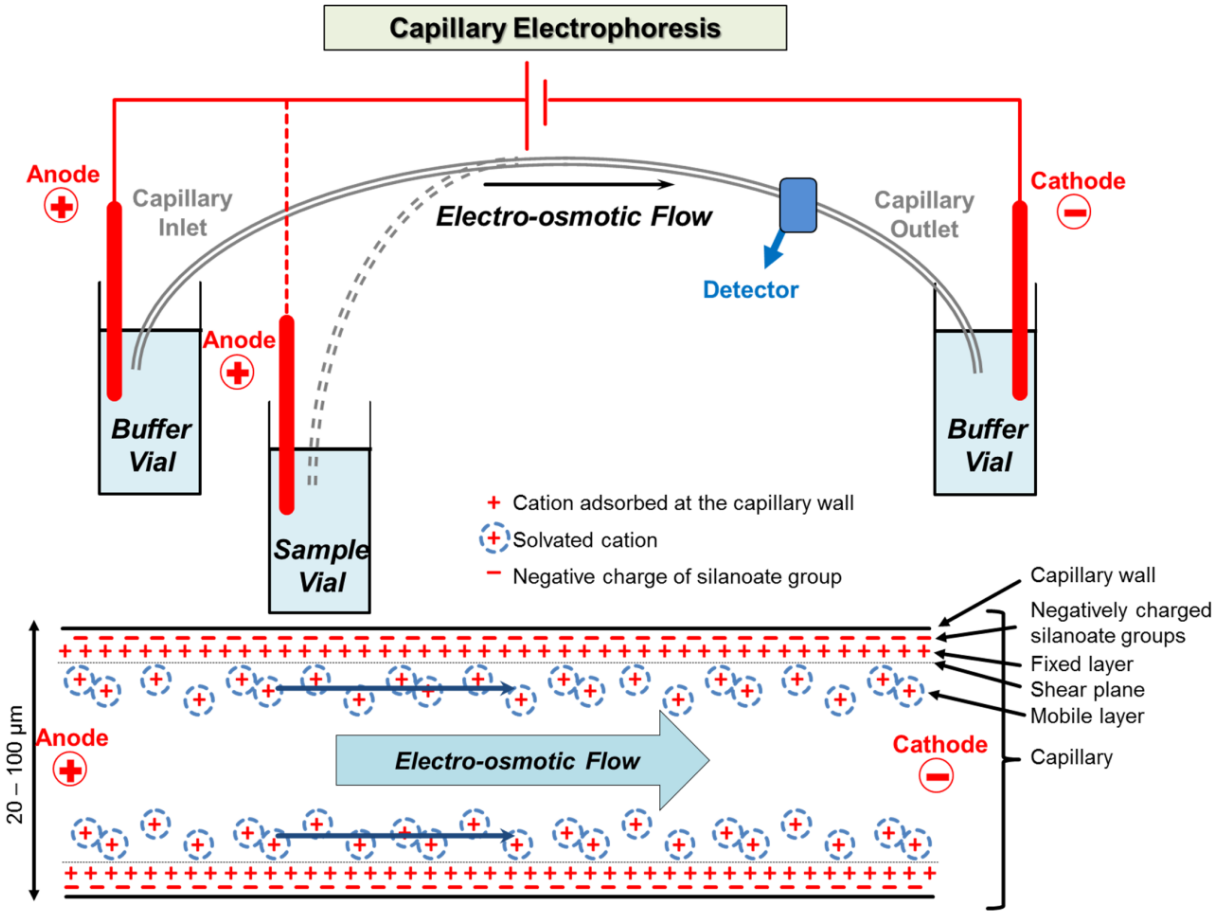

Figure 10: Principle of the Capillary Electrophoresis (CE) technique

The mode of CE is selected according to the analytes to be separated: Capillary Zone Electrophoresis (CZE), Capillary Gel Electrophoresis (CGE), Isoelectric Focusing (IEF),

Isotachophoresis (ITP), Micellar Electrokinetic Capillary Chromatography (MECC) (also reffered to as Micellar Electrokinetic Chromatography (MEKC)) and Microemulsion Electrokinetic Chromatography (MEEKC) are the main modes for capillary electrophoresis.

In the case of neutral nanoparticles for instance, a micellar pseudostationary phase (MEKC mode) or a microemulsion pseudostationary phase (MEEKC mode) can be introduced into the capillary to enable the separation. Such techniques were successfully performed for the separation and quantification of the API from nanoparticles [40] or from liposomes [41].

Size-Exclusion Chromatography (SEC) involves separating large molecules, polymers or nanoparticles by elution through a column containing a stationary gel phase of porous beads. The smaller is a particle, the larger is the diffusion volume and the slower is the retention time (see Figure 11). The elution is solely size-dependent. SEC is also named Gel Permeation Chromatography (GPC) if the eluents are organic and Gel Filtration Chromatography (GFC) if they are aqueous [42].

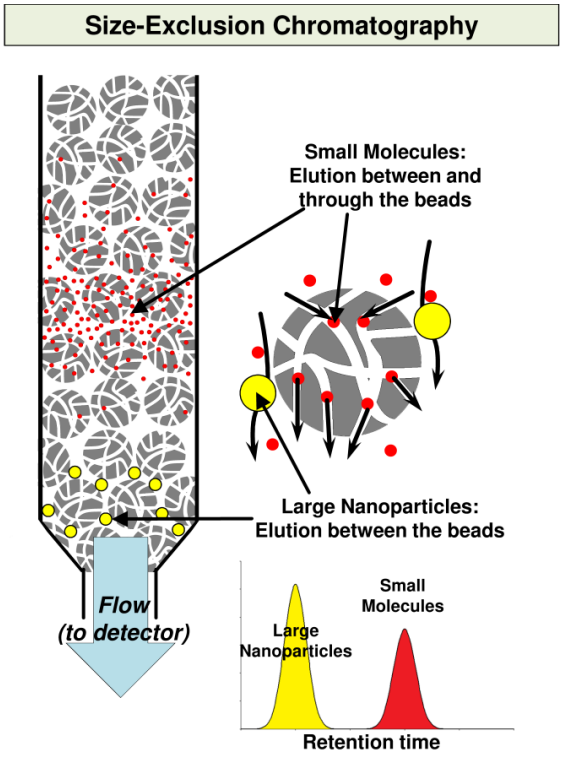

Figure 11: Principle of the Size-Exclusion Chromatography (SEC) technique

This separation technique can be coupled to many different types of detectors such as Ultraviolet detectors (UV), Fluorescence Detectors (FLD), Infrared detectors (IR), refractive index (RI), or Dynamic Light Scattering (DLS) for example. A coupling of SEC with the technique of Matrix-Assisted Laser Desorption Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) was successfully achieved to characterize the chemical composition and molecular weight of nanoparticles [43] and a coupling of SEC to HPLC for liposomes [44]. High Performance Size-Exclusion Chromatography (HPSEC) refers to SEC columns produced using materials that improve the speed and the resolution and allow higher pressure or temperature [45]. Coupling the SEC technique with a sample preparation method for the eluted nanoparticles is conceivable for instance to release and to quantify entrapped drug.

Hydrodynamic Chromatography (HDC) presents the advantage to produce less shear forces on the particles compared to the SEC technique (see Figure 12).

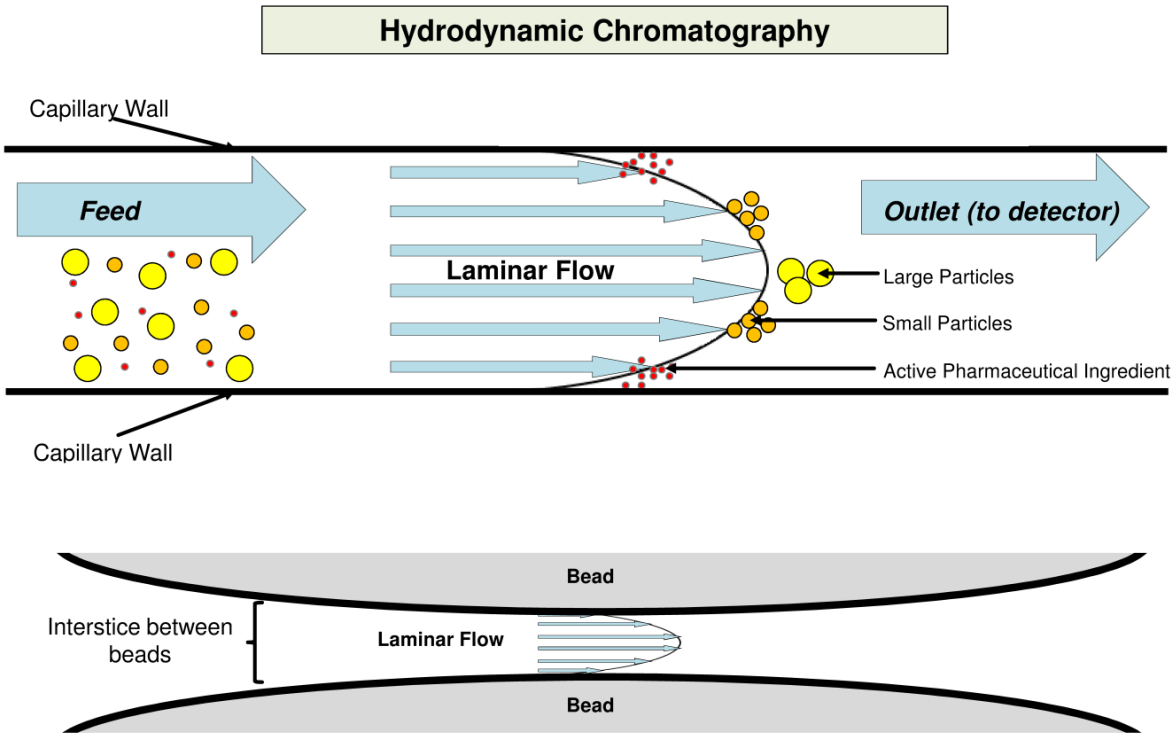

Figure 12: Principle of the Hydrodynamic Chromatography (HDC) technique. Medium circulating through capillaries (up) or between beads (down).

This technique is suitable too for the characterization of the size, distribution and molecular mass of particles and polymers. The sample is injected into an open tube or a column containing beads. The flow between the interstices of the beads or the walls of the tube is maintained laminar with a parabolic profile so that an increasing velocity gradient of the streamlines occurs from the walls until the center. The retention time consequently decreases with the size or molecular mass of the analyte. The elution ranks are the same for both HDC and SEC [46].

Solid Phase Extraction (SPE) is another separation technique based on the same principle as liquid chromatography. It consists of cartridges containing a stationary phase in contact with a mobile phase dragging the analytes reversibly bounded from the stationary phase [47]. SPE is generally considered as a very powerful separation technique [48]. In addition, the implementation of the SPE technique to separate the API from the carrier has already been investigated for liposomes [49, 50]. In the context of this dissertation, methods based on solid phase extraction were developed for lipid nanoparticles (see Chapter III).

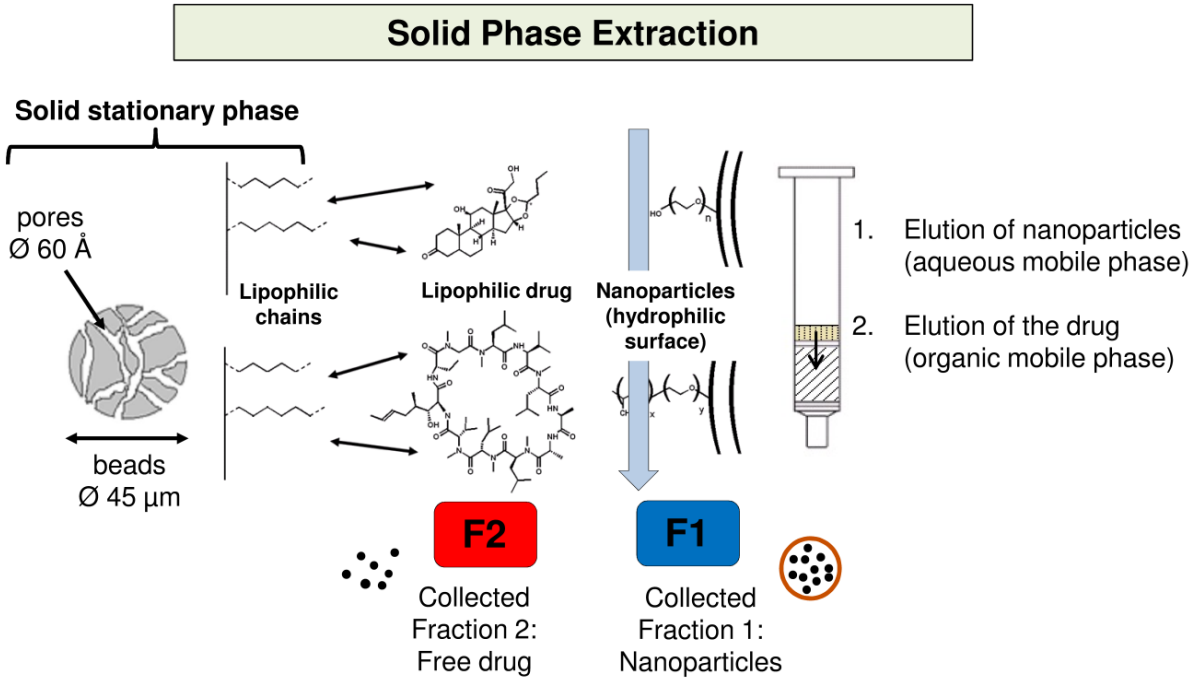

Figure 13: Principle of the Solid Phase Extraction (SPE) technique as used in Chapter III

Concurrently with physical separation methods, other techniques can be directly used for the determination of encapsulation efficiency. For instance, an ^{1}H NMR-Spectroscopy method could be developed for liposomes with very accurate and robust results [51]. Furthermore, mathematical treatments based on derivative spectrophotometry can be used as "virtual" separation methods (see Chapter I and Chapter II).

2.1.3.2 *In vitro* Drug Release Testing: State of the art for micro- and nanoparticles

According to the USP, “drug product performance may be defined as the release of the active pharmaceutical ingredient (API) from the drug product dosage form, leading to systemic availability of the API necessary for achieving a desired therapeutic response.” [23]. For conventional dosage forms, *in vitro* drug release tests are carried out to assess the drug product performance and more precisely the “bioavailability” of the API. This term covers not only the amount of API reaching the blood stream but the kinetic of the phenomena as well. Two successive phases, actually starting concomitantly, the “disintegration” and the “dissolution”, follows after administration of the product and happens before the so-called ADME scheme (Absorption, Distribution, Metabolization and Excretion). The three main phases after oral administration are described in Figure 14.

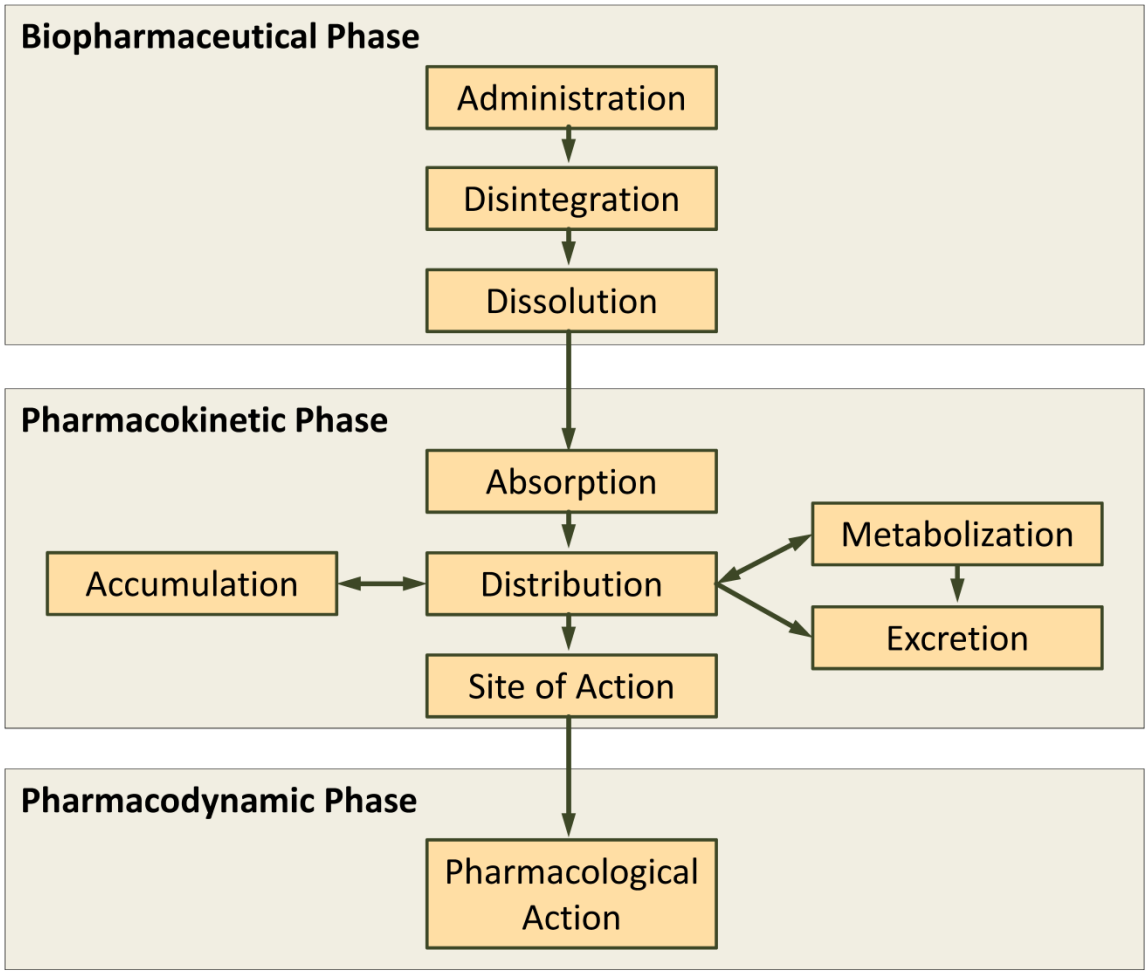

Figure 14: **Successive stages following the oral administration of a drug**

The disintegration actually corresponds to the loss of consistency, shape and finally the total dismantlement of the dosage form. This biopharmaceutical phase is investigated using disintegration tests. As concerns the dissolution, the process refers to the passage from a crystal or an amorphous form of the API or an excipient-binded form toward a molecular form of the API in solution. The following absorption phase occurs only for the molecular form of the drug. This is the reason why the dissolution rate and completion determines the bioavailability of the drug. The solubility of the API is an essential factor with an effect on the dissolution. On the other hand, many API-independent factors, like the temperature, the pH and the medium, may have a strong influence on the dissolution. *In vitro* DRT is a powerful tool to investigate the drug performance since most of the influent factors can be assessed using standardized apparatus. However, the dissolution cannot directly predict the bioavailability or biologic activity. Theoretical models based on the Fick's law have been developed to explain the dissolution mechanism. In 1897, Noyes and Whitney described the process using the following expression: $dM/dt = DS/h(C_s-C)$, where dM/dt represents the mass rate of dissolution, M the amount of drug dissolved at the time point t, D the diffusion coefficient of the drug in the solution, S the surface area of the solid, h the thickness of the diffusion layer, C_s the saturation concentration of the drug and C the concentration in the bulk solution at the time point t [52]. If the concentration C is significantly less than C_s and considering S and h constant, then the dissolution rate is proportional to the saturation concentration, i.e. the dissolved concentration increased with a first order rate over time. These conditions, so-called sink conditions, occur at the beginning of the test before the surface area is changed and for concentrations less than $1/10^{th}$ [53], $1/5^{th}$ [53] or less than $1/3^{rd}$ [23] of the saturation concentration. More complex models were developed to consider more specifically the pharmaceutical form of the drug. Furthermore, *in vitro* DRT are perfectly suited during the formulation development to identify the "critical manufacturing attributes such as the impact of ingredient properties and the impact of the manufacturing process on drug product performance." [23]. Indeed, the manufacturing of a pharmaceutical product with

very reproducible and robust drug performances is required to guarantee the safety and efficiency to the patient. Initially, *in vitro* DRT was “developed as a quality control tool to ensure drug product quality and batch-to-batch consistency.” [23]. In the context of quality control, the simpler is the apparatus, the more robust and reproducible are the studies. Several devices are described in the American, European and Japan pharmacopoeias. The rotating basket apparatus (USP apparatus 1) and the rotating paddle apparatus (USP apparatus 2) [23] are simple devices which consist of a 1,000 mL vessel containing the dissolution medium and a vertical shaft equipped either with a basket containing the dosage form or a paddle (see Figure 15). The shape and the size as well as the position of the paddle or basket are precisely standardized. The temperature of the medium is normally maintained at 37 °C (± 1 °C), and for cutaneous or ophthalmic routes, at 32 °C (± 1 °C). Under current Good Manufacturing Practices (cGMPs), apparatus suitability tests should be performed [23]. Non compendial variations of these devices are commercially available, such as for instance the Palmieri basket designed for suppositories. The pharmacopoeias describe as well the reciprocating cylinder apparatus (apparatus 3 of the USP), also known as the Bio-Dis, the flow-through cell (USP apparatus 4), the paddle-over-disk (apparatus 5), the rotating cylinder (apparatus 6) and reciprocating holder (apparatus 7) apparatuses [23]. Since May 2014, the following apparatuses were recognized by the USP for the determination of the drug release rate for semi-solid dosage forms like creams, gels or ointments: the vertical diffusion cell apparatus, also called the Franz-diffusion cell, the immersion cell apparatus and an adapter for topical dosage forms to be placed into the USP apparatus 4 [23]. The flow-through cell apparatus was initially designed for poorly soluble solid dosage forms since an open loop configuration provides a continuous stream of fresh and drug-free medium directly pumped from a reservoir to allow the maintain of the sink conditions [54] (see Figure 16).

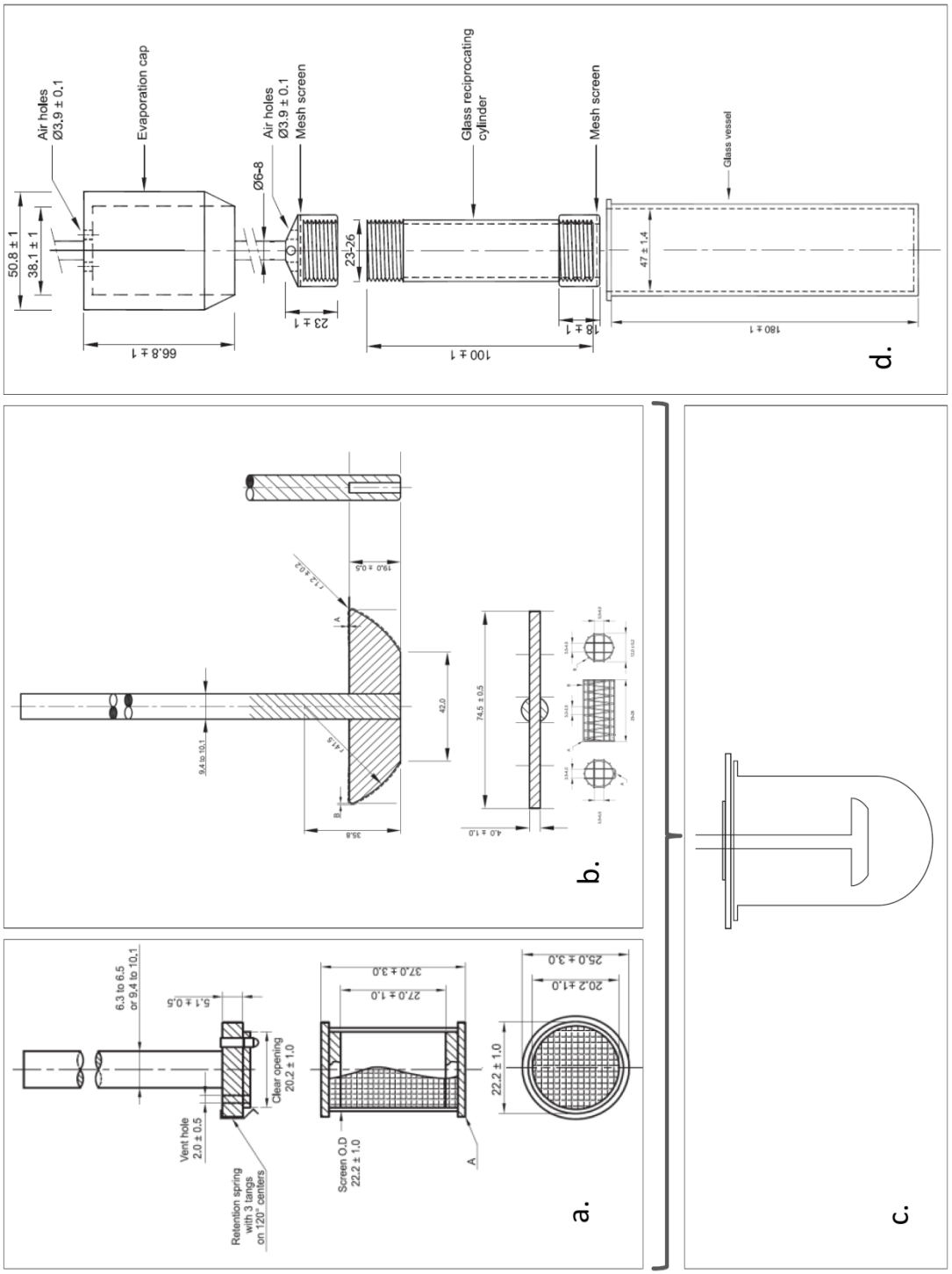

Figure 15: Dissolution apparatus for solid dosage forms, according to the European Pharmacopoeia[55]. a.: Basket stirring element of the apparatus 1 (basket apparatus). b.: Paddle stirring element of the apparatus 2 (paddle apparatus) and sinker (bottom). c.: 1 L-glass vessel with paddle (apparatus 2). Same vessels are used with baskets (apparatus 1). d.:Reciprocating cylinder (apparatus 3) and glass vessel (bottom).

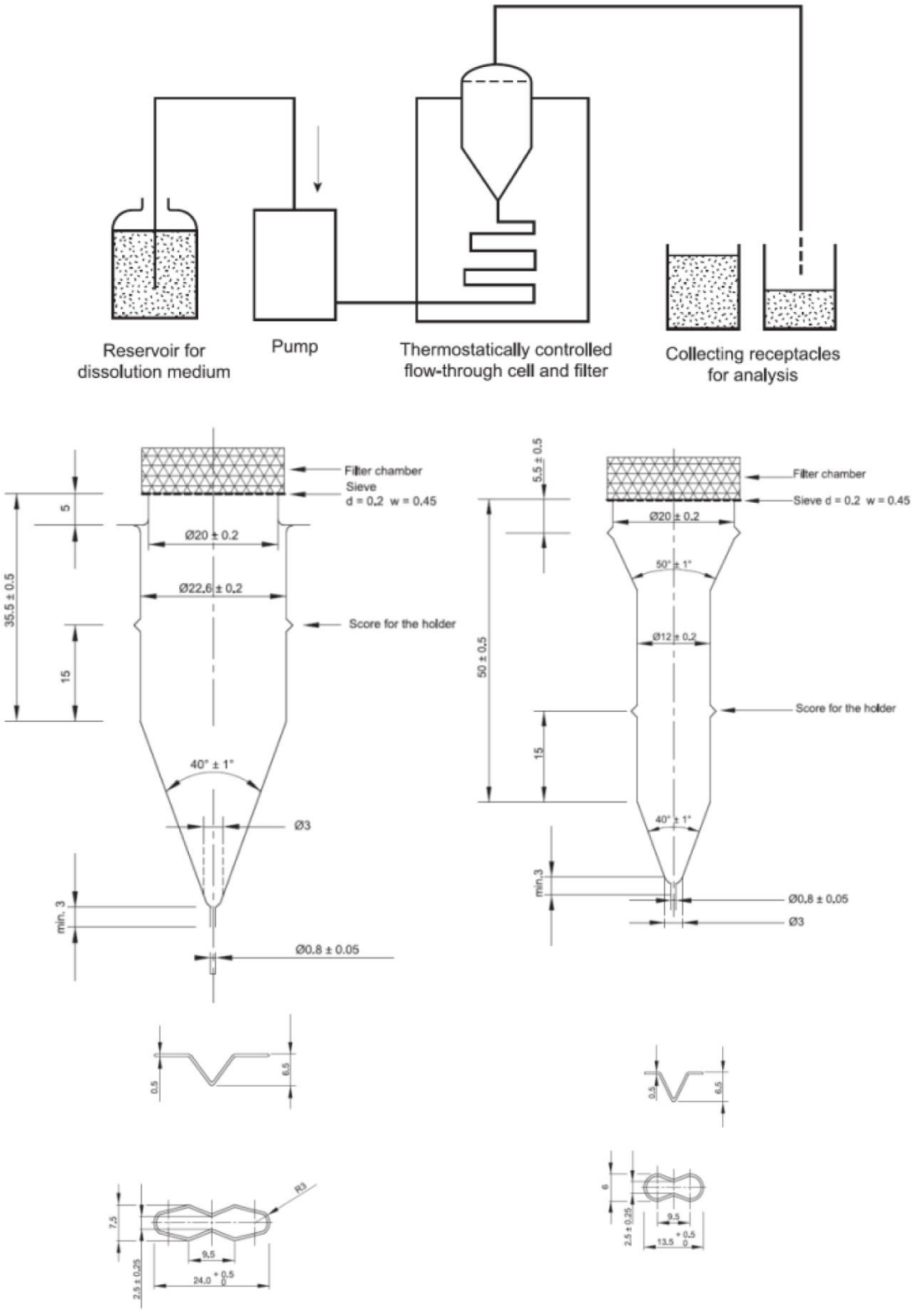

Figure 16: Apparatus 4 (Flow-through cell apparatus), according to the European Pharmacopoeia [55]. Top: schematic representation of an open loop circuit. New dissolution medium is continuously pumped into the cell so that sink conditions can be achieved. Down-left: large cell for tablets and capsules with tablet holder for large cell (bottom). Down-right: small cell for tablets and capsules with tablet holder for small cell (bottom).

Concerning micro- and nanoparticulate systems, the concept of *in vitro* DRT does not basically differ from for conventional dosage forms if these systems are intended to increase the apparent solubility and bioavailability of poorly soluble drugs. For instance nanocrystals are drug crystals manufactured at a nanoscale to increase the specific surface area and accelerate the dissolution velocity [56]. However, nanoparticles can be produced as drug carrier for targeting purpose and even be injected directly into the blood stream, bypassing the biopharmaceutical phase. Since *in vitro* DRT is basically aimed to simulate the destiny of the API during the biopharmaceutical phase, the sense of such investigations for drug carriers becomes misleading. Instead, *in vitro* tests related to the destiny of the carriers in the blood circulation and microcirculation may become relevant. Tools such as microfluidic mimetic microvessels for example might become in the future of highest interest too [57]. Anyway, in the absence of suitable procedures, researchers still perform *in vitro* DRT as analytical tool for the optimization of the manufacturing and formulation process.

As concern the drug performance assessment for nanoparticles or microparticles, most of the current methods do not involve compendial apparatus [58, 59]. The most documented procedures for *in vitro* DRT of nanoparticles involve a tightly sealed dialysis tube containing a volume of few milliliters sample. The MWCO of the selected dialysis membrane should be smaller than the particle size. The tube is placed into the dissolution medium maintained under stirring at 37 °C. The release samples are withdrawn over time and the medium may be refreshed to maintain the suitable conditions for the dialysis process [60, 61] (see in Figure 3 the configuration for the purification of nanoparticles). For that purpose, a continuous flow dialysis setup can even be achieved using pumps and the collected medium can be analyzed by HPLC [62]. Many configurations based on the dialysis principle have been documented for *in vitro* DRT. Floating dialysis tubes such as the Float-A-Lyzer® from SPECTRUM® LABORATORIES, INC are commercially available and successfully used for *in vitro* DRT [63] (see Figure 17).

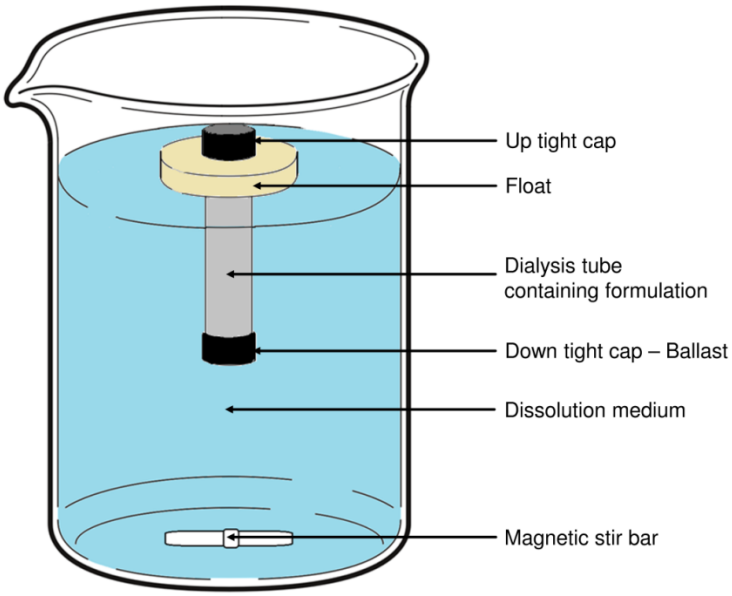

Figure 17: The Float-a-Lyser® device for *in vitro* DRT.

Dialysis tubes may be placed as well in USP apparatus 2 (paddle) by mean of an adapter [64] (see Figure 18).

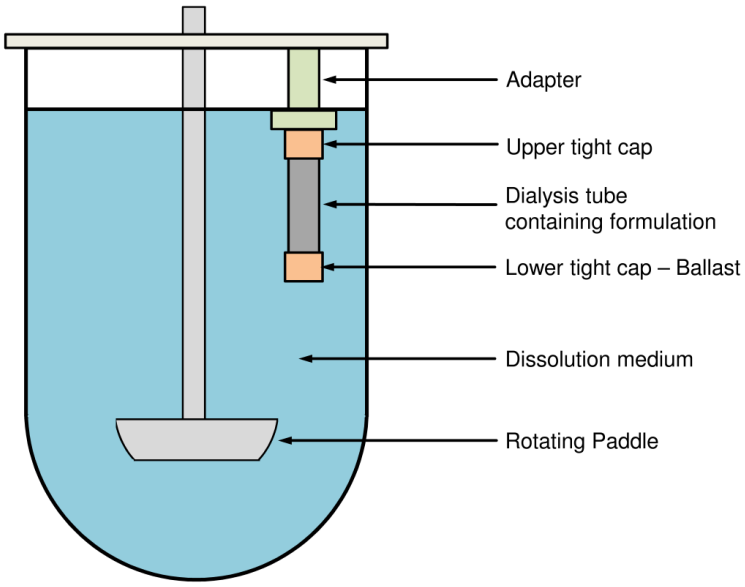

Figure 18: USP apparatus 2 (paddle) in combination with dialysis tube.

However, the sedimentation of nanoparticles may occur during the test since the samples are not agitated in the dialysis bag. Therefore, apparatuses like the USP apparatus 1 (basket) modified replacing baskets by vertical glass cylinders ended at the bottom by a

dialysis membrane may theoretically lead to more promising solutions [65, 66] (see Figure 19).

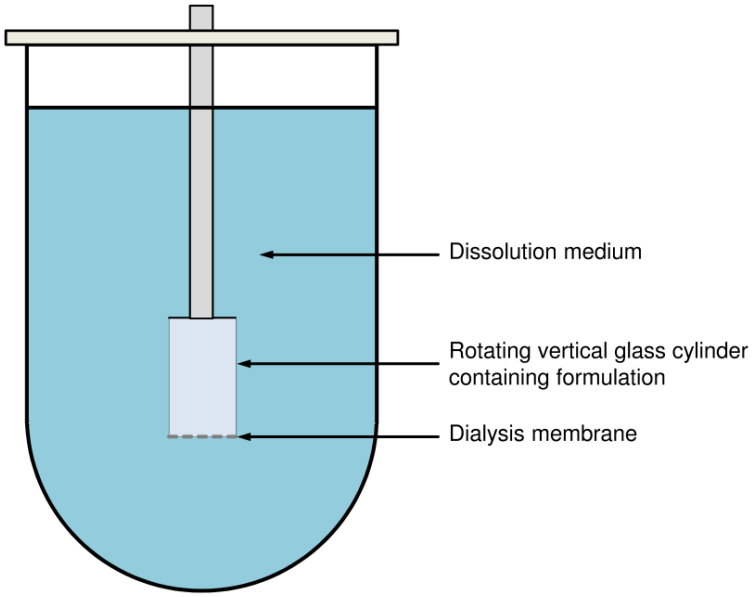

Figure 19: Modified USP apparatus 1 (basket) with vertical glass cylinders and dialysis membrane.

In addition, the standardization of the procedure is an important issue that is why methods using compendial apparatus remain the most attractive. For example, a Dialysis Sac Adaptor (DSA) has been designed to be placed in a standard cell of the USP apparatus 4 (flow-through cell) and has been tested for dispersed systems [67] (see Figure 20).

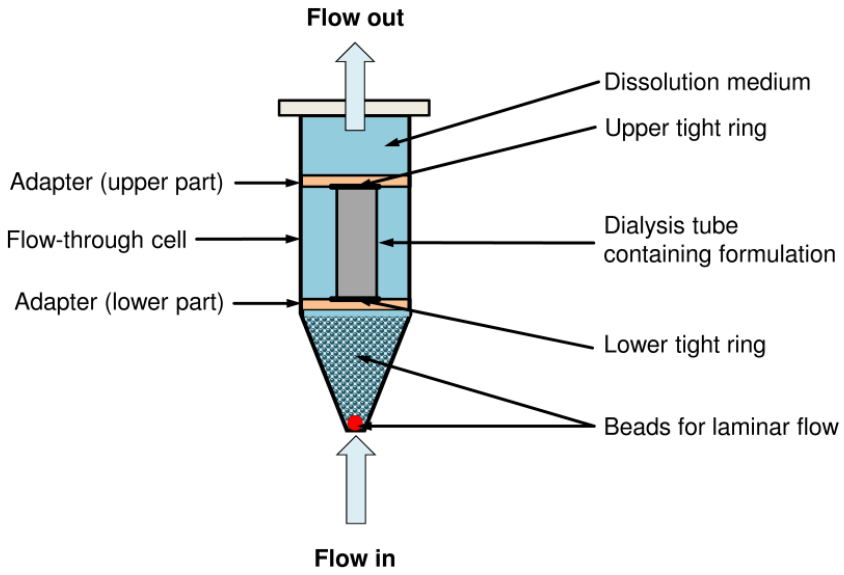

Figure 20: USP apparatus 4 (flow-through cell) and Dialysis Sac Adaptor.

Another technique using a compendial apparatus without any adaptation, since already equipped with a dialysis membrane, namely the Vertical Diffusion Cell (VDC), also called Franz-cell, has been reported for transdermal patches containing loaded nanoparticles [68]. The USP therefor points out the slope of the cumulative drug amount released plotted versus $\sqrt{t}$ (where t = time) represents the rate of drug release [23].

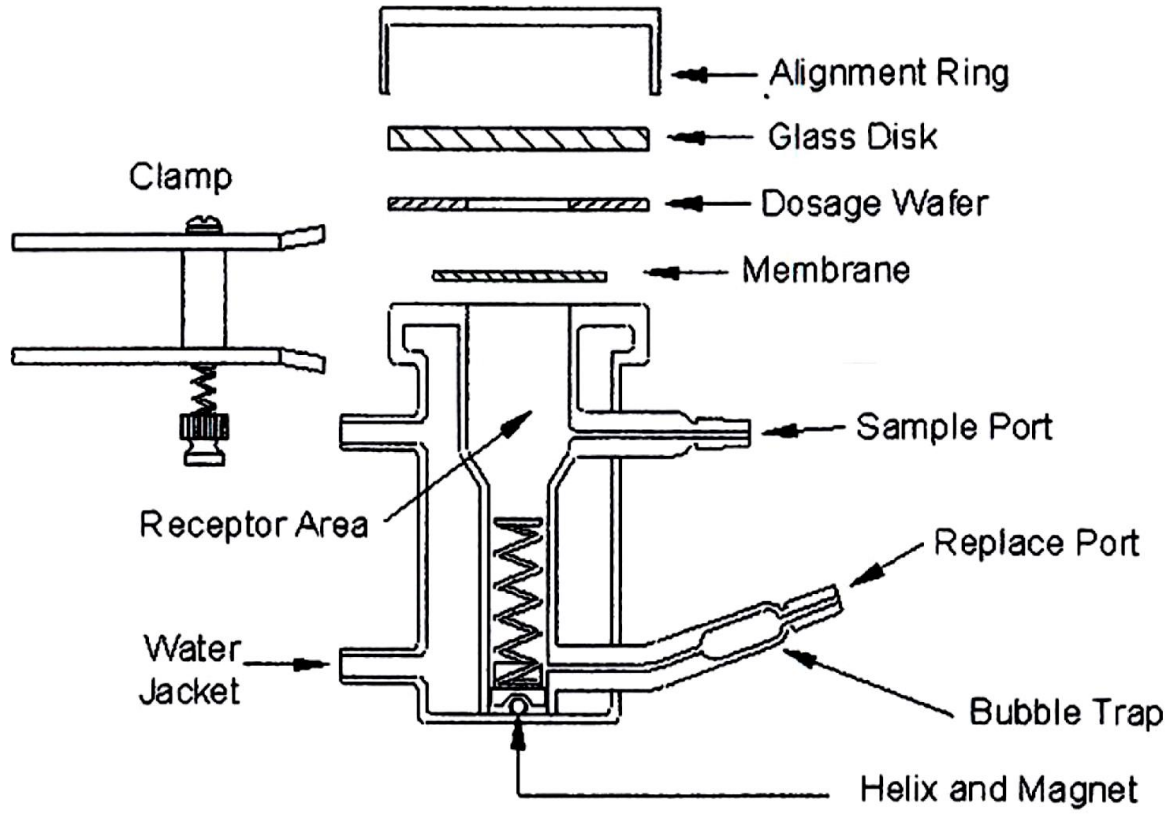

Figure 21: Vertical Diffusion Cell (VDC), also named Franz cell [69]. A dialysis membrane can be placed between the upper dosage compartment and the lower receptor compartement.

In any case, the main limitations of the dialysis-based methods are the tightness of the device, to be checked using dyes for instance, and the permeation kinetic through the membrane which may be too slow to enable DRT in some cases [70]. If the kinetic allows *in vitro* DRT, the apparent permeability constant of the API in the diffusion medium across the dialysis membrane should still be mathematically taken into account [71]. On the contrary, if the permeation kinetic limits the feasibility of dialysis methods, alternative techniques should be considered. For that matter, sampling-and-separation procedures may be required and

would consist of a withdrawal of sample at interval time points followed by a separation method for quantification, as described previously. Another option would be the *in situ* monitoring of the drug release, for instance using a Drug Selective Electrode (DSE) [72] or a fiber optics device in combination with derivative spectrophotometry, as presented in Chapter I and II.

2.2 Aim of the Work

The current transition for nanopharmaceuticals from R&D laboratories towards the pharmaceutical industry leads to many challenges, not only regulatory challenges but technical challenges too. Indeed, among the hurdles to overcome features the product characterization including quality control procedures that can be implemented in routine using reliable analytical methods.

In the first place, many publications describe *in vitro* drug release tests for nanoparticles using not standardized and home-designed dissolution devices. An important objective for this dissertation work was hence to develop suitable methods using compendial apparatus. The selected strategy to achieve this goal was firstly, for each type formulation to be investigated, i.e. PLGA-particles and Lipidot®, to identify the simplest feasible technique, and secondly, to develop new methods based on the selected technique. Lastly, the optimization and the validation of the methods were performed until the best analytical performances are reached.

For PLGA-particles, the use of fiber optics was quickly assessed as a feasible and promising solution to monitor *in situ* the drug release. However, it required the removing of interferences caused by the particles. That is the reason why mathematical separation methods based on derivative spectrophotometry were developed as well. Concerning the dissolution system, the apparatus 1 of the United State Pharmacopoeia (USP), namely the basket apparatus, was the simplest device suitable for both PLGA micro- and nanoparticles.

By contrast, *in situ* monitoring using fiber optics was not feasible for lipid nanoparticles because of their optical transparence leading to the impossibility to discriminate entrapped API from free or released API. Thereby, most of the efforts done for the lipid nanoparticles actually focused on the identification of a reliable separation method that can be at a later stage automated. The separation of the free API from the entrapped API is not always trivial, notably for Lipidot®. Consequently, this dissertation was also aimed to select, evaluate,

develop and validate the most suitable technique for lipid nanoparticles. After an arduous selection process, the Solid Phase Extraction technique appeared to be a promising way to characterize lipid nanoparticles.

Finally, all established analytical methods were implemented to evaluate the performances of new nanoformulations and hence to allow their optimization in the spirit of a regulatory submission, in the context of the EuroNanoMed Project Delivering Nano-pharmaceuticals through *Bi*ological *Ba*rriers 'BiBa' (ERA-Net EuroNanoMed Project Number 13N11846).

3 EXPERIMENTAL PART

Chapter I

In situ drug release monitoring with a fiber-optic system: Overcoming matrix interferences using derivative spectrophotometry

I *In Situ* Drug Release Monitoring with a Fiber-Optic System: Overcoming Matrix Interferences Using Derivative Spectrophotometry

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The author of the thesis made the following contributions to the publication:

- Performed the literature research
- Designed and performed the experiments
- Analyzed the data
- Wrote the manuscript

I.1 ABSTRACT

Automation takes an increasing place in all the fields of the pharmaceutical industry, such as manufacturing and quality control. In the field of *in vitro* dissolution testing, the fiber-optic system is often considered a promising tool because it allows *in situ* monitoring of the drug release without manual sampling, liquid transfer, and hence phase separation. This paper evaluates the use of fiber-optic *in vitro* dissolution testing and presents a technique to compensate for analytical interferences caused by excipients.

I.2 INTRODUCTION

To assess product performance and the quality of pharmaceuticals, *in vitro* dissolution tests are regularly carried out in the pharmaceutical industry. Over the past decades, these tests were more commonly performed manually. The tendency in industry is to reduce random error and optimize time and cost by automation. Dissolution testing is a composite procedure, with the bath as a sample preparation device, sampling and other transfer actions for liquids, filtration to obtain the portion dissolved, the chemical analysis (HPLC, spectrophotometry), and data processing at the end. All of the steps may introduce additional error, decreasing the precision of the final results. Automated systems may include automated sampling, processing of sample solutions, online quantification, and even cleaning. However, to reduce the number of steps to both accelerate the process and increase the precision of dissolution methods, alternative solutions may allow to omit the sample and filtration steps by measuring drug release directly in the vessels. Suitable probes that are connected by glass fiber to the spectrophotometer enable UV measurements at any selected time point. The goal of this paper is to review the suitability of fiber-optic systems for dissolution testing and to describe the limitations for use. An example of a fiber-optic system

with a “rod” probe is provided to expand the use. A method to compensate for the interferences by undissolved particles during *in situ* monitoring of the drug release is presented.

I.3 DESCRIPTION OF THE SYSTEM USED FOR THE EXPERIMENTS

The system comprised a UV–vis spectrophotometer (Varian Cary 50 Tablet) equipped with the Cary WinUV software and connected to an Agilent dissolution apparatus 708–DS by fiber optics. Among the different types of probes [73], the rod type was selected (Figure 22). The complete fiber-optic system is commercially available from Agilent Technologies.

Figure 22: Temperature probe (left) with optic rod probe (right) (source: Agilent Technologies).

I.3.1 Advantages

In addition to the general advantages provided by automated systems (e.g., sparing time, workforce), fiber-optic systems for *in vitro* dissolution offer following attractive features:

(1) Drug dissolution is measured *in situ*.

Firstly, the *in vitro* drug release studies become more accurate and precise as they allow observations of the phenomena as they occur in the vessel. No disturbances are introduced into the system by sampling. Sampling in the sense of dissolution kinetics implies the assumption that the aliquot withdrawn is representative of the dissolution process at a given time point and that the removal has no effect on either the aliquot or the entire system (i.e., its hydrodynamics). *In situ* monitoring does allow recording of the dissolution kinetics exactly at the site of action. As no sample solution is consumed, the sampling may be adapted to the grid needed to describe the kinetics adequately. The number of time points for analysis is not relevantly limited.

(2) Measurements can be performed for small volumes.

Possible interferences due to changes of the hydrodynamics are caused firstly by the relationship of the individual sample volume to the total volume and secondly by the total volume withdrawn as a function of sampling schedule. Fiber-optic measurements do not require sample removal. Therefore, the mechanical dissolution conditions remain unchanged throughout the experiment. The results are not biased by alterations due to volume-dependent hydrodynamics and secondary concentration changes by volume replacement with fresh medium.

(3) Transfer of sample solutions is not required.

As no transfer of sample solution is required, the time of analysis is significantly shorter, in particular in parallel experimental design:

n = 6 routine QC testing

n = 12 f2 testing

n = 24 USP stage testing

In the case of multiple sampling, the timesaving effect is relevantly greater.

(4) The use of tubing, syringes, and filters is not required.

The risk of API adsorption at surfaces of tubing, syringes, or filters is hence omitted. As no consumables are needed for sample processing, their validation is not needed, not to forget the cost-saving effects.

(5) There are no stability issues with sample solutions.

Physical instabilities as an effect of evaporation may not occur. There is no temperature gradient as for instance between the vessel (37 °C) and the spectrophotometric cuvette (25 °C). Secondary physicochemical instabilities such as temperature-dependent precipitation are avoided. As there is no time delay due to storage of sample solutions, chemical stability of sample solutions is not an issue.

(6) The chemical analyses are in real time.

In particular, in analytical method development as well as dosage form development, dissolution results are available while the test is still running. The advantage is that operating the dissolution bath and performing the chemical analysis concomitantly allows stopping experiments at any given specification or expectation time point. This advantage is valid for any online analytical coupling such as flow-through cuvette or ion-selective electrode. It is given here for completeness.

I.3.2 Particular Caution

In spite of the aforementioned advantages, using a fiber-optic system requires particular caution.

(1) Resident rod probes can cause turbulences in the vessel.

There are several reasons to leave the probes in the vessel for a time exceeding the pharmacopoeial specifications. Among others, these are to avoid entrapment of air in the optical path and crystallization of solids on surfaces. Recording of spectra also requires longer periods. However, the resident probes may bias the dissolution results. On a drug product, level validation is required.

The interferences caused by the probes can be significantly reduced if the probes are raised between data recording from the pharmacopoeial sampling point to a rest point immediately below the medium surface. As an alternative, probes may be used with hydrodynamically optimized rods. Lu et al. [73] describe alternative probes, either in the shaft of the stirrer or those adapted to the curvature of the vessel.

(2) Air bubbles adhere between the mirror and the glass fiber.

Air bubbles may form by immersing the probes into the medium. Consequently, the probes should be maintained immersed all along the test. Moreover, without any efficient degassing, air bubbles may form at the probes during a test run.

(3) Particles in suspension in the medium may form a thin layer on the probe.

Over the dissolution test, a partial buildup tends to occur on the mirror of the probe in the case of media containing many particles such as excipient aggregates or even undissolved API. The phenomenon can be avoided by both orienting the opening of the probe toward the walls of the vessel or the shaft of the paddle or basket and raising the manifold between the measurements.

(4) Excipients may cause spectrophotometric interference.

If the spectra of the API and the excipient overlap, the specificity is not given. Moreover, a simple subtraction of absorptions or the reference wavelength concept is generally inappropriate in the case where both the API and excipient concentrations increase over time. This may be solved by mathematical methods available to level out the interferences on the level of chemometry. For example, if the interfering compound has a maximal (or minimal) UV absorption within the absorption range of the API, then the drug substance can be quantified using the amplitude of the first derivative of the absorption spectrum at the zero crossing point [74]. Different derivative UV spectrophotometric methods are already described for interfering compounds with or without maximal, minimal, or constant absorption within the wavelength range of the API absorption [75, 76]. This paper focuses on the implementation of the derivative technique where the interfering compound has a constant UV absorption between two wavelengths within the absorption range of the API. Currently, this method is successfully used for *in vitro* dissolution tests (unpublished results). For interfering light scattering, derivative [77] and other corrective methods [78] are well described.

I.3.3 Limitations

The two main limitations encountered for the use of fiber-optic systems are:

(1) Very turbid suspensions cannot be analyzed.

In this case, the UV absorption of API is too weak compared with the total absorption of the suspension, thus quantification is impossible for very turbid suspensions.

(2) Solutions with compounds producing interferences that cannot be mathematically removed.

I.4 DESCRIPTION AND EVALUATION OF A DERIVATIVE SPECTROPHOTOMETRIC TECHNIQUE

I.4.1 Theory

For a mixture containing a given excipient and a drug substance, the resulting absorption spectrum of the mixture (D0) is the sum of the absorption for both substances:

$$A_{Mix}(\lambda) = A_{API}(\lambda) + A_{Exc}(\lambda)$$

Supposing that the UV absorption of the excipient is constant over a given wavelength range, $A_{Exc}(\lambda) = k \quad \forall \lambda \in [\lambda_1; \lambda_2]$, then the first derivative (D1) of the absorption spectrum of such an excipient will be close to zero within this range, further named “derivative range”:

$$\frac{dA_{Exc}(\lambda)}{d\lambda} \approx 0 \ \forall \lambda \in [\lambda_1; \lambda_2]$$

Consequently, the first derivative clears up the component brought by the drug substance by neutralization of the component brought by the excipient regardless of its concentration:

$$\frac{dA_{Mix}(\lambda)}{d\lambda} = \frac{dA_{API}(\lambda)}{d\lambda} + \frac{dA_{Exc}(\lambda)}{d\lambda} = \frac{dA_{API}(\lambda)}{d\lambda} \ \forall \lambda \in [\lambda_1; \lambda_2]$$

According to the Beer-Lambert law:

$$A(\lambda) = \varepsilon(\lambda) \cdot C \cdot l \text{ thus}$$

$$\frac{dA(\lambda)}{d\lambda} = \frac{d\varepsilon(\lambda)}{d\lambda} \cdot C \cdot l$$

The API can be quantified directly using the amplitude of D1 within the derivative range:

$$C \propto \frac{dA(\lambda)}{d\lambda} \ \forall \lambda \in [\lambda_1; \lambda_2]$$

However, the sensitivity may be decreased after the derivative operation, which is why the area under the curve (AUC) may be computed between two wavelengths within the derivative range:

$$C \propto \int_{\lambda_a}^{\lambda_b} \frac{dA(\lambda)}{d\lambda} \cdot d\lambda \quad \forall \lambda \in [\lambda_1; \lambda_2] \text{ and } \lambda_1 < \lambda_a < \lambda_b < \lambda_2$$

I.4.2 Method

A stock solution of propranolol in purified water was first diluted to prepare the following dilutions set: 10, 20, 50, 80, 100, and 120 µg/mL. A first course of absorption measurements was carried out for all solutions. The set was then spiked with the powder of excipients so that all the suspensions of the set had an excipient concentration of 0.3 mg/mL. A second course of absorption measurements was carried out for all suspensions. Finally, suspensions from 0.1 to 1.0 mg/mL excipients were prepared using the previous propranolol solution (100 µg/mL) spiked with the powder of excipients.

The baseline and blank were measured with purified water. The absorption spectra were acquired from 200 to 400 nm with a measurement interval of 0.50 nm and a measurement speed of 60 nm/min. For the “absorption method,” the amplitude of the UV absorption of the sample was extracted from the spectrum at 290 nm, whereas for the “derivative-based method,” the AUC of D1 was calculated from 290 nm and 317 nm.

The calibration curves for the two quantification methods with and without excipients were calculated and compared.

For the derivative-based method, the quantification response of the API spiked with excipients was correlated to the quantification response of the API in solution without excipients.

Finally, the recoveries of the suspensions containing different amounts of excipients were computed by the derivative-based method to the standard solution containing 100 µg/mL propranolol.

I.4.3 Validation

The UV absorption spectra and first derivative absorption spectra for propranolol with and without excipients are presented in Figure 23.

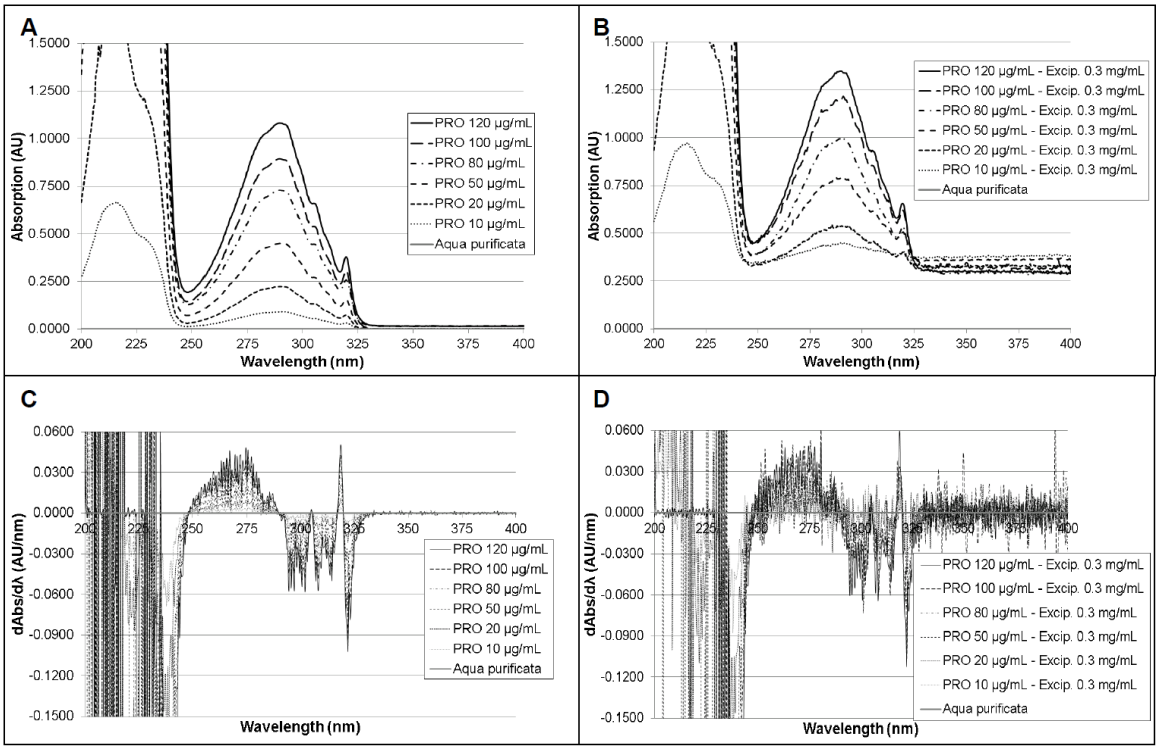

Figure 23: UV absorption and first derivative absorption spectra for propranolol (PRO) in purified water with vs. without excipients (Excip.); (A) absorption spectrum without excipients; (B) absorption spectrum with excipients. Whereas APIs and excipients are released from the drug delivery device over the dissolution test, absorptions spectra shift upward from (A) to (B). (C) First derivative absorption spectrum without excipients; (D) first derivative absorption spectrum with excipients.

The linearity of both methods was checked over the range of 10–120 µg/mL propranolol, and the determination coefficients (R2) as well as the limits of quantification (LOQ) are summarized in Table 1. The relative standard deviation (RSD) of the absorption method does not meet the specifications for R2 and LOQ. Hence, the principle was not applicable. On the contrary, the derivative-based method successfully fulfilled the linearity requirements in the presence of excipients.

Table 1: Linearity of the absorption and derivative-based methods with and without excipients.

Method	Excipient concentration (mg/mL)	R^2	LOQ (µg/mL)
Absorption	0.0	0.9998	0.07
Absorption	0.3	n.a.	n.a.
Derivative	0.0	0.9999	0.05
Derivative	0.3	0.9999	0.15

The calibration curves of the propranolol quantification in purified water in the presence of excipients are presented for both methods in Figure 24.

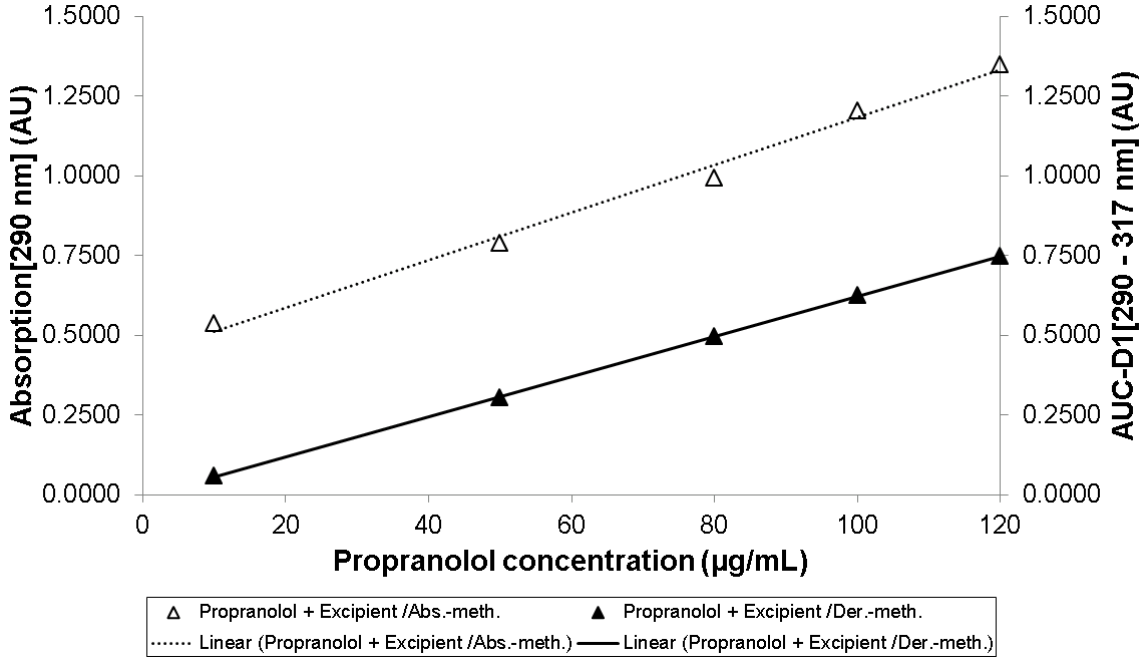

Figure 24: Linearity of the quantification methods for propranolol in purified water spiked with 0.3 mg/mL excipients; absorption at 290 nm and area under the curve (AUC) of the first derivative (D1) of the absorption between 290 nm and 317 nm.

The correlation of the quantification response with excipients to the quantification response without excipients has an R^2 of 0.9998 to 120 µg/mL propranolol for the derivative-based method (Figure 25). This proves the successful elimination of the interferences caused by excipients (specificity).

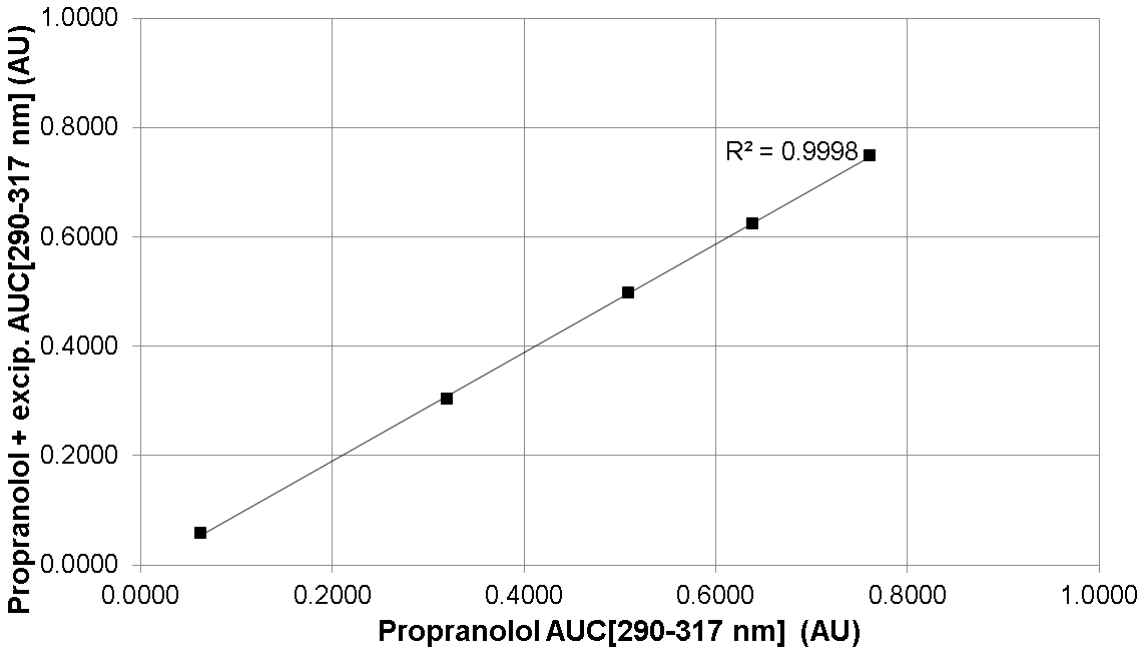

Figure 25: Correlation curve between the quantification of propranolol in purified water without and with 0.3 mg/mL excipients using the area under the curve (AUC) of the first derivative (D1) of the absorption between 290 and 317 nm.

Finally, the recoveries of the 100 µg/mL propranolol solution spiked with different excipient concentrations were 96–103 % for 0.1–0.7 mg/mL excipients and 94–103 % for 0.1–1.0 mg/mL excipients. Hence, the accuracy of the derivative-based method is proven.

I.5 CONCLUSION

Fiber-optic systems represent a suitable option to both increase the robustness of dissolution methods and facilitate the automation for *in vitro* drug release testing. The time-saving effect is remarkable. Though automation by fiber-optic systems may not be applicable to all pharmaceutical products, the range of use may be broadened by the level of data processing. The most important limitation effect by matrix interferences may be overcome by mathematical solutions, such as derivative spectrophotometric methods. The use is not limited to manufacturing industry but also includes R&D projects.

I.6 ACKNOWLEDGMENTS

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Chapter II

A new concept for *in vitro* drug release testing of micro- and nanoformulations using a fiber optic system and derivative spectrophotometry

II A new concept for *in vitro* drug release testing of micro- and nanoformulations using a fiber optic system and derivative spectrophotometry

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The author of the thesis made the following contributions to the publication:

- Performed the literature research
- Performed the experiments (excluding the particle preparation)
- Analyzed the data
- Wrote the manuscript

II.1 GRAPHICAL ABSTRACT

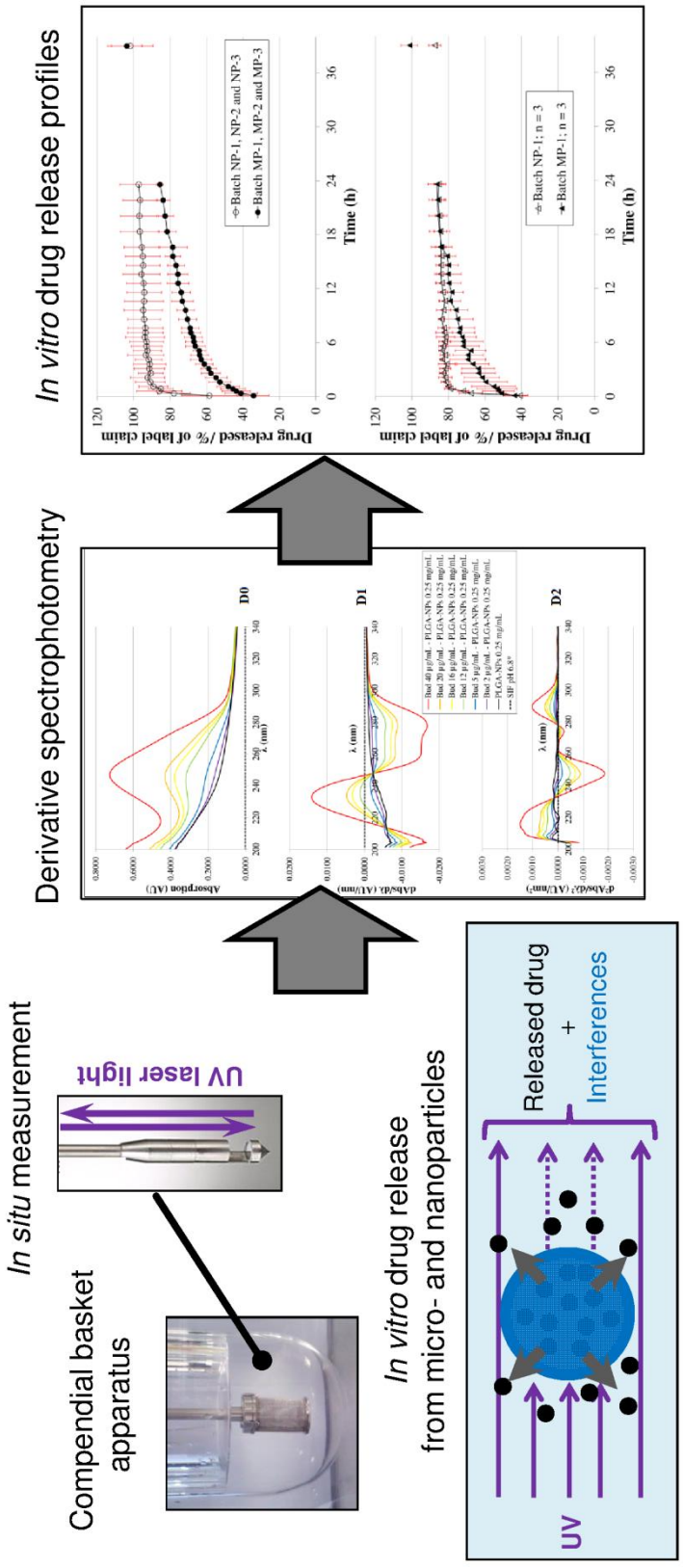

Figure 26: Graphical Abstract. A new concept for *in vitro* drug release testing of micro- and nanoformulations using a fiber optic system and derivative spectrophotometry.

II.2 ABSTRACT

Aim of the study was to explore innovative approaches to *in situ* monitoring of drug release from micro- and nanoparticles, allowing abandoning complex and time consuming separation techniques. For this purpose, the combination of derivative spectrophotometry with fiber optics and a compendial basket *in vitro* dissolution apparatus was implemented.

UV absorption spectra of poly(lactic-co-glycolic acid) (PLGA) microparticles and nanoparticles were obtained by an Agilent FiberOptic® system, allowing to mathematically compensate interferences with the released drugs. Quantification methods based on derivative spectrophotometry were first developed for microparticles loaded with either budesonide or cyclosporine A, and then extended to nanoparticles loaded with budesonide. These analytical methods were then applied to perform *in vitro* drug release tests.

The results of the validation showed a satisfying linearity, limits of quantification, accuracy and repeatability for all selected quantification methods. Consequently, *in vitro* drug release tests could be performed and the results proved that the methods were discriminative to enable the selection of the most suitable formulations.

This study demonstrates that the concept of derivative spectrophotometry in combination with fiber optics and some compendial dissolution apparatus can be used for *in vitro* drug release tests of micro- and nanocarriers without requiring cumbersome physical separation.

Keywords: nanoparticles; microparticles; *in vitro* drug release testing; dissolution; fiber optics; derivative spectrophotometry; separation techniques; PLGA; budesonide; cyclosporine A

II.3 INTRODUCTION

A recently made inventory of pharmaceutical products currently under clinical investigations or already approved for commercialization highlights the significant emergence of nanoproducts as a part of the future pharmaceutical market [79]. As the commercialization of nanoproducts requires evaluating the potential risk of such technologies, the Center for Drug Evaluation and Research (CDER) of the United States Food and Drug Administration recently pointed out the need of improvements of analytical methods for the characterization of nanoproducts [80]. At the forefront of the pharmaceutical product characterization, *in vitro* drug release studies play a key role in the establishment of *in vitro-in vivo* correlations (IVIVC) which represent a surrogate for clinical trials. The simplest methods using compendial apparatus are generally preferred, as long as they allow generating meaningful data for the investigated new formulation [81]. Furthermore, depending of the needs of the laboratory, the automation of the dissolution equipment is increasingly considered to optimize *in vitro* drug release studies in the field of pharmaceutical industry [82]. Besides economic aspects, an important concern is the optimization of the reproducibility, repeatability, precision and robustness of the analytical procedures, which requires as well the technically simplest methods possible. However, most of analytical procedures for *in vitro* drug release of nanoparticles introduce the issue of the separation which leads to rather complex methods, such for example dialysis [67, 83-85], ultrafiltration [86, 87] or biphasic dissolution media [88, 89]. As an attempt to work around the issue of separation, *in situ* monitoring using a fiber optics system in combination with compendial dissolution apparatus may be an innovative approach to develop automated methods for nanopharmaceuticals [90]. The feasibility of such a combination has already been tested for conventional dosage forms from the late 80's, using a modified paddle apparatus (apparatus 2 of the United States Pharmacopoeia (USP)) with tablets containing felodipine [91]. However, the observation of interfering spectra is often considered as a limitation to the quantification of the active pharmaceutical ingredient (API) [92]. Yet, many methods based on derivative

spectrophotometry (DSP) have been successfully evaluated to selectively quantify the drug of interest in presence of interfering components, such as pharmaceutical preparations containing several APIs [93-98], containing interfering matrix excipients [99] or even containing light-scattering components [77]. Since particles in suspension generally scatter light [100], such DSP methods are worthy of being evaluated for micro- and nanopharmaceuticals. The goal of this investigation was to study the feasibility of combining: 1) derivative spectrophotometry 2) fiber optics, and 3) some compendial *in vitro* dissolution apparatus, in order to monitor *in situ* the drug release from micro- and nanoparticulates systems. The interfering UV absorption spectra of poly(lactic-co-glycolic acid) (PLGA)-microparticles (MPs) and PLGA-nanoparticles (NPs) were compared. In a previous work [101], it has been shown that the released API could be quantified selectively in presence of the API still entrapped inside the opaque polymeric matrix of the particles. Quantification methods based on DSP were hence developed and validated according to ICH guideline Q2A [102] for budesonide (BUD) spiked with placebo 5 µm MPs, then as well as for a second API, cyclosporine A (CSA) spiked with the same MPs. The concept was extended to budesonide spiked with placebo 150 nm NPs. However, another previous study performed using propranolol as model drug had already shown that the concept of DSP could not be extended for this nanoformulation if the API absorbs below 260 nm [101] which, however, applies for cyclosporine A. Finally, drug content determination and *in vitro* drug release tests were performed for BUD-loaded PLGA-MPs, CSA-loaded PLGA-MPs and Bud-loaded PLGA-NPs using the compendial basket apparatus (apparatus 1 of the USP) and a fiber optics system.

II.4 MATERIALS AND METHODS

II.4.1 Materials

The fiber optic system was provided by Agilent Technologies and was equipped with a UV/Vis spectrophotometer Cary 50 Tablet and a 1 mm probe type “rod”. The *in vitro* drug release tests were performed using a compendial basket apparatus (apparatus 1 of the USP) type 708-DS Dissolution Apparatus from Agilent Technologies equipped with 1 L-vessels and 100 mesh-baskets. Rotating paddles (apparatus 2 of the USP) were used for the infinity tests. Cyclosporine A (CSA) and budesonide (BUD) were provided by RTC™. The placebo and API-loaded 5 µm spray-dried PLGA-microparticles were provided by the Helmholtz Institute of Saarbrücken as well as the placebo and BUD-loaded 150 nm PLGA-nanoparticles. The acetonitrile hypergrade for LC-MS LiChrosolv® (ACN) was provided by Merck. The highly purified water – aqua valde purificata (AVP), was produced using a Milli-Q system type Q-POD® from Millipore. The simulated intestinal fluid pH 6.8 without pancreatin (SIF 6.8) and the phosphate buffer solution pH 3.0 (PB3) were prepared according to Ph. Eur. 7.5. The filters 0.45 µm GHP Acrodisc Glass Fiber were provided by Pall Life Sciences. The ultrasonic bath was a Sonorex Digital 10 P from Bandelin.

II.4.2 Methods

II.4.2.1 Comparison of the interferences caused by nanoparticles vs. microparticles

An ultrasonic bath was used for 20 min at room temperature to disperse either the micro- or the nanoparticles from the placebo powders in the medium SIF 6.8. The weight concentration was 0.3 mg/mL of either MPs or NPs. The UV absorption spectra of the suspensions were measured with the fiber optics system between 200 and 350 nm using the medium SIF 6.8 as baseline.

II.4.2.2 Development of derivative quantification methods

The quantification methods were developed from calibration curves of API dilution sets spiked with nanoparticles (only in the case of BUD), with microparticles (for both BUD and CSA) and without nano- nor microparticles (BUD and CSA). More precisely, a stock suspension containing 0.60 mg/mL of placebo PLGA-microparticles was prepared dispersing the powder of MPs in SIF 6.8 using an ultrasonic bath for 20 min at RT (suspension M). Another stock suspension containing 0.50 mg/mL of placebo PLGA-nanoparticles was prepared in the same way (suspension N). A stock solution containing 2000 µg/mL cyclosporine A was prepared dissolving the drug in ACN (stock A). This stock solution was further diluted 1:10 using SIF 6.8 (solution A). Another stock solution containing 2000 µg/mL budesonide was prepared in the same way (stock B) and diluted as previously (solution B). Two dilution sets of drug (CSA and BUD) without micro- or nanoparticles were prepared diluting respectively the solutions A and B from 2 µg/mL to 16 µg/mL cyclosporine A and from 2 µg/mL to 40 µg/mL budesonide using SIF 6.8. Two other dilution sets containing the same amount of drug (CSA and BUD) and 0.30 mg/mL PLGA-MPs were prepared as previously spiking the dilutions with the suspension M. A third dilution set of budesonide, containing 0.25 mg/mL PLGA-NPs, was prepared as previously spiking the dilutions with the suspension N. According to this protocol, no dilutions contained more than 2 % ACN, v/v. The UV absorption spectra (D0) of the dilution sets were acquired from 200 nm to 350 nm with a scan rate of 60 nm/min using SIF 6.8 as blank and baseline. The first derivatives (D1) of the absorption spectra were calculated for each dilution. The second derivatives (D2) were calculated as well only for the dilutions containing budesonide spiked with nanoparticles and not spiked. A wavelength range was graphically selected so that the signal due to the interferences is removed without affecting the signal of the drugs (see the selected ranges in Table 2). Then the areas under the curves (AUC) of D1 and D2 within the selected wavelength range were calculated for each concentration in order to build the AUC-based calibration curves. The coefficients of determination (R^2) and the limits of quantification

(LOQ) where calculated. The correlation between the quantification of budesonide without micro- and nanoparticles and the quantification of budesonide spiked with micro- and nanoparticles was checked using the Student's t-test.

Table 2: Quantification methods. BUD-MPs: budesonide-loaded microparticles; BUD-NPs: budesonide-loaded nanoparticles; CSA-MPs: cyclosporine A-loaded microparticles. AUC of D1: area under the curve of the first derivative absorption spectrum; AUC of D2: area under the curve of the second derivative absorption spectrum. The areas were used as quantification parameter, i.e. proportional to the drug concentration.

Formulation	BUD-MPs	BUD-NPs	CSA-MPs
Parameter	AUC of D1	AUC of D2	AUC of D1
Wavelength (nm)	249 - 300	279 - 312	215 - 250

II.4.2.3 Drug content determination

The determination of the drug content was performed for three batches of each following formulation: budesonide-loaded PLGA-microparticles (BUD-MPs), cyclosporine A-loaded PLGA- microparticles (CSA-MPs) and budesonide-loaded PLGA-nanoparticles (BUD-NPs). Prior to the test, a solvent mixture containing 50 % (v/v) ACN and 50 % (v/v) PB3 was prepared (ACN:PB3). A given amount of particles was weight in an Erlenmeyer-flask (25 mg for BUD-MPs and for CSA-MPs and 20 mg for BUD-NPs) and a given volume of ACN:PB3 was added (30 mL for BUD-MPs and for CSA-MPs and 10 mL for BUD-NPs). The samples were further agitated and placed 20 min in an ultrasonic bath. After filtration, the drugs were quantified using the DSP methods previously developed. The reference solutions for the quantification were prepared by dilution of the stock solutions stock A and stock B using ACN:PB3 until 124.1 µg/mL for CSA and 61.7 µg/mL for BUD. The baseline and blank were performed using ACN:PB3.

II.4.2.4 *In vitro* drug release tests

The *in vitro* drug release tests were performed for three batches of each BUD-loaded formulation (repeatability of the manufacturing process) and one batch was investigated in triplicate (repeatability of the analytical method). Concerning the CSA-loaded formulation, the tests were performed for one batch in duplicate. Prior to the tests, the dissolution medium (SIF 6.8) was degassed using an ultrasonic bath for 15 min. The formulations were weighted directly in the baskets. The tests were performed using the drug release methods described in Table 3.

Table 3: *In vitro* drug release methods

Formulation	BUD-MPs	BUD-NPs	CSA-MPs
Apparatus	Basket	Basket	Basket
	100 mesh	100 mesh	100 mesh
Amount	30 mg	120 mg	30 mg
Dissolution medium	SIF pH6.8	SIF pH6.8	SIF pH6.8
Volume	500 mL	500 mL	500 mL
Temperature	37 °C	37 °C	37 °C
Rotation speed	75 rpm	75 rpm	75 rpm

The UV absorption spectra were acquired at a scan rate of 60 nm/min from 240 nm to 330 nm and from 200 nm to 260 nm for BUD and CSA formulations, respectively. A last test was finally carried out to force the release of the API by a strong physical stress on the formulation in order to reflect the cumulated release at the infinity time point. This infinity test was performed after 48 h using paddles rotating at 250 rpm for 1 h above the baskets removed and placed on the bottom of the vessels. The methods for the quantification of the APIs are summarized for each formulation in Table 2. The reference solutions for the quantification were prepared by dilution of the stock solutions stock A and stock B using SIF 6.8 until 8.33 µg/mL and 5.0 µg/mL for CSA and BUD, respectively. The baseline and blank were performed using SIF 6.8.

II.5 RESULTS AND DISCUSSION

II.5.1 Comparison of the interferences caused by placebo nanoparticles vs. microparticles

According to the UV absorption spectra of the suspensions presented in Figure 27, both MPs and NPs could interfere with any APIs absorbing in the investigated wavelength range. The interferences caused by the microparticles formed a relative straight line above 215 nm. This signal could consequently be properly described above 215 nm by a zero- or first-degree polynomial function that could theoretically be removed by a first- or second-order derivative. However, the analytical response decreased with the order of the derivative that is why the first order was preferred when feasible. Concerning the nanoparticles, the shape of the spectrum was in relation to 1/λn, where λ is the wavelength and n the degree of the light scattering (between 2 and 4). An approximation of this signal between two wavelengths could be done with a first-degree polynomial function that could be removed by a second-order derivative.

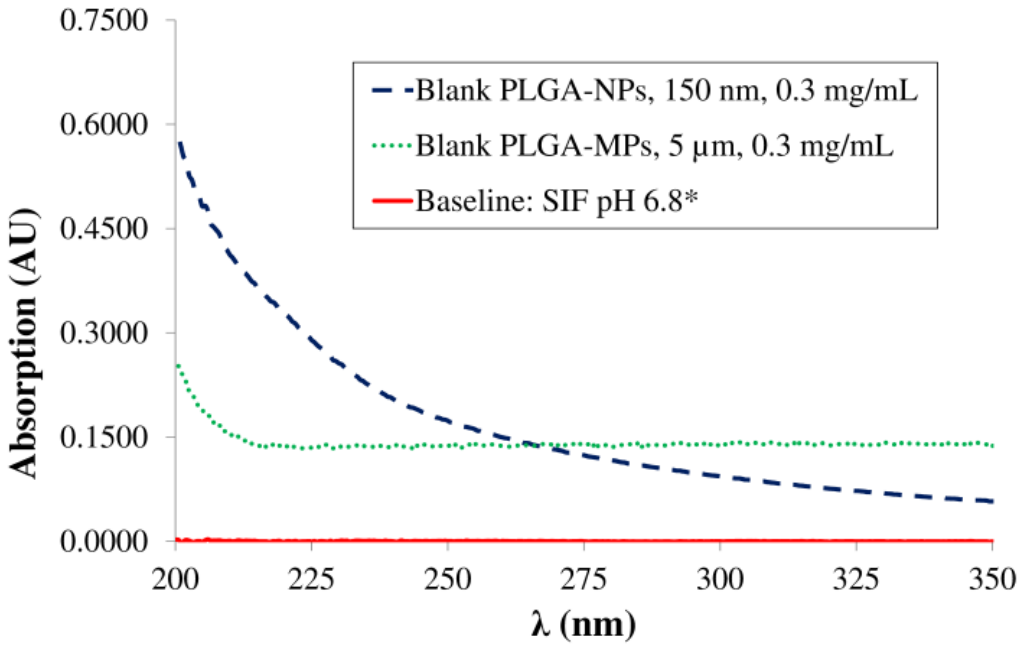

Figure 27: UV-absorption spectra of PLGA-nanoparticles and PLGA-microparticles in SIF pH 6.8 (Simulated Intestinal Fluid, without pancreatine), n = 3.

II.5.2 Saturation concentrations and sink conditions

The dilution sets of BUD and CSA without particles were slightly turbid in the investigated concentration ranges. For higher concentrations, precipitates were observed suggesting that the saturation concentration in SIF 6.8 was reached above the investigated concentration. Indeed, the aqueous solubility of these APIs is very low: 40 µg/mL at 25 °C for BUD [103] and 42 µg/mL at room temperature for CSA [104]. Consequently, the amounts of the dosage form were adjusted so that the maximal concentration of released API was less than a third of the saturation concentration in order to maintain sink conditions as suggested in the USP [23].

II.5.3 Development of derivative quantification methods

According to the spectra of the dilution sets of BUD and CSA spiked with PLGA-particles presented in Figure 28, the following derivative quantification methods could be selected for evaluation: area under the curve (AUC) of D1 between 249 and 300 nm (AUC-D1[249-300]) for budesonide spiked with microparticles (BUD-MPs), AUC-D2[279-312] for budesonide spiked with nanoparticles (BUD-NPs) and AUC-D1[215-250] for cyclosporine A spiked with microparticles (CSA-MPs). The results of the validation of the three selected methods presented in Table 4 confirm the suitability for quantifying the drug released from the formulations. However, an excellent correlation was established between the measured concentration of budesonide alone versus in presence of micro- as well as nanoparticles, respectively (Figure 29).

Table 4: Validation of the derivative quantification methods and corresponding formulations

Method	AUC-D1[249-300]	AUC-D2[279-312]	AUC-D1[215-250]
Formulation	BUD-MPs	BUD-NPs	CSA-MPs
PLGA Range (mg/mL)	0.00 – 0.30	0.00 – 0.25	0.00 – 0.30
API Range (µg/mL)	0 – 20	0 – 40	0 – 16
R^2	0.9999	0.9991	1.0000
LOQ (µg/mL)	0.23	0.41	0.50

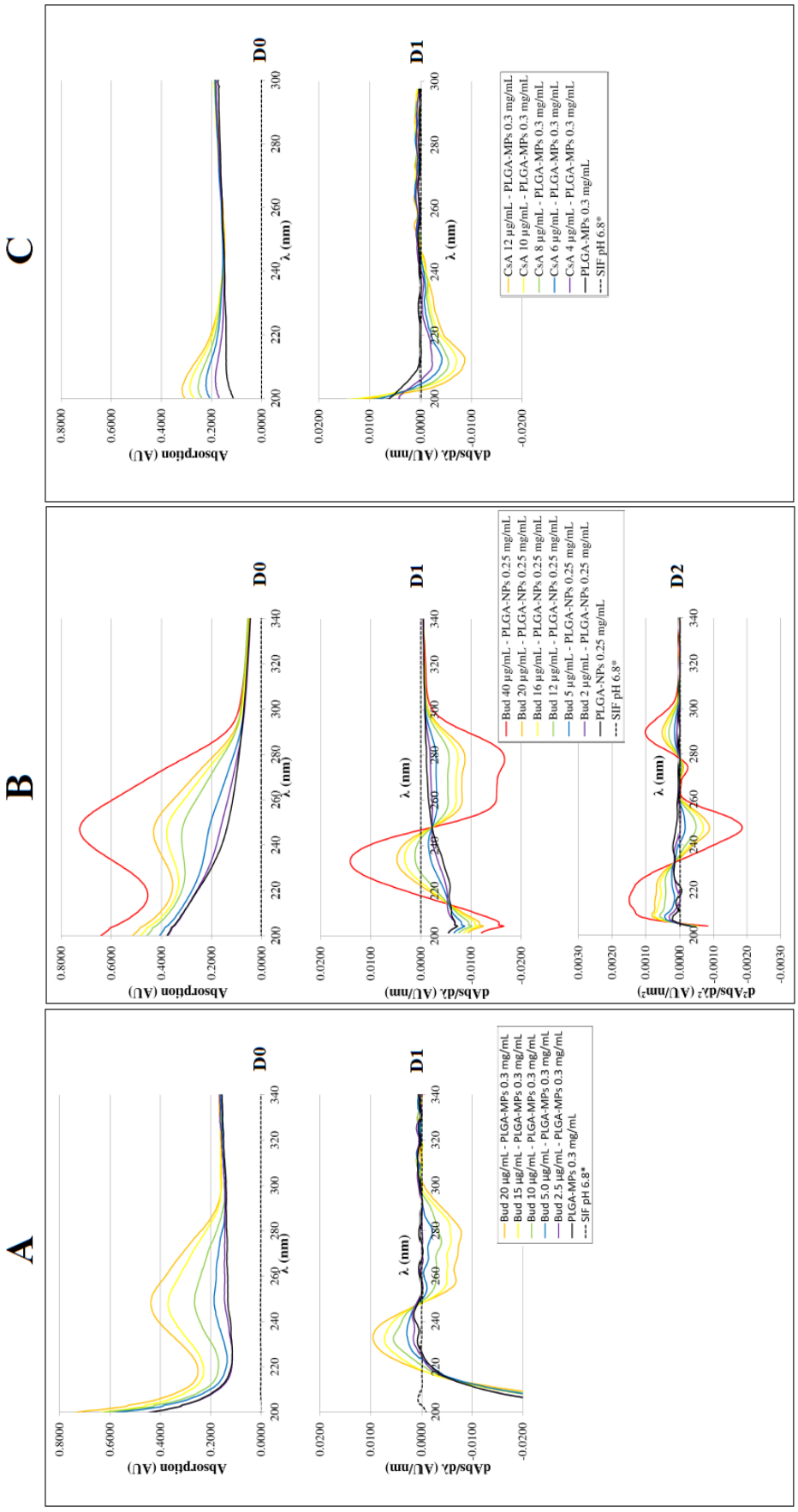

Figure 28: **A: UV-Absorption (D0) and first-order derivative (D1) spectra of the BUD dilution set in SIF 6.8 spiked with placebo PLGA-MPs. The interferences caused by the particles could be removed using D1 between 249 and 300 nm. B: UV-Absorption (D0), first-order derivative (D1) and second-order derivative (D2) spectra of the BUD dilution set in SIF 6.8 spiked with placebo PLGA-NPs. The interferences could be removed using D2 between 279 and 312 nm. C: UV-Absorption (D0) and first-order derivative (D1) spectra of the CSA dilution set in SIF 6.8 spiked with placebo PLGA-MPs. The interferences could be removed using D1 between 215 and 250 nm.**

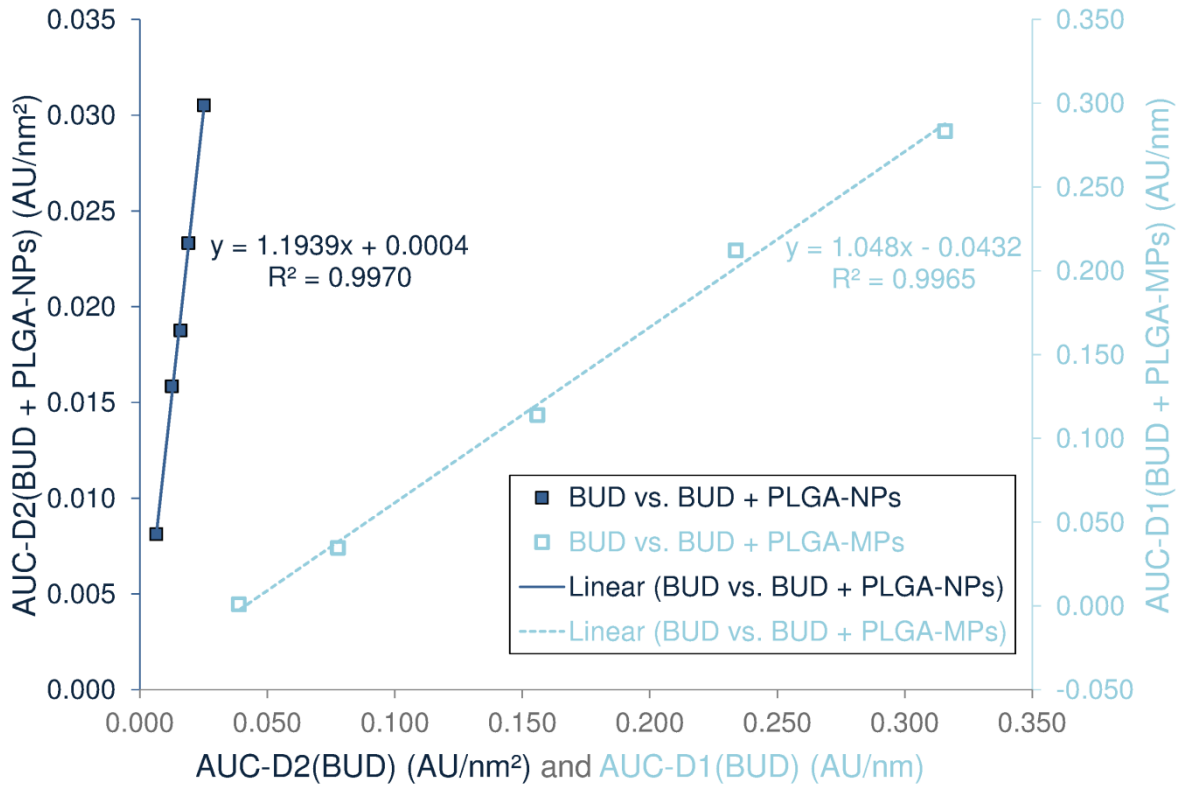

Figure 29: **Light blue curve: correlation between the AUC of D1[249-300] for budesonide versus budesonide spiked with PLGA-microparticles. Unilateral Student's t-test: $p < 0.0005$. Dark blue curve: correlation between the AUC of D2[279-312] for budesonide versus budesonide spiked with PLGA-nanoparticles. Unilateral Student's t-test: $p < 0.005$. According to result of the Student's t-test, the value of the quantification parameters (i.e. AUC of D1[249-300] and AUC of D2[279-312]) do not significantly differ in presence or in absence of micro- and nanoparticles. As a consequence, in the selected ranges of concentrations, the drugs can be quantified regardless to the amount of micro- and nanoparticles.**

II.5.4 Drug content determination

The calculated mean drug contents, relative standard deviations (RSD), recovery ranges and recoveries to the expected values based on the weight are given for all the investigated formulations in the Table 5.

Table 5: Results of the drug content determination for BUD-MPs, BUD-NPs and CSA-MPs. The theoretical drug content is related to the amount of drug introduced in the manufacturing process. The experimental drug content differs especially for nanoparticles because of a purification step aimed to remove unentrapped drug.

Formulation	Batch #	Drug content (µg/mg)	RSD (%)	Recovery range (%)	Recovery to the theoretical drug content (%)
BUD-MPs	Batch 1 (n = 3)	75.4	1.1	98 – 101	100.2
	Batch 2 (n = 1)	76.4	-	-	101.4
	Batch 3 (n = 1)	80.1	-	-	102.4
	Batches 1, 2 and 3	77.3	3.2	97 – 104	101.3
BUD-NPs	Batch 1 (n = 3)	28.6	2.8	97 – 103	68.6
	Batch 2 (n = 1)	28.1	-	-	70.4
	Batch 3 (n = 1)	31.7	-	-	81.9
	Batches 1, 2 and 3	29.5	6.7	95 – 108	73.6
CSA-MPs	Batch 1 (n = 3)	144.8	2.8	98 – 104	102.9
	Batch 2 (n = 1)	144.4	-	-	102.6
	Batch 3 (n = 1)	152.0	-	-	102.2
	Batches 1, 2 and 3	147.1	2.9	98 – 104	102.5

The results showed a satisfying repeatability and accuracy both between batches (consistency of the manufacturing process) and within batches (performance of the drug content determination methods). However, the tests performed between batches were less repeatable for nanoparticles as for microparticles. Moreover, since the terminal step of the manufacturing process of the nanoparticles was a purification step, the recovery to the theoretical value of the drug content was not complete contrary to microparticles. Nevertheless, including the values of previous unpublished investigations performed on other batches of the same type of formulation, the recovery to the expectation was actually practically complete for nanoparticles as well.

II.5.5 *In vitro* drug release tests

The drug release tests performed either in triplicate (budesonide) presented consistent trends with acceptable standard deviations (Figure 30), The tests performed using three batches showed a narrow standard deviation for BUD-microparticles but a larger deviations were observed for BUD-nanoparticles. As a consequence, the drug release tests were discrimitative enough to reveal a better manufacturing reproducibility for BUD-microparticles than for BUD-nanoparticles. In addition, the method could successfully discriminate the release kinetics of the micro- and nanoparticles and pinpoint the release mechanism. Indeed, a burst release (40 % release after 5 min) followed by a sustained release was observed for BUD-MPs whereas a much stronger burst release (60 % release after 5 min) was observed for BUD-NPs. Since the specific surface increases for smaller particles, the burst effect was stronger for the nanoparticles and suggested a large amount of drug adsorbed at the surface of the particles. The developed *in vitro* drug release methods successfully allowed the selection of the most suitable formulation for further development: BUD-MPs.

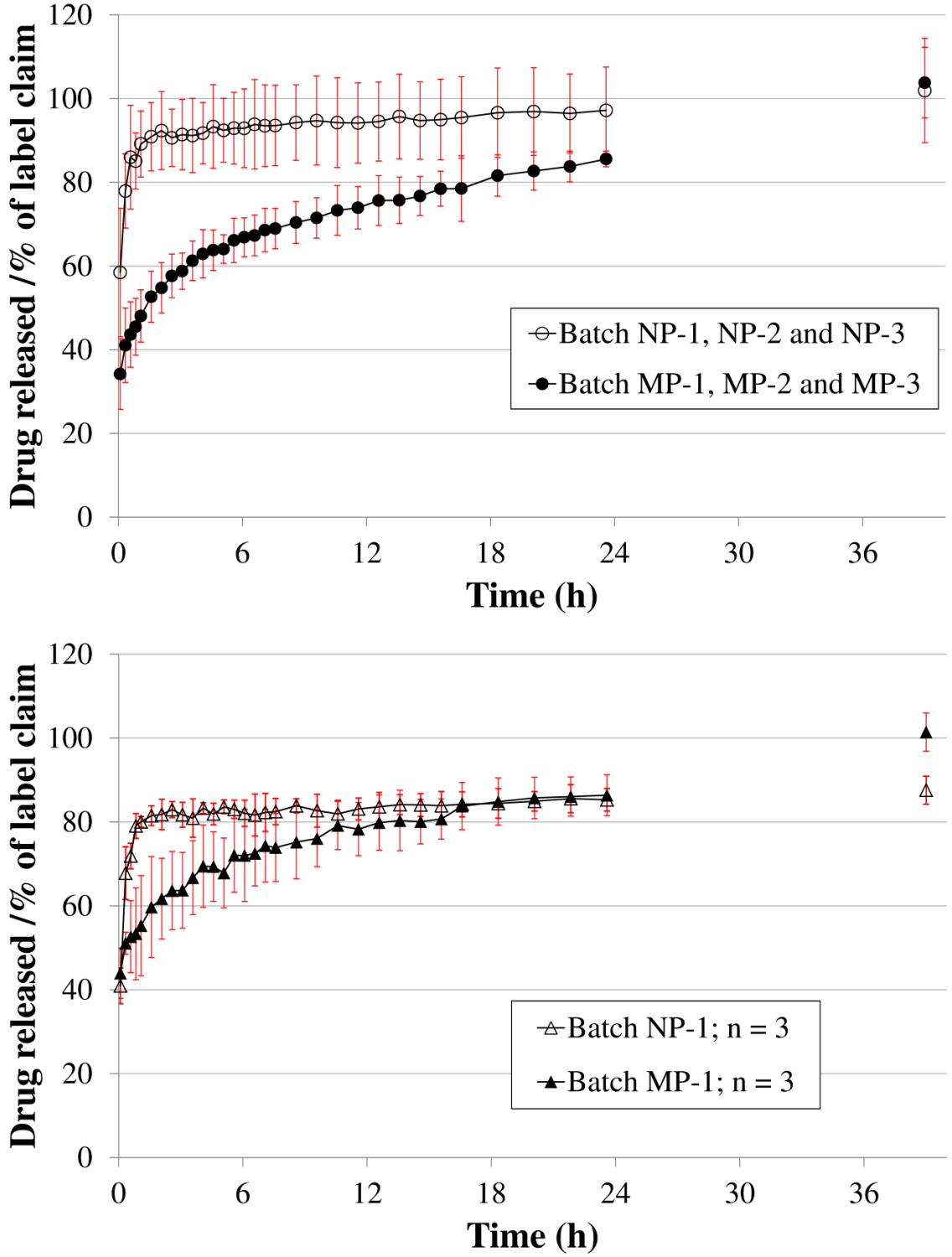

Figure 30: ***In vitro*** **drug release profiles of the mean values and standard deviations for BUD-MPs and BUD-NPs. The last time point above 36 h corresponds to the infinity test. The drug release is expressed in percentage of the experimental drug content, as previously determined. Up: Repeatability between three batches (MP-1, MP-2 and MP-3 for BUD-MPs and NP-1, NP-2 and NP-3 for BUD-NPs). Down: Repeatability within batch (MP-1 in triplicate for BUD-MPs and NP-1 in triplicate for BUD-NPs).**

The Figure 31 corresponds to the results for CSA-loaded PLGA-MPs. Contrary to the drug release profile obtained with BUD-nanoparticles, no significant burst effect was observed for this formulation containing microparticles, which remains in line with the previous observations. Kinetic differences based on the loaded drug were revealed by the drug release test, since cyclosporine A loaded microparticles presented a slower kinetic than budesonide loaded microparticles. According to these results, the CSA-MPs formulation represents the most hopeful candidate.

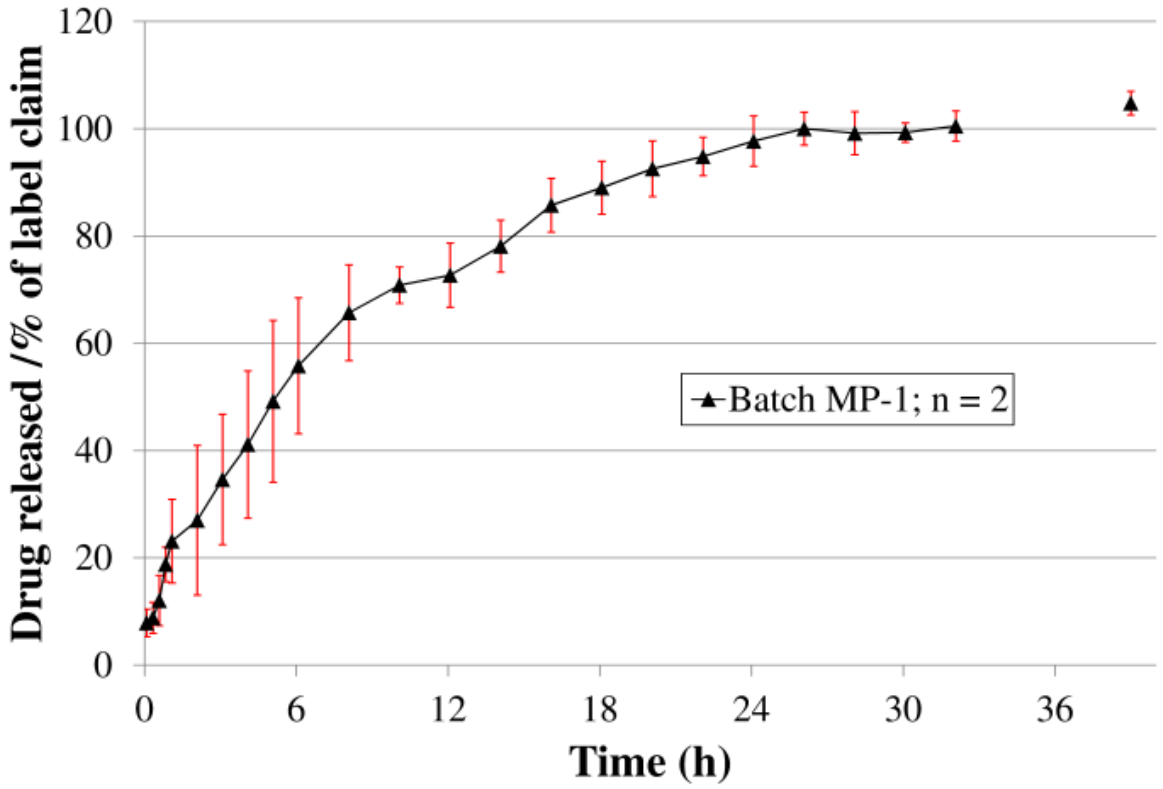

Figure 31: ***In vitro*** **drug release profiles of the mean values and standard deviations for one batch of CSA-MPs tested in duplicate. The last time point above 36 h corresponds to the infinity test. The drug release is expressed in percentage of the experimental drug content, as previously determined.**

II.6 CONCLUSION

This investigation on the combination of derivative spectrophotometry (DSP) with fiber optics and a compendial *in vitro* dissolution apparatus led to encouraging results. Indeed, quantification methods could be properly developed and validated for two model drugs, budesonide and cyclosporine A, and two particulate formulations, PLGA-nanoparticles and PLGA-microparticles. These methods were finally implemented to evaluate the *in vitro* drug release performances for the tested formulations. It could be shown that the release profiles for microparticles showed a much more pronounced retardation than for nanoparticles which showed a strong burst effect, suggesting that the latter might require some further optimization to reach suitable pharmaceutical properties. Concerning the repeatability of the manufacturing process, the results were in line with the expectations for such formulations in a development phase. Again, the repeatability was somewhat better for microparticles than for nanoparticles.

Overall, this study demonstrates that the use of DSP to compensate for UV-absorption interferences caused by particles represents a valid alternative to manual sampling and physical separation methods. In this context, automation becomes more simple, time and costs are saved and the repeatability is improved. In principle, this concept can be extended to any kind of interferences, except for cases of very high turbidity or lack of mathematical solutions. Innovative drug delivery systems, such as micro- and nanoparticles, are thus not necessarily incompatible with compendial equipment and the regulatory challenges caused by the lack of analytical tools can be overcome.

II.7 ACKNOWLEDGEMENTS

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Chapter III

Development of an innovative separation method for lipid nanoparticles based on solid phase extraction

III Development of an innovative separation method for lipid nanoparticles based on solid phase extraction

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The author of the thesis made the following contributions to the publication:

- Performed the literature research
- Designed the experiments
- Performed the experiments (excluding the particle preparation and the particle size determination)
- Analyzed the data
- Wrote the manuscript (excluding the particle preparation)

III.1 ABSTRACT

Purpose

Contrary to physical characterization techniques for nanopharmaceuticals (shape, size and zeta-potential), the techniques to quantify the free and the entrapped drug remain very few and difficult to transpose in routine analytical laboratories. The application of Solid Phase Extraction (SPE) technique was investigated to overcome this challenge.

Methods

The separation of free and entrapped drug by SPE was quantitatively validated by High Performance Liquid Chromatography. The developed protocol was implemented to characterize cyclosporine A-loaded 120 nm-sized lipid nanoparticles (LNPs, Lipidot®) dispersed in aqueous buffer. The colloidal stability was assessed by Dynamic Light Scattering (DLS).

Results

Validation experiments demonstrated suitable linearity, repeatability, accuracy and specificity to quantify residual free, entrapped and total drug. For the investigated LNPs, the method revealed a very limited shelflife of the formulation when stored in an aqueous buffer at 5 °C and even more at elevated temperature. Nevertheless, the DLS measurements confirmed the stability of nanoparticles during Solid Phase Extraction (SPE) in a suitable concentration range.

Conclusions

Solid phase extraction (SPE), when successfully validated, represents a valuable tool for drug development and quality control purposes of lipid-based nanopharmaceuticals in an industrial environment.

Keywords: Solid-phase extraction; Lipid nanoemulsion; Nanoparticles; Cyclosporine; Separation techniques

III.2 INTRODUCTION

In the scientific literature, nanomedicines are often considered as a promising field that could offer many advantages over small molecules. Many nanoproducts are currently under clinical trial or have already been approved, such as Abraxane® (paclitaxel) Ferumoxytol® (iron supplement) or Amphotec® (amphotericin B) for instance. However, the path until the final status of "approved" appears to be harder as hoped [105]. The complexity of the nanotechnologies for human health is such that time consuming missteps are unfortunately very common [106]. In particular, suitable techniques for the physicochemical characterization of the product are of the highest importance to reliably interpret results of the next studies (*in vivo* tests, toxicity…). The shape, the size or the zeta-potential of nanoparticles are key parameters for which many techniques [107] are already available and thoroughly investigated. On the contrary, far fewer techniques can be implemented in routine to investigate the distribution of the Active Pharmaceutical Ingredients (API) within the different compartments of the nanoformulation, essentially to distinguish between the free and entrapped drug. Dialysis methods are common for this purpose but the interpretation of the results is often not so obvious [108]. Other separation techniques like Cross-Flow-Filtration (CFF), Field-Flow Fractionation (FFF), Ultracentrifugation (UC), Ultrafiltration (UF), Capillary Electrophoresis (CE), Size Exclusion Chromatography (SEC) or Hydrodynamic Chromatography (HDC) have been proposed in the scientific literature [109]. Yet, simple and reliable methods, as required for quality control purposes, may not be generally feasible for any nanosystem.

For the present study, the Solid Phase Extraction (SPE) technique was selected because it generally allows at a later stage the full automation of the analytical procedure and hence, a strong reproducibility. Moreover, SPE has already been successfully applied for liposomes [41, 110], suggesting that it may be suitable for lipid nanoparticles (LNP) as well. This paper deals with the development of SPE methods to quantify separately free and entrapped cyclosporine A in a patented nanocarrier: Lipidot® [111]. The colloidal stability of the 120 nm-

sized LNPs passed through SPE-cartridges was verified by monitoring the particle size. The subsequent quantification of the API was performed by High Performance Liquid Chromatography (HPLC). The developed protocol as presented in Figure 32 consisted of a tripartite SPE-method including a Total Drug Content Determination method (TDCD), an Entrapped Drug Content Determination method (EDCD) and a Free Drug Content Determination method (FDCD).

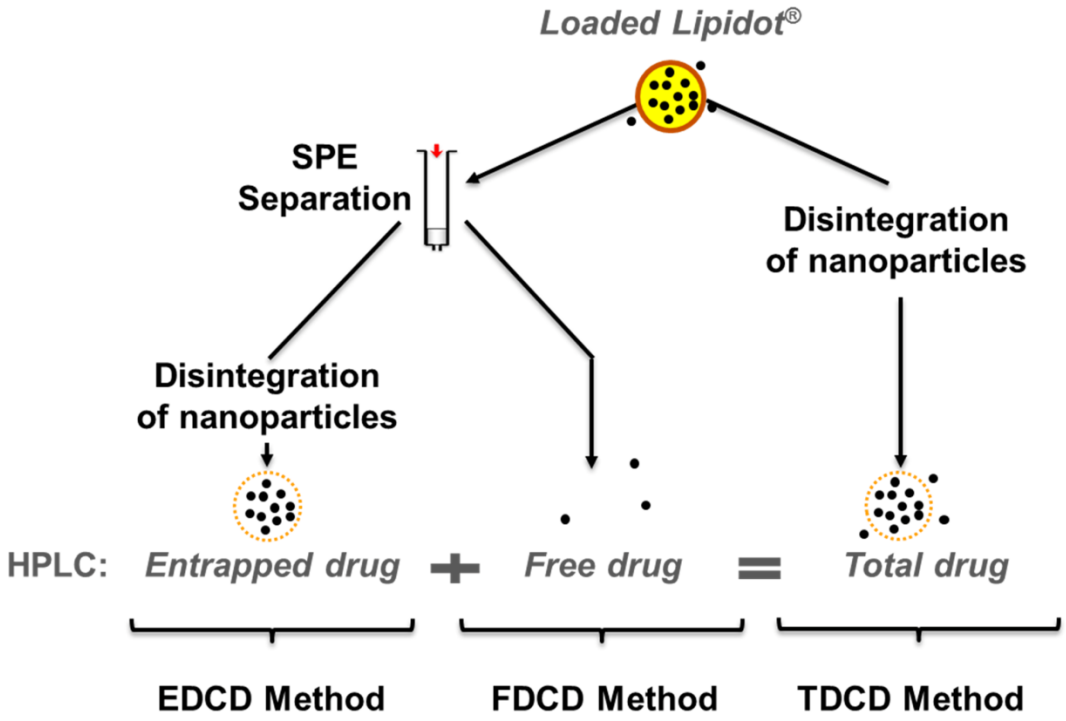

Figure 32: Simplified process description of the tripartite SPE-method

After validation of the analytical procedure according to the ICH guideline Q2A [102], the protocol was implemented for the Lipidot® formulation. Finally, a 48 h-stress test at elevated temperature and stirring speed was performed to evaluate the stability of the nanoformulation.

III.3 MATERIALS AND METHODS

III.3.1 Materials

Suppocire NB™ was purchased from Gattefosse S.A. (Saint-Priest, France). Myrj™ S40 (PEG 40 stearate, 1,980 Da) and Super Refined Soybean Oil were obtained from Croda Uniquema (Chocques, France). Lipoid® S75-3 (soybean lecithin at 69 % of phosphatidylcholine) provided by Lipoid GmbH (Ludwigshafen, Germany). All these excipients are pharmaceutical grade and used as received. Acetonitrile (ACN) and methanol (MeOH) were provided by LiChrosolv®, orthophosphoric acid 85 % by BDH Prolabo® and trifluoroacetic acid 99.9 % by EMD Millipore Corporation. All reagents were HPLC-grade. The cyclosporine A (CSA) was supplied by RTC Pharma. Highly purified water was produced using a Milli-Q® Gradient A10 from EMD Millipore Corporation. Lipidots are formulated using a VCX750 Ultrasonic processor from Sonics (Newtown, USA) equipped with a 3 mm-diameter microtip. The balance was an AX205 from Mettler Toledo, the water bath was the type 19 of Julabo, the centrifuge was a Minispin from Eppendorf and the vortex was a Vortex-Genie 2 from Scientific Industries. The quantifications of the active pharmaceutical ingredient (API) were performed by high performance liquid chromatography (HPLC) using an Agilent 1100 Series system from Agilent Technologies with a degasser, oven and DAD-detector. The HPLC-column was an XTerra® RP-18 (5 µm x 150 mm x 4.6 mm) from Waters. The separation of the drug from nanoparticles was carried out using a solid phase extraction (SPE) system from Supelco: Visiprep™ 12-Port Vacuum Manifolds. The SPE-cartridges Supelclean™ LC-18 SPE tube (1 mL, 60 Å pore size, 45 µm particle size) were provided by Supelco. The nanoparticle size experiments were performed using a Zetasizer Nano ZS from Malvern Instrument equipped with a 532 nm green laser source. The stress test was carried out using the Manual Diffusion Test System equipped with a 7 mL vertical diffusion cell provided by Hanson Research.

III.3.2 Lipidot® formulation

The formulation of conventional Lipidot® has been previously described elsewhere [112, 113]. The lipid phase was prepared by mixing lipophilic ingredients: wax, oil and Lipoid S75-3 phospholipids, whereas the aqueous phase was composed of the hydrophilic PEG surfactants, MyrjS40, solubilized in 1X PBS aqueous buffer. After homogeneous melting at 45 °C, both phases were crudely mixed and sonication cycles are performed during 5 min. The purification step was carried out overnight using dialysis (1X PBS, MWCO: 12 kDa, regenerated cellulose membrane, Spectra/Por®). Lipid nanoparticles (LNP) were finally formulated at a total concentration of lipids of 60 mg/mL and then filtered through a 0.22 µm cellulose Millipore membrane. For 120 nm-sized formulation, the lecithin/PEG surfactant weight ratio was 0.21 with a surfactant/core weight ratio of 0.43. Concerning the manufacturing of the CSA-loaded LNPs, an appropriate amount of CSA in absolute ethanol (45 µL of 63.7 mg/mL of CSA stock solution) was initially added to the oily phase, and then the solvent was evaporated under argon flow. The nanoparticles encapsulating CSA were then formulated as above described. CSA drug was initially loaded until a concentration of 210 µg/mL and a total lipid concentration of 60 mg/mL. The blank nanoparticles were prepared without drug corresponded to the placebo formulation.

III.3.3 Preparation of the SPE and HPLC mobile phases

The solution of trifluoroacetic acid 0.5 % (v/v) (hereafter named TFA), was prepared diluting trifluoroacetic acid 99.9 % with highly purified water. The solution of ACN:H3PO4 5 mM (75:25; v/v) (hereafter named mobile phase A) and the solution of MeOH:H3PO4 5 mM (75:25; v/v) (Mobile phase B) were prepared mixing phosphoric acid 5 mM solution with respectively ACN and MeOH. The solution of phosphoric acid 5 mM was prepared by dilution of orthophosphoric acid 85 % with highly purified water.

III.3.4 Principle of the tripartite SPE-method for lipid nanoparticles

The samples formerly stored at 5 °C were left without handling until equilibration at room temperature. As described in Table 6, media were successively introduced into the SPE-cartridges for cleaning and equilibration prior to introduction of the samples of formulation. The nanoparticles were eluted in a first fraction (F1) adding TFA as eluent into the SPE-cartridge. The free drug was eluted in a second fraction (F2) using ACN as a second eluent. The pressure in the vacuum chamber during the elution of LNPs (Step 3) was adjusted, resulting in a flow rate of 1 mL/min.

The nanoparticles eluted in F1 could further be disintegrated to release entrapped drug. Briefly, the disintegration step consisted in mixing one volume of eluted nanoparticles with two volumes of ACN. The mixture was vortexed and centrifuged at 10,000 rpm for 10 min. The released drug in supernatant was quantified using a suitable HPLC method (EDCD method).

The free drug eluted in F2 could be directly quantified by HPLC (FDCD method).

The total drug content was determined for a sample without being passed through SPE but directly prepared as previously described in the disintegration step. The released entrapped drug and the free drug were quantified by HPLC (TDCD method).

Table 6: SPE overall plan. ACN: Acetonitrile; TFA: Trifluoroacetic acid 0.5 % (v/v); Sample: Nanoformulation to be tested; NPs: nanoparticles; API: Active Pharmaceutical Ingredient; VF1: volumetric flask #1; VF2: volumetric flask #2. The volumetric flasks were made to the marks after elution using the same medium as used for elution.

Step	Medium	Volume	Pressure	Fraction	Container	Function
1	ACN	≈ 6 mL	≈ - 50 kPa	F0	Waste	Cleaning
2	TFA	≈ 4 mL	≈ - 50 kPa			Equilibration
3	Sample	1 mL	≈ - 20 kPa	F1	5 mL-VF1	Elution of NPs
4	TFA	≈ 3 mL	≈ - 50 kPa			
5	ACN	≈ 4 mL	≈ - 50 kPa	F2	5 mL-VF2	Elution of API

III.3.5 Assessment of the colloidal stability of Lipidot® following SPE protocol

Size distribution of Lipidot® was investigated by dynamic light scattering (DLS) to monitor the colloidal integrity of the nanoparticles after being applied SPE method. Prior to SPE, the sample of placebo Lipidot® was diluted using TFA until 3, 6, 10, 15, 30 and 60 mg/mL LNPs. Each dilution of the set was further eluted by SPE as described in Table 6 until step 4. The fractions F1 were collected and the particle sizes were assessed using the Zetasizer Nano ZS. The solution of TFA was used as blank and placebo Lipidot® as negative control. Before measurement, samples were diluted in TFA to a dispersed phase weight fraction of 1 mg/mL in order to avoid multiple scattering effects. All samples were prepared in duplicate and analyzed in triplicate. The Z-average diameter (Size, nm) and polydispersity index (PdI) of the lipid nanoparticles were extracted from the second cumulant of the correlation function of the intensity distribution. Each result was the mean of three independent measurements performed at 25 °C, at a fixed angle of 173 °.

III.3.6 SPE separation methods and validation by HPLC analysis

The quantitative validation of the SPE method for the FDCD was performed for a set of placebo Lipidot® diluted with highly purified water solutions spiked with different proportions of cyclosporine A. A CSA stock solution was prepared in duplicate dissolving the powder of CSA in ACN until a concentration of 2842 µg/mL. A first CSA dilution set was then prepared by dilution of the stock solution using ACN until 284, 426, 710, 852, 1136, 1421 and 1705 µg/mL CSA. The dilution set was further diluted with a dilution factor of 50 using highly purified water, so that the resulting dilution set contained 2 %, (v/v) ACN, i.e. less than the limit of 5 % above which LNPs disintegrate. The sample of placebo Lipidot® was added until a concentration of 9.6 mg/mL, i.e. until a LNP dilution factor of 4:25 (dilution set designated by “CSA + placebo Lipidot® for FDCD” in Table 7). In addition, another dilution set (“CSA for FDCD” in Table 7) was prepared without placebo Lipidot®. Each sample of both the dilution sets of CSA solutions and CSA-spiked placebo LNPs was investigated in triplicate by solid

phase extraction according to the validation plan presented in Figure 33 and using the SPE protocol previously described in Table 6.

Table 7: Dilution sets prepared for the validation of the tripartite-SPE method. For the EDCD method, the data given in the table do not correspond to entrapped CSA because of the technical impossibility to spike drug inside nanoparticles. The value of the entrapped concentrations (from 0.00 to 2.27 µg/mL) were hence calculated from the concentration of CSA spiked in ACN (from 0.00 to 3.41 µg/mL), i.e. using a factor 1.5. For the TDCD method, the data were calculated as for the EDCD method.

Dilution set	**CSA for FDCD**							
Dilution label	0%	20%	30%	50%	60%	80%	100%	120%
CSA (µg/mL)	0.00	5.68	8.53	14.21	17.05	22.74	28.42	34.10
LNPs (mg/mL)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dilution set	**CSA + placebo Lipidot® for FDCD**							
Dilution label	0%	20%	30%	50%	60%	80%	100%	120%
CSA (µg/mL)	0.00	5.68	8.53	14.21	17.05	22.74	28.42	34.10
LNPs (mg/mL)	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60
Dilution set	**CSA + placebo Lipidot® for EDCD**							
Dilution label	0%	20%	30%	50%	60%	80%	100%	120%
CSA (µg/mL)	0.00	0.38	0.57	0.95	1.14	1.52	1.89	2.27
LNPs (mg/mL)	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60
Dilution set	**CSA + placebo Lipidot® for TDCD**							
Dilution label	0%	20%	30%	50%	60%	80%	100%	120%
CSA (µg/mL)	0.00	0.38	0.57	0.95	1.14	1.52	1.89	2.27
LNPs (mg/mL)	1.92	1.92	1.92	1.92	1.92	1.92	1.92	1.92

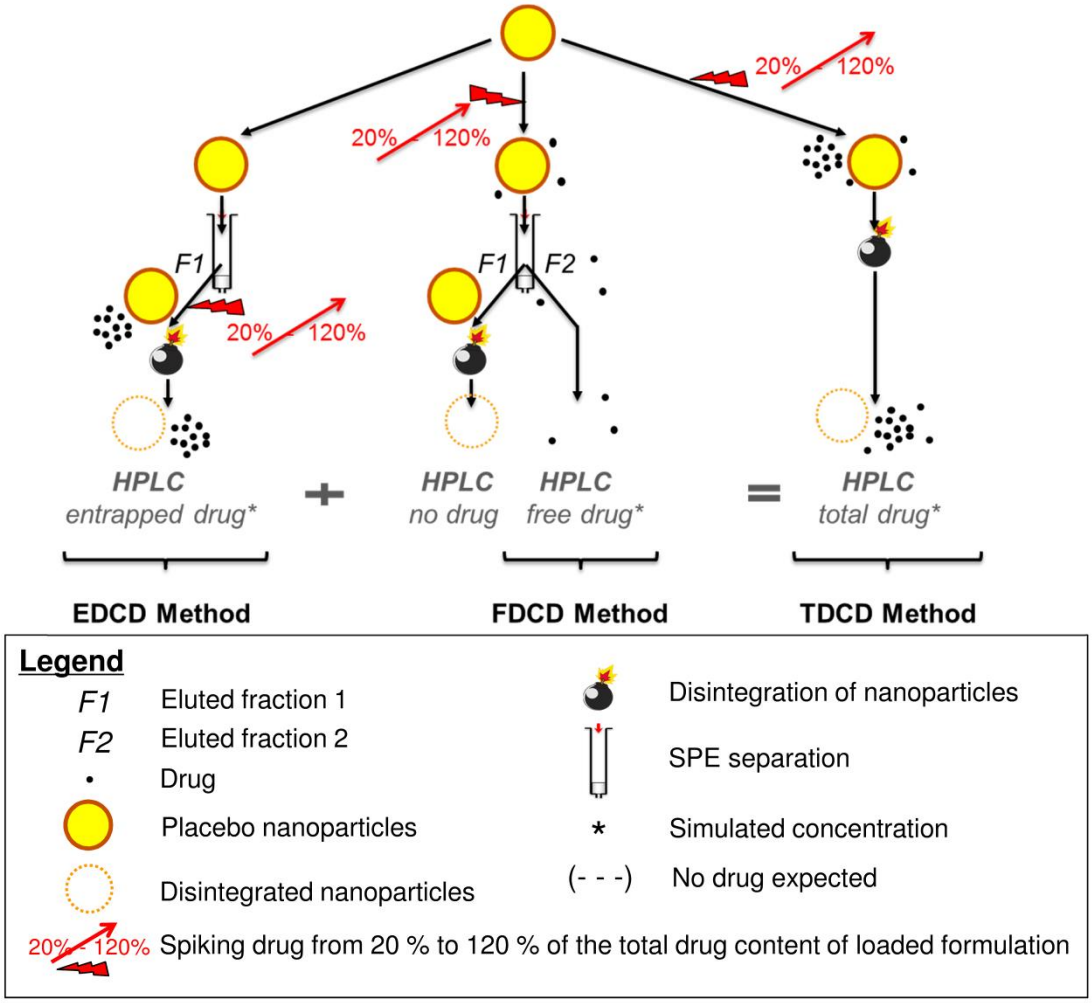

Figure 33: Validation plan for the tripartite SPE method.

III.3.6.1 Validation FDCD method

The FDCD method further consisted in maintaining the fractions F2 of eluted CSA in a water bath for 5 min at 37 °C. Aim of this step was to dissolve the precipitate formed by co-eluted excipients present in the extern phase of the Lipidot® formulation. The CSA was quantified by HPLC using the setups for FDCD (see in Table 7), compatible with the presence of co-eluted excipients. The specificity of the CSA separation was assessed from the fraction F1: the eluted LNPs were disintegrated as previously described and analyzed by HPLC to verify the absence of CSA in this fraction. The HPLC method used the setups for EDCD and TDCD

(see in Table 7), compatible with the presence of excipients released after disintegration of the nanoparticles.

Table 8: Setups of the HPLC methods used for FDCD, EDCD and TDCD

Parameters	Setups for FDCD	Setups for EDCD and TDCD
Column	XTerra®, RP-18 (5 µm x 150 mm x 4.6 mm)	
Elution mode	Isocratic	
Mobile phase	ACN:H_3PO_4 5 mM (75:25, v/v)	MeOH:H_3PO_4 5 mM (75:25, v/v)
Flow	1.0 mL/min	1.2 mL/min
Wavelength detection	205 nm	
Temperature	55 °C	
Injection volume	30 µL	50 µL
Run time	7 min	10 min
Retention time	3.10 min (± 0.05 min)	6.55 min (± 0.10 min)

III.3.6.2 Validation EDCD method

Concerning the EDCD method, the validation focused on the step following the elution of LNPs in F1, since spiking placebo Lipidot® with entrapped CSA was not feasible. Placebo LNPs were diluted using highly purified water until a concentration of 9.6 mg/mL LNPs (i.e. LNP dilution factor 4:25) and were further investigated in triplicate according to the overall SPE-plan presented in Table 6, until step 4. Afterward, 0.5 mL of F1 were mixed with 1 mL of a dilution set of CSA in ACN containing 0.57, 0.85, 1.42, 1.71, 2.27, 2.84 and 3.41 µg/mL drug (dilution set designated by “CSA + placebo Lipidot® for EDCD” in Table 7). The resulting mixtures were shaken using the vortex to disintegrate the placebo LNPs and centrifuged for 10 min at 10,000 rpm to precipitate the excipients and to only collect the CSA in supernatant solution. The HPLC setups used for EDCD are presented in Table 8.

III.3.6.3 Validation TDCD method

The TDCD method was validated by diluting the placebo Lipidot® using TFA until equivalent concentration of LNP to Fractions F1 in EDCD methods (1.92 mg/mL LNPs). As used for the validation of the EDCD method, 0.5 mL of this latter diluted solution were mixed afterward with 1 mL of the same dilution set of CSA in ACN (dilution set designated by "CSA + placebo Lipidot® for TDCD" in Table 7). The mixtures were vortexed and centrifuged as previously described for the EDCD method and then analyzed using the same HPLC method (see Table 8).

III.3.7 Implementation of the tripartite SPE method for a sample of cyclosporine A-loaded Lipidot® formulation and stress test

The SPE protocol was implemented for a sample of cyclosporine A-loaded 120 nm-sized Lipidot® as previously validated. After equilibration at room temperature and dilution of the sample (dilution factor 4:25 in highly purified water), the FDCD and the EDCD methods were implemented directly whereas for the TDCD method, a dilution in TFA with factor 1:5 was applied. All samples were prepared five times (n = 5).

The protocol was implemented as well for the nanoformulation without the dilution step in highly purified water, namely 1 mL of Lipidot® product was placed in the SPE cartridge at step 3 (see Table 6) for the FDCD and EDCD methods and the product was directly diluted in TFA (dilution factor 1:5) for the TDCD method.

Regarding the stress conditions, 7 mL of cyclosporine A-loaded Lipidot® formulation were placed in the acceptor compartment of the vertical diffusion cell (VDC) used without membrane as 1-compartment cell. The stress test was carried out for 48 h at 37 °C with a magnetic stirring of 500 rpm. The stressed product was analyzed as previously without the dilution step in highly purified water.

III.4 RESULTS AND DISCUSSION

III.4.1 SPE technique applied to Lipidot®

The developed protocol is based on the reverse-phase SPE concept, relying on the Van der Waals interactions between a hydrophobic analyte and a hydrophobic solid stationary phase (SPE-cartridge). This phenomenon leads to a stronger retention of the lipophilic analyte whereas the lipid nanoparticles are eluted faster by a hydrophilic mobile phase due to their outer hydrophilic PEG shell. The separation between the API and LNPs is further enhanced by the significant size difference of the two entities. Indeed, the stationary phase consists of beads containing pores smaller than the nanoparticle diameter but larger than the API as molecular entity. Based on these two mechanismes, PEG-shell lipid nanoparticles loaded with hydrophobic drugs or contrast agents can be eluted in a first step by using TFA as a hydrophilic mobile phase. In a second step, the retained drug can be eluted using ACN as a more lipophilic mobile phase. This technique is consequently suitable to separate and quantify either the remaining non-entrapped drug fraction after a given manufacturing process, to detect a leakage of drug during storage on the shelf, or even to monitor drug release during an *in vitro* performance test. Moreover, such separation of the API from the particles takes only few minutes whereas other techniques, like e.g. dialysis, may require several days, and thus might be of the same time scale as the expected drug release from the carrier. Contrary to polymer or inorganic nanoparticles, considering the metastable character of lipid nanoparticles when dispersed in aqueous buffer, techniques based on separation-precipitation cannot be applied. Methods requiring organic solvents would destabilize the interface of droplets resulting in a biphasic system (oil and water parts). Consequently, solid phase extraction turned out to be the most suitable separation method for lipid nanoparticles.

III.4.2 Assessment of the colloidal stability of Lipidot® following SPE protocol

As colloidal nanoemulsions, Lipidot® are composed of lipid droplets surrounded by lecithin and coated with PEG surfactant with a particle size range from 50 to 120 nm to render colloidal stability, reported with a shelf-life in suspension over one year [112]. Because their interaction with the stationary phase materials could potentially affect the integrity of the nanoparticles it was necessary to confirm their colloidal stability after the SPE process to validate its use as a separation method. Such experiment was consequently performed using a Zetasizer Nano ZS after SPE process. It was firstly shown that the median size and Polydispersity Index (PdI) for different dilutions of LNPs in TFA (control dilutions) did not significantly differ one to another. This finding demonstrated that acid medium did not destabilize the LNPs. Secondly, obvious differences between the particle size of the LNPs eluted in TFA after SPE and the control-LNPs diluted in TFA were observed for the concentrations 3 mg/mL LNPs (no peak observed after SPE) and 6 mg/mL LNPs (delayed peak corresponding to larger particle sizes after SPE) (Figure 34 and Table 9). This suggests that nanoparticle concentration of 6 mg/mL and below should not be used.

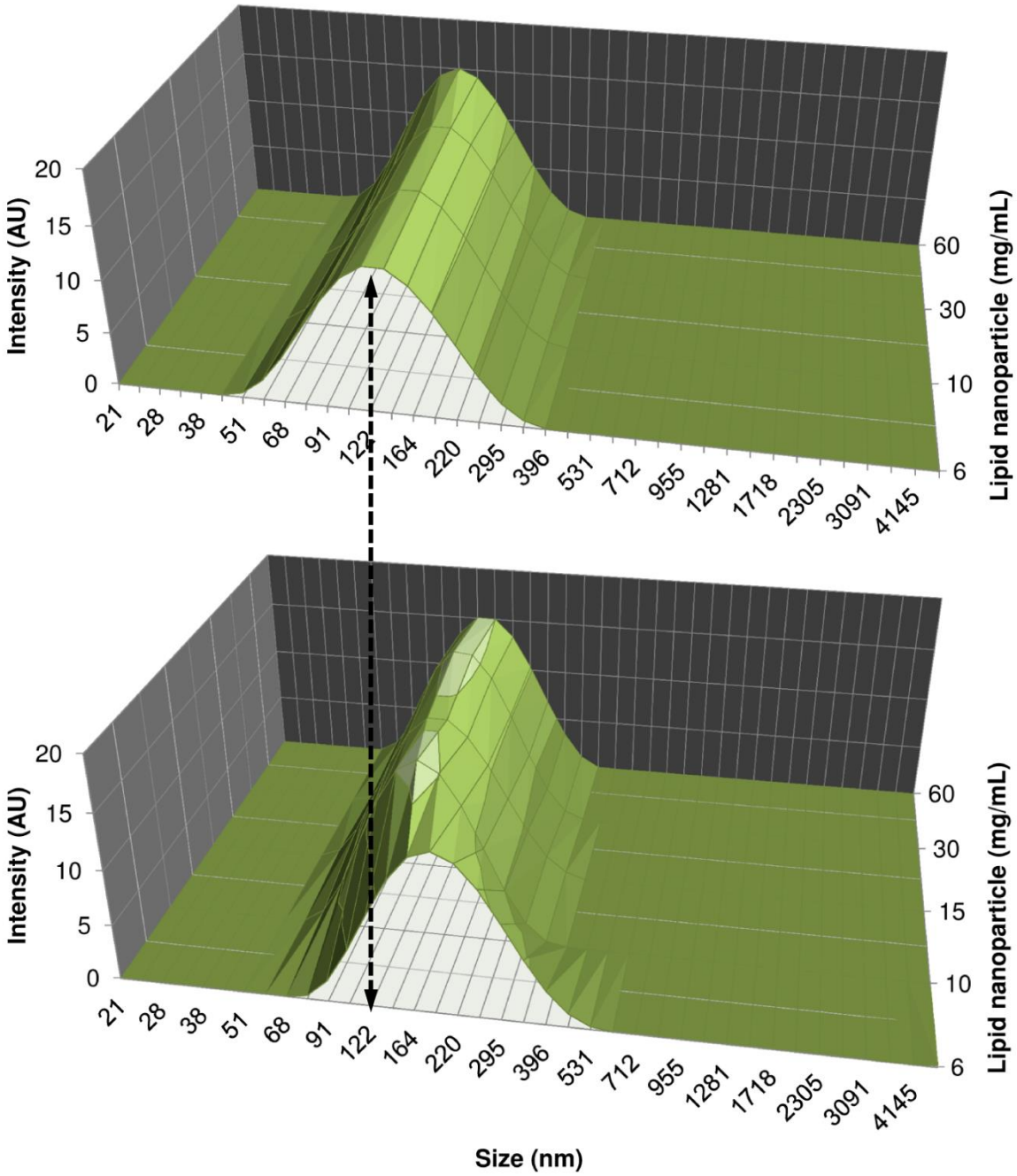

Figure 34: Particle distribution for different concentrations of Lipidot® samples before and after SPE. The control set of LNPs (top) was diluted until same concentration as the set of eluted LNPs through SPE (bottom). The size distribution remained constant for the whole control set (6, 10, 30 and 60 mg/mL). After SPE, the modal size remained not affected for the concentrations higher than 10 mg/mL but was shifted towards higher values at 6 mg/mL.

Table 9: Particle size (nm) and polydispersity index (PdI) for different concentrations of Lipidot® samples prepared by solid phase extraction (SPE) or not (Control)

	Particle size (nm)		PdI	
LNP concentration (mg/mL)	**Control**	**SPE**	**Control**	**SPE**
3	n. a.	2849.0	n. a.	0.747
6	123.3	169.3	0.125	0.129
10	124.8	137.0	0.117	0.097
15	n. a.	130.9	n. a.	0.141
30	124.1	125.8	0.114	0.106
60	124.4	123.8	0.125	0.106

III.4.3 Validation of the tripartite SPE method

The tripartite SPE method actually consists of preparative and analytical methods for the quantification of the total API (TDCD), the entrapped API (EDCD) and the free API (FDCD). The dilution set of CSA prepared without LNPs for the validation of the FDCD method was practically not turbid in the selected cyclosporine A concentration range. However, for concentrations greater than 35-40 µg/mL (before SPE), the turbidity increased, precipitates were observed and the validation test failed (RSD >> 5 %). The validation of FDCD succeeded using highly purified water as dilution medium until 34.10 µg/mL (see results in Figure 35 and in Table 10). The linearity and repeatability were slightly better in presence than in absence of Lipidot®, obviously reflecting the improved solubility of cyclosporine A by this formulation.

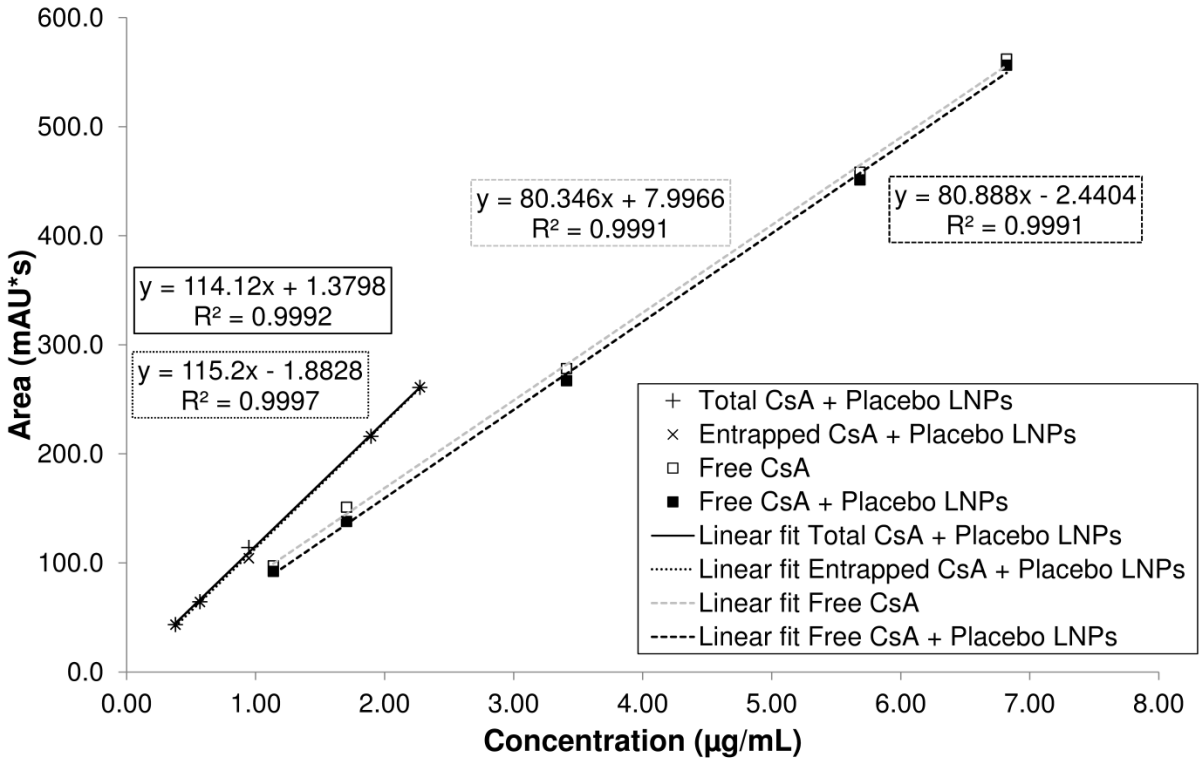

Figure 35: Linearity of the tripartite SPE method: measured HPLC area related to the CSA concentration for the TDCD, EDCD and FDCD methods. The given concentrations correspond to the samples after SPE.

Table 10: Validation of the tripartite SPE method: The given concentrations correspond to the samples prepared for HPLC analysis and should be multiplied by a factor 5 for FDCD and a factor 15 for EDCD and TDCD to calculate the concentration of the sample before preparation. Linearity performed with 20, 30, 60, 100 and 120 % of the simulated free API concentration, 100 % corresponding to 5.684 µg/mL CSA for FDCD method and to 1.920 µg/mL CSA for both EDCD and TDCD methods. * Results for CSA without placebo Lipidot®; ** Results for placebo Lipidot® spiked with CSA.

Parameters	FDCD*	FDCD**	EDCD**	TDCD**
Linearity				
• Range (µg/mL)	1.14 – 6.82	1.14 – 6.82	0.38 – 2.27	0.38 – 2.27
• R^2	0.9991	0.9991	0.9992	0.9997
• LOQ (µg/mL)	0.41	0.17	0.26	0.16
• LOD (µg/mL)	0.14	0.06	0.09	0.05
• RSD (%)	3.97	1.71	2.56	1.67
Repeatability (n = 3)				
• CV(%) at 20 %	5.59	2.13	0.93	1.33
• CV(%) at 100 %	1.15	3.88	1.58	1.06
• CV(%) at 120 %	1.95	1.68	1.02	1.56
Accuracy (n = 3)				
• 20 %	99.9 – 111.7	98.3 – 103.0	100.9 – 102.8	100.9 – 103.3
• 100 %	98.4 – 100.8	94.7 – 102.1	99.8 – 103.0	100.5 – 102.6
• 120 %	99.3 – 103.0	99.2 – 103.1	100.1 – 103.0	100.4 – 103.4
Specificity	No interfering peaks were observed at the retention time of the API			

For the EDCD and TDCD methods, the results of the validation were satisfying regardless of the very low selected concentrations. The areas corresponding to CSA in the chromatograms (see Figure 35 and Figure 36) were low for the EDCD and TDCD methods but still acceptable. All relevant peaks were separated with a sufficient resolution and peak symmetrie for a reproducible integration. Both methods presented very wide and high injection peaks attributed to excipients released after destruction of LNPs and immediately eluted by HPLC. However, further peaks were observed for TDCD but not for EDCD, suggesting that other excipients of the formulation were not eluted in the fraction F1 but instead co-eluted with CSA in the fraction F2. Additional peaks were actually observed for FDCD, but did not affect the quantitation of the drug related peaks.

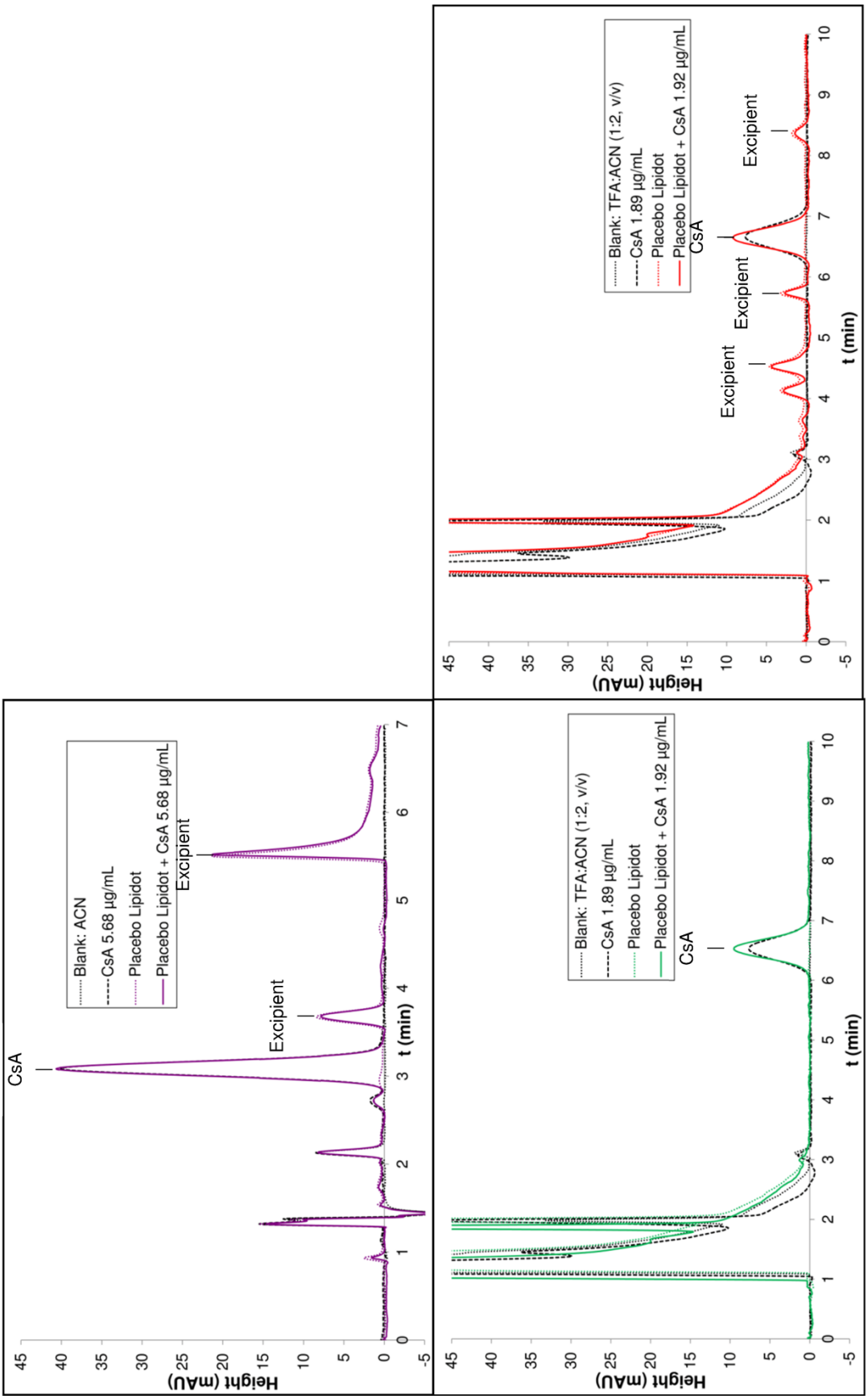

Figure 36: Chromatogram of cyclosporine A quantified using the FDCD method (violet), EDCD method (green) and TDCD method (red)

III.4.4 Tripartite SPE method for a Lipidot® nanoformulation – Stress test

A protocol of characterization based on the SPE technique was previously designed, developed and validated using placebo lipid nanoparticles. Its implementation was also performed using the loaded Lipidot® formulation. The tripartite SPE method was able to determine the concentrations of entrapped CSA (88.9 µg/mL), free CSA (67.5 µg/mL) and total CSA (163.8 µg/mL). Moreover, comparing the experimental total CSA concentration to the theoretical concentration (210.0 µg/mL), an encapsulation efficiency of the manufacturing process could be calculated (78 %) to highlight the loss of non-encapsulated drug removed over intensive dialysis during the manufacturing process. The protocol was further implemented using a diluted formulation of Lipidot® in order to assess the influence of a dilution on the release of CSA. The free CSA represented 41 % of the total concentration for the samples prepared without dilution, vs. 82 % for the diluted samples. The entrapped CSA was quantified as well and represented 54 % for undiluted sample vs. 11 % for diluted samples. The methods for free and entrapped CSA could successfully reveal a dilution-effect and were almost complementary. However, according to the results summarized in Table 11, the repeatabilities (coefficient of variation) were very satisfying for the total and free CSA content determination method but not optimal for the entrapped CSA. Consequently, when the complementarity between the three methods was not completely achieved, the unidentified amount should be to be related to the entrapped CSA (see Figure 37). As concern the results of the stress test at 37 °C and 500 rpm, a proportion of 68 % free CSA was observed after the stress test vs. 41 % free CSA without the test. The result suggested a sustained release potential for the Lipidot® formulations. All the results of the tests performed for CSA-loaded Lipidot® formulation are given in Table 11 and Figure 37.

Table 11: Total, entrapped and free CSA concentrations for the loaded Lipidot® formulation. Dilution 1:1: sample not diluted prior SPE separation; Dilution 4:25: sample diluted prior SPE separation; Dilution 1:1 Stress test: sample stressed over 48 h, not diluted prior SPE. Experiments performed for n = 5. *: n = 4. **: n = 3. CV: coefficient of variation.

	Dilution 1:1 Mean (CV)	Dilution 4:25 Mean (CV)	Dilution 1:1 Stress test Mean (CV)
Total CSA (µg/mL)	163.8 (1.1 %)	155.5 (1.1 %)	178.8 (1.1 %)
Entrapped CSA (µg/mL) % /Total CSA	88.9 (8.5 %) 54	17.6 (15.3 %)** 11	39.0 (15.3 %) 22
Free CSA (µg/mL) % /Total CSA	67.5 (6.3 %)* 41	127.1 (5.0 %) 82	121.5 (4.0 %) 68
Total - (Entrapped + Free) (µg/mL) % /Total CSA	7.4 5	10.8 7	18.3 10

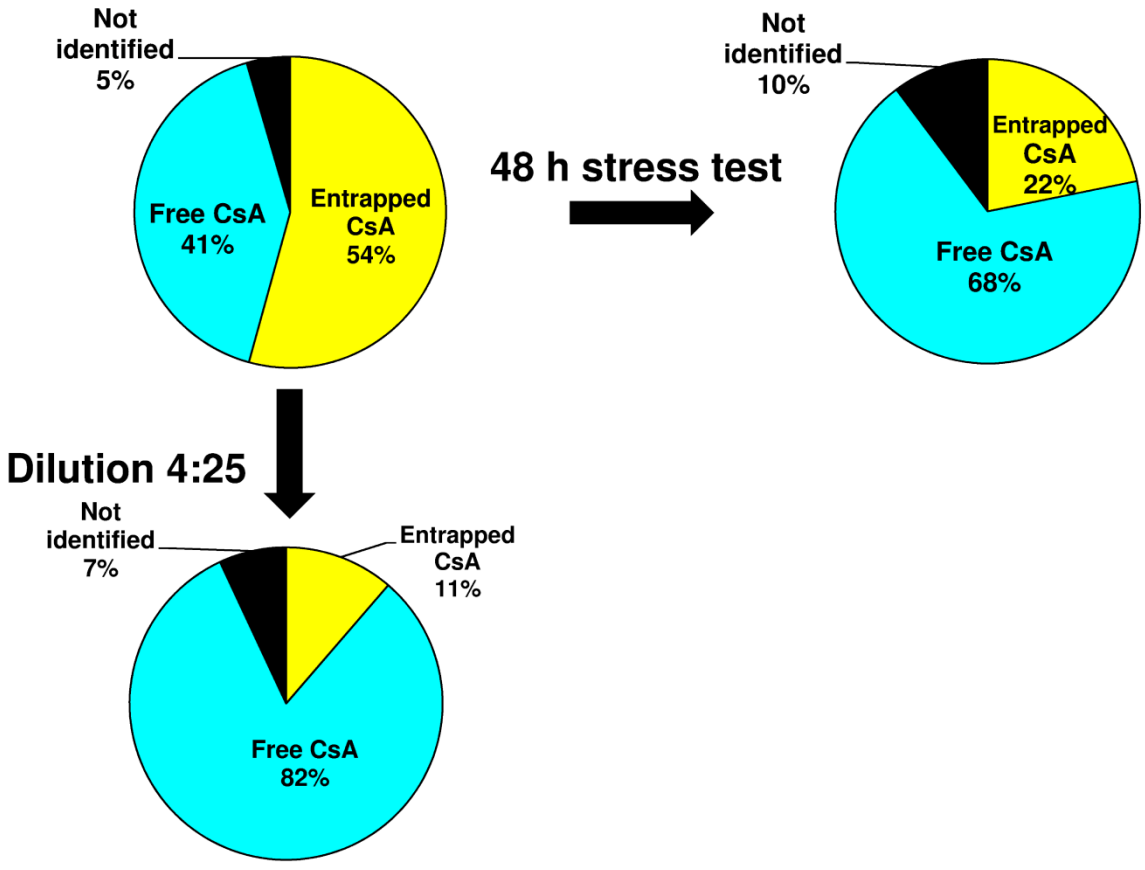

Figure 37: Total, entrapped and free CSA concentrations for the loaded Lipidot® formulation. Top-left: Dilution 1:1 (sample not diluted prior SPE separation). Bottom-left: Dilution 4:25 (sample diluted prior SPE separation). Top-right: Dilution 1:1 Stress test (sample stressed over 48 h, not diluted prior SPE).

III.5 CONCLUSION

An accurate, repeatable, fast and automatable method based on SPE wasdeveloped for the separation and quantification of non-entrapped and entrapped cyclosporine A in the lipid nanoformulation Lipidot®. The results obtained by DLS have shown that nanoparticles were actually eluted in a first fraction. Furthermore, the validation of the technique by HPLC resulted in very satisfying results revealing an excellent analytical performance of the method and usability for quality control purposes. At this stage of product developement, the SPE technique clearly demonstrated the complexity of the Lipidot® formulation as well as necessary further improvements towards a pharmaceutical product. Besides testing drug release and leakage during storage, respectively, the technique would also allow to quantify the amount of API removed during the purification step of the manufacturing process and thus the encapsulation rate and efficacy. Apart from the considerations related to the specific formulation, which served as an example for the study, the concept of SPE presents itself as very promising tool for drug development and quality control purposes of lipid-based nanopharmaceuticals in an industrial environment.

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4 CONCLUSION

As previously mentioned, inflammatory bowel disease concerns millions people in the world, the clinical consequences can be very severe, the etiology remains poorly known and the current treatments are often either not sufficient enough or accompanied with strong adverse side effects. This is the reason why safe and efficient new treatments are needed. Passive targeting using nanocarriers represents exactly the strategy of interest. In this context, analytical tools to assess the manufacturing process of candidate formulations and their *in vitro* drug release performances are desired.

In the scientific literature, methods are available for *in vitro* drug release testing but most are based on home-designed dissolution devices, typically including dialysis systems, and are generally not suitable to achieve quality control tests required for regulatory submissions. Moreover, in the particular case of lipid nanoparticles and because of its metastable character, no reliable techniques are available for the assessment of the formulation drug load. The present dissertation attempted to provide analytical tools based on standardized compendial apparatuses and that presents suitable analytical performances with the possibility of automation.

Concerning the polymeric drug carriers, namely the PLGA-nanoparticles and PLGA-microparticles, a drug content determination method and *in vitro* drug release testing methods were developed. The drug content determination method was based on the use of a solvent to release the whole amount of API from the polymeric matrix. The validation concluded to a narrow accuracy (98 – 101 % for budesonide microparticles, 97 – 103 % for budesonide nanoparticles and 98 – 104 % for cyclosporine A microparticles). *In vitro* drug release methods for polymeric micro- and nanoparticles could be further developed using the combination “fiber-optic system – derivative spectrophotometry – compendial basket dissolution apparatus”. The fiber-optic system presented the advantage to monitor *in situ* the drug release so that sampling, filtration and sample preparation were not required anymore. The derivative spectrophotometry was necessary to correct the interferences produced by the nano- or microparticles and to quantify the released drug. The basket apparatus was the

simplest compendial apparatus for the *in vitro* assessment of the performances for these formulations. As a result, robust methods completely automatable were performed for all three polymeric formulations. The resulting drug release profiles showed a strong burst effect for the nanoparticles but a moderate burst release followed by a sustained release for the microparticles. To sum up, the developed analytical methods revealed that polymeric microparticles of PLGA were suitable as such for pharmaceutical development but nanoparticles require further optimization, for instance by coating. *In vitro* DRT remains in this context the most suitable tool to assess the performances of new formulations.

As concern the lipid drug carriers, i.e. the Lipidot®, methods to determine the total drug content and to separate free residual or released drug from nanoparticles were successfully established. The total drug content determination method (TDCD) was based on the use of a solvent mixture to disintegrate the nanoparticles and to release the whole amount of API trapped in the carrier. This method was aimed to be used in combination with a separation technique: the solid phase extraction (SPE). The SPE leaded to a first fraction containing only free drug (free drug content determination method: FDCD) and a second fraction containing only entrapped drug in nanoparticles. Isolated lipid nanoparticles can further be disintegrated using the same method to release and to quantify specifically the entrapped drug (entrapped drug content determination method: EDCD). This “tripartite-method” was favorably validated and presented an accuracy of 95 – 102 % for the FDCD method and 100 – 103 % for both the EDCD and TDCD methods. As for the *in vitro* drug release method developed for polymeric drug carriers, the SPE method was aimed to be at a later stage completely automated for routine analysis. The tripartite-SPE-method was implemented for a sample of Lipidot® before and after a stress-test and after a dilution step. According to the results, a large amount of drug was either already released before analysis or not entrapped after manufacturing (42 %). Moreover, after dilution of the formulation, a much larger amount of free drug was unexpectedly observed (83 %). Further investigations are required to confirm this dilution-induced release and to identify the causes of this effect. However, the

formulation was stable enough to maintain entrapped a significant amount of API during a 48 h-lasting stress-test. Indeed, 42 % of entrapped drug remained entrapped after the test. As a conclusion, a powerful analytical tool for the characterization of Lipidot® was established and is now available for the optimization of the manufacturing process (entrapment efficiency), of the purification process and of the storage conditions. This characterization method can be used to investigate the hypothetical dilution-induced drug release and more generally as sample preparation for *in vitro* drug release tests or drug-load stability tests. The preliminary studies performed for a loaded formulation already revealed that Lipidot® present promising release properties, namely the appropriate balance between stability and release required for any drug carrier system.

Finally, the present dissertation enabled the use of a standard compendial dissolution apparatus for the assessment of *in vitro* performances of nanopharmaceuticals. In addition, this work provided an original solution for lipid nanoparticles for which no reliable method was available to date to quantify selectively free or released drug and entrapped drug. The investigated techniques showed adequate analytical performance for quality control purposes in an efficient industrial context, including the potential to be automated at a later stage. The optimization of the investigated nanoformulations was highlighted by the developed analytical tools to be necessary for the pharmaceutical development of drug nanocarrier platforms. At a final stage, the commercialization of such therapeutic platforms may be a promising alternative treatment for diseases, such as the inflammatory bowel disease.

5 LIST OF ABBREVIATIONS

λ	Wavelength
^{1}H-NMR	Proton Nuclear Magnetic Resonance
ACN	Acetonitrile
ADME	Absorption, Distribution, Metabolization and Excretion
AF4	Asymmetric Flow Field-Flow Fractionation
ANUC	Analytical Ultracentrifugation
API	Active pharmaceutical ingredient
AU	Analytical Ultracentrifugation
AUC	Area under the curve
AVP	Aqua valde purificata (Highly purified water)
BUD	Budesonide
CD	Crohn Disease
CE	Capillary Electrophoresis
CFF	Cross-Flow-Filtration
cGMP	current Good Manufacturing Practices
CSA	Cyclosporine A
CV	Coefficient of Variation
CZE	Capillary Zone Electrophoresis
D0	Zero-order derivative
D1	First-order derivative
D2	Second-order derivative

DAD	Diode Array Detector
DLS	Dynamic Light Scattering
DRT	Drug Release Testing
DSA	Dialysis Sac Adaptor
DSE	Drug Selective Electrode
DSP	Derivative Spectrophotometry
EDCD	Entrapped Drug Content Determination
EPR	Enhanced Permeation and Retention
EOF	Electro-Osmotic Flow
F0	Fraction 0
F1	Fraction 1
F120	Formulation containing 120 nm-sized lipid nanoparticles
f2	Similarity Factor
F2	Fraction 2
F50	Formulation containing 50 nm-sized lipid nanoparticles
FDA	Food and Drug Administration
FDCD	Free Drug Content Determination
FFF	Field-Flow Fractionation
FLD	Fluorescence Detector
GFC	Gel Filtration Chromatography
GPC	Gel Permeation Chromatography

H_3PO_4 5 mM	Phosphoric acid solution 5 mM
HDC	Hydrodynamic Chromatography
HF5	Hollow-Fiber Flow Field-Flow Fractionation
HPLC	High Performance Liquid Chromatographie
HPSEC	High Performance Size-Exclusion Chromatography
IBD	Inflammatory Bowel Diseases
IR	Infrared
IVIVC	*In vitro-in vivo* correlation
LNP	Lipid Nanoparticle
LOD	Limit of Detection
LOQ	Limit of Quantification
mAb	monoclonal Antibody
MALDI	Matrix-Assisted Laser Desorption Ionisation
MEKC	Micellar Electrokinetic Chromatography
MEEKC	Microemulsion Electrokinetic Chromatography
MeOH	Methanol
MF	Microfiltration
MP	Microparticle
MS	Mass Spectrometry
MWCO	Molecular Weight Cut-Off
n. a.	Not available/not applicable

NP	Nanoparticle
OROS	Osmotic-controlled Release Oral delivery System
PB3	Phosphate buffer pH 3.0
PBS	Phosphate Buffered Saline
PdI	Polydispersity Index
PEG	Polyethylene Glycol
Ph. Eur.	European Pharmacopoeia
PLGA	Poly(lactic-co-glycolic acid)
PRO	Propranolol
QC	Quality Control
R&D	Research and Development
R^2	Determination coefficient
RI	Refractive Index
RP	Reverse Phase
rpm	Revolutions per minute
RSD	Relative Standard Deviation
RT	Room Temperature
SCD	Spin Centrifugation-Dialysis
SEC	Size Exclusion Chromatography
SF3	Sedimentation Field-Flow Fractionation
SF4	Symmetric Flow Field-Flow Fractionation

SIF 6.8	Simulated intestinal fluid pH 6.8 without pancreatin
SMEDDS	Self-Micro-Emulsifying Drug Delivery System
SPE	Solid Phase Extraction
SPLITT	Split Flow Thin Cell Fractionation
TDCD	Total Drug Content Determination
TFA	Trifluoroacetic acid 0.5 % (v/v)
TFF	Tangential-Flow-Filtration
ThFFF	Thermal Field-Flow Fractionation
TOF	Time-Of-Flight
UC	Ultracentrifugation
UF	Ultrafiltration
USP	United State Pharmacopoeia
UV	Ultraviolet
VDC	Vertical Diffusion Cell (Franz cell)
VF1	Volumetric flask for the elution of the fraction F1
VF2	Volumetric flask for the elution of the fraction F2

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SCIENTIFIC OUTPUT

SCIENTIFIC OUTPUT DIRECTLY FOLLOWING FROM THIS THESIS

Research papers in peer-reviewed journals

Guillot, A.; Couffin, A.-C.; Draheim, C.; Sejean, X.; Navarro, F.; Limberger, M.; Lehr, C.-M., Development of an innovative separation method for lipid nanoparticles based on solid phase extraction. *Pharm Res*. (Submitted)

Guillot, A.; Draheim, C.; Limberger, M.; Hansen, S.; Lehr, C.-M., A new concept for *in vitro* drug release testing of micro- and nanoformulations using a fiber optic system and derivative spectrophotometry. *Int. J. Pharm.* (Submitted)

Guillot, A. L., M.; Kraemer, J.; Lehr, C.-M., *In Situ* Drug Release Monitoring with a Fiber-Optic System: Overcoming Matrix Interferences Using Derivative Spectrophotometry *Dissolution Technol.* **2013,** *20* (2), 15-19.

Oral presentations

Guillot, A. In *In vitro dissolution testing for nano-/microparticles using the Agilent's FiberOptic® system*, 4th Annual World Drug Delivery & Formulation Summit, Berlin, Germany, 2013.

http://www.ddfevent.com/pdf/AlexisGuillot.pdf (accessed November 10, 2014)

Poster presentations

Guillot, A.; Couffin, A.-C.; Draheim, C.; Sejean, X.; Navarro, F.; Limberger, M.; Lehr, C.-M. Analyzing lipid nanoparticles by Solid Phase Extraction, In *CRS Annual German Chapter Meeting*, Muttenz, Switzerland, 2015.

Guillot, A.; Draheim, C.; Limberger, M.; Hansen, S.; Lehr, C.-M. In *Derivative spectrophotometry with a fiber optic system to monitor in situ the drug released from loaded PLGA-nanoparticles*, 10th International Conference and Workshop on Biological Barriers, Saarbrücken, Germany, 2014.

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