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CHIMERA AGGLOMERATES FOR EXTRAVASCULAR DELIVERY OF PARTICULATE DRUGS

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CHAPTER 1

1.1. Introduction

Due to unfavourable biopharmaceutical and/or pharmacokinetics properties, several active compounds are not suitable for administration through routes different than the parenteral one. These compounds, although very potent and of great therapeutic significance, are not able to reach an effective concentration at the physiological site of action, as a consequence of absorption, distribution, half-life or stability problems. Often biopharmaceutical problems have been faced modifying the chemical structure of the compounds. Structural modifications can improve the unfavourable molecule characteristics that hinder rapid dissolution, satisfactory stability and proper absorption, but generate a new chemical entity.

More recently, the development of new products has been achieved by means of strategies not involving a chemical modification of the primary molecule. Pharmaceutical technology, for example, can make a substance rapidly soluble, stable and suitable for transmucosal absorption. Actually, pharmaceutical technology, masking the chemical structure of the molecule, allows the drug to pass unmodified through those steps critical for its bioavailability. Furthermore, pharmaceutical technology does not generate new drugs but new dosage forms. A completely new drug is burdened by toxicology and clinical studies that can produce serious economic concerns. On the contrary, the manufacturing of a product containing an already known active compound formulated as an innovative dosage form makes available a new product at relatively low costs, revaluating active compounds well established in the clinical practice.

Technology is able to optimize the biological availability of drugs. Every active substance needs a dosage form, essential for using the active to exploit its therapeutic action. For these reasons, new formulations contribute not only to the therapeutic effectiveness of the product, but also to the socio-economic and industrial aspects. A classic example of this approach is the controlled release of drugs that deals with dosage forms able to prolong the action of an active compound after single administration. These products allow the drug to be released when is needed, the use of different administration routes and the attainment of specific body targets. In addition, these new

medicines sometimes reveal new pharmacological properties of already known drugs.

It is well known that the effect of a compound after extra-vascular administration is related to its bioavailability. Bioavailability is a quantitative concept: it measures the rate and the amount of active substance reaching the site of action after absorption in comparison with direct injection of the active into the bloodstream. The aim of the biopharmaceutical development is to obtain a dosage form with a foreseeable bioavailability, possibly related to its performance *in vitro* (*in vivo-in vitro* correlation). For example, peptides, hormones and antibiotics are important active compounds difficult to administer by extra-vascular route. Often their solubility, stability and permeability significantly affect the biopharmaceutical development of an extra-vascular dosage form.

A modern approach for the biopharmaceutical development of these substances is to make use of nano/microcarriers to modify their unsuitable properties. Thus, nano/microtechnologies may optimize the formulation of “problematic” substances.

Micro or nanoparticles administration requires the preparation of a drug product. Usually they are administered dispersed in an aqueous form by injection, even if solid forms for administration by nasal, pulmonary, transdermal and oral routes have been proposed. The most important characteristic determining their use in pharmaceuticals is the size: powders having particles larger than 100 microns flow easily and pharmaceutical manufacturing processes are facilitated, whereas below 100 microns they are increasingly difficult to handle. Nanoparticles are probably the most attractive delivery system in pharmacy due to the possibility to alter the absorption and distribution of the encapsulated drug. However, the benefit of using drugs in small particles is reduced by the inherent difficult manipulation of the small particles during solid dosage form manufacturing.

The transformation of nano/microparticles in solid dosage forms may involve processes such as granulation, but the formation of granules could lead to non-reversible modifications of particle original size. Therefore, microparticulate drug products having at the same time small size for the “delivery phase” and large size for the “fabrication phase” are sought for. A solution to this pharmaceutical paradox could be the construction of large particles that are capable to recreate the smaller ones only at the

administration site. There are few examples of this concept in inhalation such as the soft pellets preparation and, recently, the “Trojan” particles [1, 2].

1.1.2. Paediatric oral drug delivery

The development of formulations which are appropriate for children can present significant challenges to the pharmaceutical scientists. Unlike in adults, where oral solid dosage forms such as tablets or capsules will be acceptable to the majority of patients, potential paediatric patients may include neonates, newborn, toddlers, young children and adolescents will have widely varying needs [3]. The European Committee for Medicinal Products for Humane Use (CHMP) has published a document that splits the group of “children” into two subgroups: preschool children between 2 and 5 years of age, and school-going children between 6 and 11 years of age. Generally, childhood is characterized by periods of rapid growth, maturation and development. Paediatric practice requires a range of dosage forms that are acceptable at different ages and abilities and a range of strengths or concentrations allowing administration of the correct age-related dose [4]. The age at which children take tablets or capsules is a factor of importance to their safety so that inadvertent inhalation and choking are avoided but is also of great importance to manufacturers. Other important problems are the taste and the swallowing of the oral formulations. The last problem is very predominant when the tablet is gastroresistant, i.e. for the treatment of inflammatory bowel disease. In fact, the split of the tablet determinates the losing of the coating and as a consequence the drug did not reach the inflammed region in the colon. The development of multiple dosage forms for different ages are particular pharmaceutical challenges. In fact, multiparticulates dispersed in liquid or semi-solid material, such as cream or pudding, are administrated to children at the age of six months.

Moreover, taste is important; if the children spit out a bitter formulation, there is a risk of incomplete ingestion and consequently a reduction of the dose. Solid multiparticulates coated with taste-masking films or with a sweetening excipient, such as sugar, are the formulations of choice.

1.2. AIM OF THE WORK

Particle agglomeration is a process able to change the microparticle size in reversible way. Drug-containing primary nano- or microparticles can be prepared for oral or transmucosal (buccal, nasal) administration. These primary particles will be further transformed into bigger and free-flowing chimerical agglomerates. Soft agglomerates can be prepared processing the primary microparticles by tumbling or sieving. “Chimera agglomerates” are particle soft clusters that in water reconstitute the primary microparticles dispersion. They characterized by low crushing strength, but enough resistant to be processed. The structure and properties of agglomerates depend on the composition of microparticles. Moreover, the main feature of agglomerates is the capability of recovering the size of the primary particles at the administration site by use of water. For these reasons, the agglomerates can be used for preparing extemporaneous formulations. In particularly, they are adapted for preparing formulation for children or elder people that have swallowing difficulties.

In this PhD thesis the soft agglomerate technology was exploited for the formulation of products containing drugs for the therapeutical applications in patients uncaple to swallow solid dosage forms.

In particulary, a mesalazine gastroresistant multiparticulate system for paediatric administration, since the dosage is very high and, as a consequence, the tablets should have a big size.

It was also studied an extemporaneous formulation of artemisinin- clindamycin agglomerate combination for the treatment of uncomplicated malaria.

Finally, the manufacturing of sodium levothyroxine agglomerates for buccal/nasal administration in the treatment of hypothyroidism was investigated.

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CHAPTER 2

DEVELOPMENT OF MESALAZINE MULTIPARTICULATE DOSAGE FORMS FOR COLON DELIVERY IN PAEDIATRIC PATIENTS

2.1. INTRODUCTION

2.1.1. Inflammatory bowel diseases

Inflammatory bowel disease (IBD) is a localized and chronic inflammation of unclear etiology affecting the small and large intestine. IBD is comprised of two specific conditions: ulcerative colitis [1] and Crohn's disease [2]. Crohn's disease and ulcerative colitis primarily affect young adults, but in the 15% – 25% of cases initial disease starts in childhood (<18 years old) [3]. Both these pathologies occur with equal frequency in children in the first 8 years of life, whereas in older children Crohn's disease is much more common than the other one.

Ulcerative colitis typically emerges with continuous inflammation extending from the rectum to anus, including some times the entire colon. It causes swelling and scaring in the colon tissues and it may also be called colitis or proctitis. Nevertheless, unlike ulcerative colitis which only affects the colon, Crohn's disease can affect the entire gastrointestinal tract from the mouth to the anus with discontinuous local ulceration. Crohn's disease is characterized by transmural, mainly submucosal, inflammation which is discontinuous in nature and often associated with epithelioid granulomas. In Crohn's disease the inflammation involves the full thickness of the bowels, whereas in ulcerative colitis only the inside layer of the bowels is usually affected.

Both Crohn's disease and ulcerative colitis predispose to cancer with ulcerative colitis to a greater extent. In accordance with the unknown etiology of IBD, the treatment is aimed at reducing or eliminating symptoms and preventing episodes of relapse.

The major hypotheses regarding the etiology of IBD have included infection, allergy to dietary components, immune responses to bacterial or self-antigens and environmental causes. Infectious causes include pathogenic microbial antigenic triggers that haven't been identified yet [4]. Defective colonic mucosa and abnormal intestinal epithelial permeability may increase the access of luminal dietary and bacterial products to the mucosa [5]. Bernestein et al. suggested that the environmental causes have been proposed in relationship to the prevalence of IBD [6]. However, there has been so far little evidences that any particular food components play a primary role in the disease

etiology. For these reasons, Bonen and Cho supported the theory of multifactorials that caused the IBD [7]. The overall principle in the pathophysiology of ulcerative colitis is the dysregulation of the normal immune system against an antigenic trigger. The aggregate effect of genetic, environmental, and other processes is a sustained activation of the mucosal immune response. A state of altered immune regulation leading to prolonged mucosal inflammatory response, that involves a recruitment of leukocytes from the gut vasculature in IBD.

The mucosa in patients affected by IBD pathology may be dominated by CD4+ non-T helper lymphocytes generating a humoral immune profile. Defective colonic mucosa allows the access of luminal dietary and bacterial products to the mucosa[8]. Proinflammatory mediators, or cytokines, are also known to play an important role in ulcerative colitis, with evidence to support increased levels of IL-1, IL-6, IL-8 and tumour necrosis factor (TNF-a) [9].

The most common clinical course in patients with ulcerative colitis is chronic with intermittent symptoms. Moreover, approximately 10% of cases present acute fulminating signs [1]. Between episodes, patient may be free of symptoms. Symptoms are related to the extent of the disease, with common clinical features being intermittent rectal bleeding, tenesmus, crampy pain, passage of mucous, and mild diarrhea. In paediatric IBD patients nutritional issues and failure to thrive are significant problems. Although grown failure is a common problem in children with Crohn's disease, it can also be an early indicator of disease flare [10].

Instead, when the disease is severe, more systemic features can be seen. These include fevers, weight loss, severe abdominal pain, anemia, and malnutrition. Based on clinical and endoscopic findings the disease is characterized as to its severity and extents, all of which considerably diminish the quality of life (QoL) of the patients, especially for children and adolescents. Truancy, social isolation and extreme behaviour problems (i.e., depression) are particularly common in younger patients [10].

2.1.2. Rationale for targeting drugs to the colon

The challenge of targeting drugs to the colonic region of the gastrointestinal tract have involved the scientists over the past two decades. Current pharmacotherapy for colonic disorders is generally inefficient, requiring the need for surgical intervention in some patients. Through the introduction of new therapeutic agents which would improve the therapy, there is much that can be done from the perspective of drug delivery. The targeting of drugs specifically to the colon using new and improved delivery strategies would provide significant clinical benefits.

Site-specific drug delivery systems would ensure the direct treatment at the disease site with possible reduction of the administered dose and associated systemic adverse effects.

Additional interest in colonic targeting has stemmed from the potential of this region as an absorption site of drugs that entry into the systemic circulation, may have an improved bioavailability. This region of the colon is recognized having a somewhat less hostile environment, in terms of mucosal digestive enzymes, with less diversity and intensity of activity with respect to the stomach and the small intestine. Additionally, the colon shows a longer retention time and appears highly responsive to agents that enhance the absorption of orally unmodified peptide drugs. As the large intestine, the colon is relatively free of peptidases, then special delivery systems will have a large chance to get their drug sufficiently absorbed after peroral application [11].

2.1.3. Colonic Anatomy and Physiology

The gastrointestinal tract is essentially a hollow muscular tube, which acts as taking nutrients and eliminating waste by physiological processes such as secretion, motility, digestion, absorption and excretion. On the basis of function and morphology, the gastrointestinal tract is divided into the mouth, pharynx, oesophagus, stomach, small intestine and large intestine (Figure 2.1). The large intestine is approximately 1.5 m in length and it extends from the ileocaecal junction to the anus. It is divided into four sections: caecum, colon, rectum and anal canal. The colon is further subdivided into ascending, transverse, descending and sigmoid regions. The colon is involved in the

fermentation of polysaccharides and proteins, absorption of water and electrolytes, and the formation, storage and elimination of faecal material. As a consequence of the function of the colon, the colonic environment is generally viscous in nature. This could impact on the performance of drugs and delivery systems in this region of the gut. Due to rapid absorption of water in the ascending colon it is estimated that the human colon contains only 220 g of wet contents. Further studies suggest that the volume of the contents in the ascending colon is 50–150 mL[12]. The human colon also contains 200–300 mL of gas, in the form of nitrogen, carbon dioxide, methane and hydrogen, confined to the ascending and transverse colon.

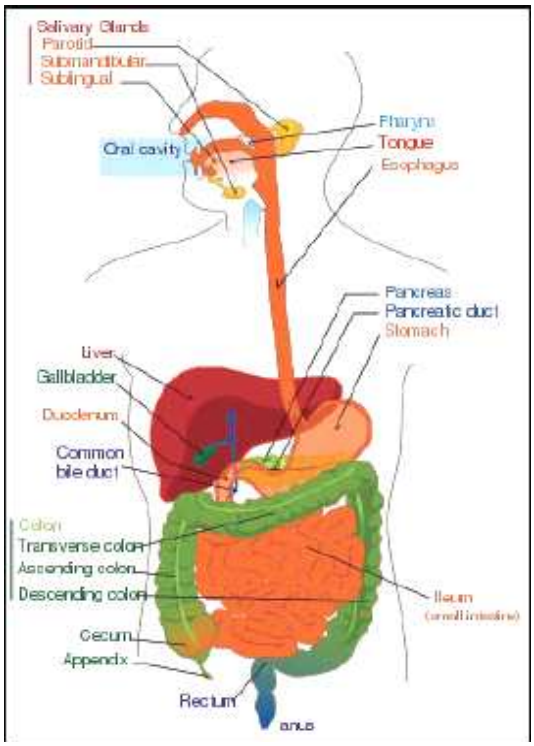


Figure 2.1. Human gastrointestinal tract [13].

The colon has a near neutral pH; however, in patients with IBD luminal pH of the distal intestine can be lower (pH ranging from 5.0 to 7.0 and in same cases also pH of 2.0 or 3.0) with respect to the pH values in healthy volunteers. In addition, the colon environment is consisting of viable microflora, that are involved in the fermentation of polysaccharides and proteins that have escaped digestion in the upper gut. In fact, the

stomach and the small intestine contain roughly 10^3-10^4 colonic forming unit (CFU)/ml, instead the number increases rapidly in terminal ileum and colon until 10^{11}-10^{12} (CFU)/ml [2]. Moreover, over 400 different species are present. Colonic bacteria are predominantly anaerobic in nature and they secrete enzymes that are capable of metabolising endogenous and exogenous substrates, such as carbohydrates and proteins, that escape digestion processes in the upper gastrointestinal tract [12].

The materials, that remain unaltered to the conditions present in the stomach and small intestine but are susceptible to bacterial fermentation in the colon, could be utilised as carriers for drug delivery to the colon. Moreover, the other consequence of bacterial fermentation is the faecal bulking, which increases transit of the colonic contents, nitrogen utilisation in the gut and formation of short chain fatty acids, which provide a useful energy supply. The colon has a significantly smaller surface area (1.3 m^2) than the small intestine (200 m^2); it displays also a 'tighter' paracellular pathwayand elevated levels of the efflux trans porter P-glycoproteinpotentially limiting drug transport across the epithelium.

2.1.4. Strategies for targeting drugs to the colon

As the colon represents the most distal segment of the gastrointestinal tract, targeting the drug to this region of the gut can be problematic. Although the rectal route can be used to gain access to the colon via the administration of suppositories and enemas, such formulations rarely succeed in spreading beyond the descending colon, with little or no drug reaching the proximal colon. Moreover, the rectal route is not convenient or acceptable for most patients. The oral route is therefore the preferred via of administration. After oral administration, conventional dosage forms normally dissolve in the stomach fluids and the absorption of the drug from these regions of GIT depends upon physicochemical properties of the active principle. On the other hand, this kind of drug release is extremely unfavourable in conditions where localized delivery of the drugs in the colon is required or in conditions where a drug needs to be protected from the hostile environment of the upper GIT.

The delivery of drugs to the colon is valuable in the treatment of diseases of colon as IBD, carcinomas or infections, because high local drug concentration can be achieved. In this case, side effects that occur because of release of drugs in the upper GIT or unnecessary systemic absorption are minimized. Several pharmaceutical approaches that can be exploited for the development of colon targeted drug delivery systems are categorized in four categories:

1) Time-based delivery systems.Time-dependent formulations are designed to release their loaded drug after a predetermined lag time. Depending on the size, shape and density of the dosage form and the feed status of the individual, residence time in the stomach can range from few seconds to a number of hours. In contrast, the transit through the small bowel is relatively consistent at 3±1 h, irrespective of formulation and dietary factors [2]. On the basis of this principle, a number of systems have been developed, with one of the earliest being the Pulsincap™ device. The device consists of an impermeable capsule sealed at one end with a hydrogel plug. On contact with gastrointestinal fluids, the plug hydrates and swells and, after a pre-set lag time, ejects from the capsule body, enabling drug release[14].

2)pH-based systems. The pH-dependent systems exploit the generally accepted point of view that pH of the human GIT increases progressively from the stomach (pH 1-2 which increases to 4 during digestion), small intestine (pH 6-7) at the site of digestion and it increased to 7-8 in the distal ileum [11]. Most commonly used pH-dependent coating polymers are methacrylic acid copolymers, known as Eudragit® (registered trademark of Rhom Pharmaceuticals, Darmstadt, Germany). The polymers form salts and dissolve above pH 5.5 and higher depending on the type of Eudragit®.

Since the colon is rich in microorganisms that are involved in the process of reduction of dietary components or other materials, the polysaccharide polymers, which show degradability as a function of colonic flora, can be exploited in the designing of formulations for colonic drug delivery [15].

3) Enzyme-based systems. In this case it is present a linkage between drug and carrier in such a manner that upon oral administration the moiety remains intact in the stomach and the small intestine. This approach chiefly involves the formation of prodrug, which is a pharmacologically inactive derivate of a parent drug molecule that requires

spontaneous or enzymatic transformation in the biological environment to release the active drug.

The first bacteria-sensitive system developed for colonic delivery was sulfasalazine, a prodrug consisting of the active ingredient mesalazine linked by an azo-bond to a carrier molecule, sulfapyridine [12].

Moreover, enzymatic hydrolysis of a wide range of substrates has been used to deliver drugs to specific sites in the GIT. Amino acid, glycoside, glucuronide, sulfate, dextran, and cyclodextrin conjugates have all been prepared [15, 16].

4) Pressure-based systems. The variations of pressure along the lumen of the GIT is used to trigger drug release. The pressure, which is generated via muscular contractions of the intestinal wall for grinding and propulsion of luminal contents, varies in intensity and duration throughout the gastrointestinal tract. In particular, due to the reabsorption of water from the large intestine, the viscosity of the luminal contents raises establishing an increase of the intestinal pressure. The device developed by Takaya and co-worker [17] exploited this mechanism and is called pressure-controlled colon delivery capsule (PCDC) system. Formulations susceptible of changes in pressure are prepared from capsule-shaped suppositories coated with ethylcellulose. The materials used in preparation of the suppositories are polyethylene glycols (PEGs), which are selected on the basis of their characteristic to melt at body temperature. The system behaves as a balloon once the PEG liquefies. In the upper intestine, there is sufficient fluidity to maintain the integrity of the balloon and no drug release occurs, while in the large intestine the pressure induced by peristalsis directly affects the ethylcellulose balloon leading to rupture and subsequent drug release.

2.1.5. Mesalazine, first line therapeutic treatment of IBD

The approach to the therapy of IBD has been dependent on severity of symptoms with frontline therapy being aminosalicylates. Mesalazine, also known as mesalamine or 5-aminosalicylic acid (5-ASA), is a first-line anti-inflammatory drug principally used to induce and maintain remission to acute bowel disease such as Crohn’s disease and mild to moderate ulcerative colitis (Figure 2.2).

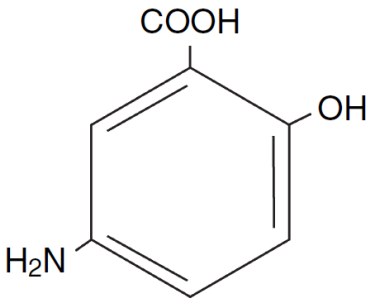


Figure 2.2. Chemical structure of 5-aminosalicylic acid (5-amino-2-hydroxybenzoic acid).

Mechanism of action. Despite new treatment options, mesalazine is still the cornerstone of IBD therapy probably due to its therapeutic effectiveness, associated with the mild adverse effect profile. A variety of different mechanisms have been proposed by which aminosalicylates work in IBD. A number of potential targets for 5-ASA action have been suggested, among which there is the peroxisome proliferator-activated receptor-γ (PPAR-γ), which is known to be involved in IBD inflammation. Indeed, 5-ASA can act as a synthetic agonist of PPAR-γ [20]. However, additional mechanisms of action, independent from PPAR-γ activation, have also been proposed. These include the inhibition of cyclooxygenase and lipoxygenase pathways to reduce the production of prostaglandins and leuokotrienes, respectively; the antiproliferative effects of nuclear factor-kappa B activation by tumour necrosis factor alpha (TNF-alpha) and interleukin-1 (IL-1) reducing intestinal cell transcription of inflammatory mediators [21]; the inhibition of platelet activating factor and production of oxygen radicals and other anti-inflammatory factors [22]. As the mesalazine is a zwitterion and belongs to a group of medicines named aminosalicylates, is also an antioxidant that traps oxygen free radicals that play a significant role in the pathogenesis of inflammatory bowel disease. All these properties are significant in reducing the acute symptoms of the IBD[23].

Besides its therapeutic role in ulcerative colitis, 5-ASAs have been observed to have properties of chemoprevention for colon cancer. Since colorectal cancer has a higher incidence in patients with inflammatory bowel disease than the general population, there

has been great interest in determining whether antinflammatory drugs used to treat IBD have an effect on reducing colorectal cancer rates.

Administration of mesalazine. As 5-ASA is believed to exert a direct effect on the colonic mucosa through a variety of anti-inflammatory mechanisms, direct application of this agent to the colonic mucosa is required. There are a number of strategies for achieving this delivery to the target tissue. Rectal administration of gels, foams and enemas containing 5-ASA is effective for administering the active drug directly to the rectum, sigmoid or left colon. Indeed, recent studies in patients with extensive IBD have shown that combination of both oral and rectal therapies may maximize 5-ASA concentration throughout the colon and the therapeutic effect is superior to oral therapy alone [24]. However, patients often dislike rectal formulations because of difficulty with this mode of administration and problems with discomfort, retention and leakage. Moreover, the use of suppositories in a particular class of patients, as the children, is quite popular in Western Europe (i.e., Germany, France or Spain), while it is uncommon in UK or in US. Therefore, the frequency of the use and the experiences of physicians with rectal drug administrations may differ significantly in each country [25]. In contrast, the commercial oral 5-ASA formulations are more acceptable to patients than the rectal formulations.

On the other hand, oral administration of 5-ASA presents a different challenge as the majority of oral 5-ASA dosage forms. However, if the 5-ASA formulation is taken in uncorrect manner (i.e. breaking a coating table), the mesalazine is completely absorbed from the stomach and proximal small intestine, leaving little or no 5-ASA to reach the colon (Figure 2.3) [26].

Mesalazine absorbed from the lumen of the gastrointestinal tract is subject to first-pass metabolism in epithelial cells of the intestinal mucosa and in the liver. The formed metabolite N-acetylmesalazine (N-Ac-5-ASA) is eliminated via the renal route, while only trace amounts of unchanged parent drug can be detected in urine [27].

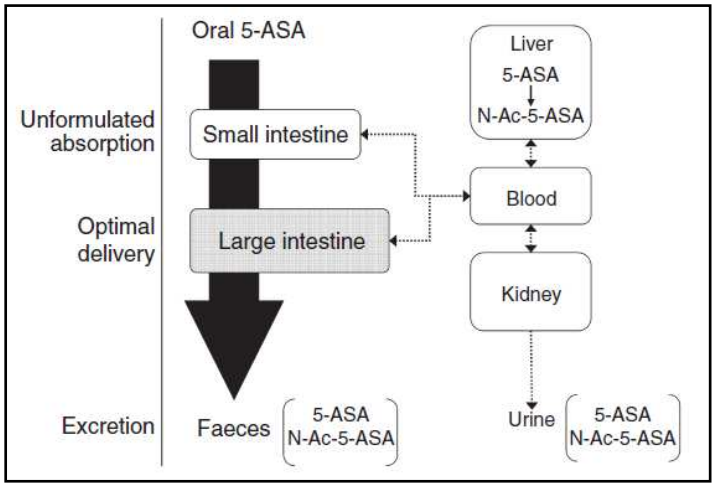


Figure 2.3. Proposed metabolic pathway of 5-ASA after oral administration. The shaded area (large intestine) indicates the site of topical action. Unformulated 5-ASA is absorbed rapidly from the small intestine, many current formulations are designed to delay release of 5-ASA until the terminal ileum or proximal colon [20].

Different preparations have been designed in accordance to delivery site depending where the highest activity of disease is located. Sulfasalazine was the first 5-ASA to be used for the treatment and maintenance of symptoms of IBD as pointed above [28]. The azo-bonded sulfapyridine molecule protects the active drug until bacterial azoreductase cleavage in the colon by colonic bacterial enzymes to produce the two active products. Sulfapyraidine is metabolized by the liver and excreted in the urine whereas the 5-ASA component is acetylated by the colonic epithelium.

The original indication for 5-ASA was for rheumatoid arthritis, however it was subsequently found to be efficacious in ulcerative colitis. Misiewicz et al published the first placebo controlled maintenance trial in 1965 randomizing patients to receive sulfasalazine or placebo for one year. Seventy three percent of patients taking placebo relapsed compared to 21% taking the active drug, thus showing sulfasalazine to be highly efficacious for the treatment of ulcerative colitis [29].

Due to the allergic reactions and the number of dose-dependent adverse effects of sulfasalazine, more tolerable mesalamine-based drugs void of the sulphur component have been developed. These new formulations are composed of 5-ASA, the active moiety of sulfasalazine, without the poorly tolerated sulfapyridine carrier molecule. The

newer generation aminosalicylates allow for targeted delivery with reduced side effects compared to sulfasalazine:olsalazine (Dipentum; Celltech Parmaceuticals, Inc., Rochester NY, USA) and balsalazide (Salix Pharmaceuticals, Inc., Morrisville, NC, USA) are prodrugs, where a molecule of 5-ASA is bound covalently through an azo linkage to another 5-ASA and the release of the 5-ASA is due to bacterial azo-reduction in the colon.

An alternative strategy to delivery 5-ASA is to coat the formulation with a gastro-resistant film that prevents5-ASA release until luminal conditions approach pH 7(normally in the terminal ileum). Acrylic polymers (Eudragit®) that dissolve at pH above 6 have been commonly used for this purpose. This concept is based on the assumption that gastrointestinal pH increases on passing from the small intestine to the colonic tract [12].

Dew et al. [30] were the first group to utilise gastrointestinal pH as a trigger for drug release in the distal gut by applying the enteric polymer Eudragit® S to the capsule dosage forms. Eudragit®S is a copolymer of methacrylic acid and methyl methacrylate that dissolves above pH 7. The transit and disintegration of the coated capsule dosage form was investigated in a group of convalescent patients using x-ray imaging, demonstrating that the capsule and the drug release was observed in the distal gut. A subsequent study involving Eudragit®S-coated tablets was conducted in patients with ulcerative colitis [31]. The results from these studies provided the basis for the development of the Asacol® MR delayed-release mesalazine tablets (Procter & GamblePharmaceuticals, Cincinnati, OH, USA), consisting of a Eudragit®S-coated tablets formulation containing 5-ASA. The other coated mesalazine product in this class is Pentasa® (Ferring Pharmaceuticals, Saint-Prex, Switzerland), that works independently from pH environment. Drug release occurs via diffusion through a water-insoluble ethylcellulose film coating and, hence, the drug release occurs throughout the gastrointestinal tract.

Recently, Multi Matrix System (MMX mesalazine) was designed by Giuliani SpA (Milan, Italy). This delivery system uses lipophilic and hydrophilic matrices enclosed within a gastroresistant pH-dependent coating to facilitate the prolonged exposure of the colonic mucosa to 5-ASA. This system permits to release gradually the drug. In fact, after the disintegration of the gastroresistant coating at pH 7 or above, intestinal fluids

interact with the hydrophilic matrix and therefore the tablet can swell and form an outer, viscous gel mass, that permit to release 5-ASA [32].

Tolerability. 5-aminosalicylates have been shown to be safe in short term use with a dose-response efficacy without dose-related toxicity for oral delayed-release mesalazine for doses of 1.6 g or 2.4 g/day. Moreover, there are sufficient data to demonstrate long-term safety of mesalazine at doses of up to 5 g daily[33]. The result of a retrospective trial demonstrated that oral delayed-release mesalazine was reported to be well tolerated in children [34].

The most commonly reported adverse events with the 5-ASA formulations include headache, gastrointestinal symptoms such as diarrhea, bloating and nausea. Other rare side effects include interstitial nephritis, hepatitis, pericarditis, pancreatitis, pneumonitis, dermatitis, myocarditis, and hematological disturbances[4]. Considering that IBD affects younger population, it is also reasonable to evaluate its safety profile in pregnancy. There has been no evidence of teratogenic effect or fetal toxicity with mesalazine placing it into a FDA category B[35]. In a work reported by Habal et al. 18 out of 19 pregnancies under mesalazine medication resulted in full-term delivery with no fetal abnormalities [36].

2.2. AIM OF THIS SECTION

Due to elevate dosage of 5-ASA the commercial tablets have high dimensions. Tablets could be a problem for a pediatric administration because the children are not adults, where oral dosage from such as tablets or capsules are well accepted. In the case of paediatric patients, the solution is to crush the solid dosage form. In this case, the problem remains the swallowing of the solid dosage form linked to a bitter taste of the formulations.

In this part of the thesis, a gastroresistant multiparticulate system able to delivery of 5-ASA directly to the inflammatory area was studied. The formulation has to be easily dispersible in the beverages (like water, milk or juice) or liquid fluid (like soap) to facilite the administration to children or elder people.

In order to prepare and optimize an appropriate gastroresistance formulation several techniques for the manufacturing of microparticles were investigated. In particular, the attention was focused on spray-drying, granulation, spray-congealing and agglomeration processes. The samples were characterized in terms of morphology, solid state and drug release properties.

2.3. MATERIALS AND METHODS

2.3.1. Materials

Mesalazine was kindly supplied by Doppel (Cortemaggiore, Italy). The batches employed were TFR07122 and 0822. Several excipients were used: hydropropylmethilcellulose (Methocel 4K CR Premium EP, supplied by Colorcon, USA); sodium carboxymethylcellulose (Acef, Italy); chitosan (ChitoClear FG90, provided by Primex, Iceland); stearic acid supplied by ACEF and Carlo Erba Reagenti, Italy); amidated pectin (Pectin Amid CF020, provided by Herbstreith & Fox KG., Germany); sodium alginate (Carlo Erba S.p.a., Italy); modified starch (Amprac 01, supplied by Rofarma S.r.l., Italy); calcium chloride (Fluka, Germany); carnauba wax (Produits Roche S.A. France), Eudragit S®100 and Eudragit®L (Rhom Pharma, Germany). Mannitol was donated by Lisapharma (Como, Italy) and lecithin (Lipoid S45) was supplied by Lipoid AG (Ludwigshafen, Germany). All other chemicals were of analytical grade.

2.3.2. Methods

2.3.2.1. Production of microparticles spray dried

Spray-drying is a technology that allows to obtain a dehydrated product beginning from a liquid (solution, suspension or emulsion). This technique is not used only in pharmaceutical process, but also in the alimentary and industrial sections. It is a speedy, time-saving and gentle method of obtaining even smaller quantities of substances in powder form. The advantages of this technique compared with other methods of drying (such as for example freeze drying) are the production rate and the short process time. The very rapid residence time and the cooling effect resulting from evaporation make it possible to treat even temperature-sensitive products in a gentle manner. In addition, not only the spray-dryer make possible to obtain a powder directly from a solution but many other processes can be accomplished in one stage such as:

1) modification of particle size;
2) agglomeration of nanoparticles;
3) drying of suspensions;
4) particle coating;
5) immobilization of liquids and solid materials in a matrix;
6) manufacture of microparticles.

Initially, the scope was the manufacture of 5-ASA gastroresistant microparticles and the production of the powder was carried out by using a “Mini Spray-Dryer Büchi” mod. 191 (Büchi Laboratoriums-Technik, Switzerland) (Figure 2.4). The formulations and parameters of spraying are reported in the Table 2.III and 2.IV, respectively.

Figure 2.4.“Mini Spray-Dryer Büchi” instrument [37].

The yield (%) of the spray-dried process is reported as a percentage of the product weight over the total weight of starting components of the spray-dried system.
Moreover, the excipient microparticles were produced using the spray drying technique. Mannitol was dissolved in 92 mL of water. Lecithin was dissolved in 8 mL of ethanol at 40°C and mixed with mannitol solution giving an opalescent mixture. The solid concentration was 2% (w/v); mannitol and lecithin ratios used were 85:15 (w/w).

The solution was spray-dried under the following conditions: inlet temperature 95°C; outlet temperature 42-45°C, feed rate 5 ml/min, nozzle diameter 0.7 mm, drying air flow 600 L/h.

2.3.2.2. Production of microcapsules

The spray congealing is the technique used to manufacture the 5-ASA microcapsules. This technique (alternatively called spray chilling or spray cooling) is gaining considerable attention, especially from the point of view of safety and rapidity. In fact, the spray congealing technique atomizes a solution or a dispersion of the drug into a molten carrier, which melts at a relatively low temperature (45– 75°C). The process overcomes the problem of residual solvents and the atomized droplets quickly solidify due to their exposure to an ambient air flow. Recent developments in spray congealing apparatus include the use of congealing chambers with integrated bag filters for cooling and separation of product in one unit. The performance of the spray congealing process strictly depends on the atomization efficiency of the molten mixture, which may be sprayed using different types of devices, traditionally divided into rotary or centrifugal atomizers. The new pneumatic nozzle developed by Prof. Rodriguez has been identified with the acronym WPN (Wide Pneumatic Nozzle) because of the wide (4.5 mm) orifice opening (the inner diameter is usually around 1 mm) [38]. The scheme is shown in Figure 2.5. Briefly, WPN is a two-fluid atomizer which acts in an unusual configuration: the air enters in radial direction, while the fluid enters axially and it proceeds along a rectilinear path. The air cross the fluid with an inclination of 45° and then the mixture is sprayed. In practice, the atomizer is a particular kind of venturimeter that takes advantage of the Venturi effect.

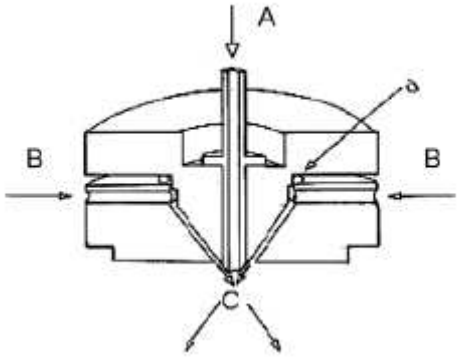


Figure 2.5. Scheme of the WPN (not in scale). (A) Material aspiration (feeding), (B) air inlet, (C) atomization of the fluid, (a) o-ring [38].

This phenomenon takes place when a fluid (the melted mixture) is flowing through a pipe (called Venturi tube) and it is forced through a narrowing. This constriction causes a drop in pressure in the fluid flowing in the pipe; consequently, the fluid is sucked from the top of the tube (point A) towards the nozzle. At the same time the fluid velocity increases due to the conservation of energy: the gain in kinetic energy is supplied by the drop in pressure (described by Bernoulli's equation). The fluid is delivered from a thermostated reservoir, placed above the point A, to the nozzle by both the force of gravity and the effect of Venturi. The inlet air flux (B) is obtained by three holes placed one from the other at 120°; when the air comes in contact with the fluid, the atomization (C) occurs. The WPN requires modest air consumption (1–3 bar depending on the desired particle size) and it generates an uniform spray pattern; the spray stream is delivered with a 90° angle which is symmetric to the nozzle cone. The microparticles are then collected at the bottom of a cooling chamber (1.80 m in height and 75 cm in diameter). In this device, the atomization air is not heated due to the presence of two resistors connected to an inverter which heats the nozzle. The output of the inverter was feed-back regulated through a suitable electric circuit by the signal of a thermocouple embedded into nozzle, thus keeping the temperature of the nozzle at the pre-set value ±1 °C (20 °C above the melting point of the carrier).

The 5-ASA microcapsules were manufactured in two steps through this technique. In the first step cores containing 5-ASA were produced. The mesalazine was dispersed in a solution of Eudragit®L in isopropyl alcohol prepared under stirring at the temperature of 70°C. Then, the carnauba wax (m.p. 85°C) was added to the dispersion and the temperature was raised up until 95°C to evaporate the isopropyl alcohol and to melt the lipid. Finally, the melted mass was sprayed through the WPN nozzle at 3.0 bar pressure. A second batch was prepared spraying at 3.0 bar pressure the 5-ASA dispersed in melted carnauba wax at the temperature of 90°C obtaining the drug cores without Eudragit®L.

In the second step the microcapsules were manufactured. The cores were dispersed in a low melting point lipid as stearic acid (m.p. 69-70°C) at the temperature around 70°C. At this temperature the microsphere did not melt and remain well dispersed in the liquid mass. The dispersion was sprayed with the WPN nozzle at 1.2 bar pressure.

The second batch of cores without Eudragit®L was also dispersed in the melted stearic acid at the temperature at 55°C and sprayed at 1.2 bar pressure to obtain microcapsules.

2.3.2.3. Preparation of agglomerates

Since the lipidic nature of the microcapsules, they were not well dispersed in the liquid to be administered. Hence, the 5-ASA lipid microparticles and excipient microparticles (2:1, 4:1, 6:1 and 8:1) were agglomerated in Turbula apparatus (Wab, Basel, Switzerland) using bakelite cylindrical jar (diameter 5.0 cm, length 4.4 cm). During the blending process two inox balls (diameter 1 cm) were introduced in the jar. The process lasted 45 minutes.

2.3.2.4. Determination of drug loading

To evaluate the content of 5-ASA in the spray-dried microparticles, a amount of microparticles were added to simulated intestinal fluid (SIF) at pH 7.4 under stirring at room temperature until complete dissolution. When the lipophilic excipients were present in the formulation, the drug content was determined by adding the samples to

SIF at pH 7.4 and heating up to 70°C or to 85°C to melt the lipophilic carrier as stearic acid or carnauba wax, respectively. The process was carried out under magnetic stirring for 5 hours to extract completely the 5-ASA. Finally, the solution was filtered with microcellulose filter (Minisart RC, 0.45µm, Sartorius, Goettingen, Germany) and then assayed by UV(UV-Vis spectrophotometer, Lambda25, PerkinElmer®, Massachusetts, USA) at 330 nm. The analysis was performed in triplicate for each batch formulation. Blank runs showed absence of significant interference with the spectrophotometric measurement.

2.3.2.5. Study of morphology

Morphology and appearance of the spray-dried microparticles, microcapsules and agglomerates were evaluated by Scanning Electron Microscopy (JSM 6400, Jeol Ltd., Tokyo, Japan). Each sample was sputter-coated with gold using a vacuum evaporator and examined at 15 kV accelerating voltage. Moreover, the lipid microcapsules and the agglomerates were examined both under an optical stereomicroscope (magnification 40x) (Citoval 2, Jena, Germany) connected to a video camera (JVC, Tokyo, Japan) and Scanning Electron Microscopy (SEM). Finally, the surface elemental composition of agglomerates was assessed using the Scanning Electron Microscope equipped with an EDS detector for X-ray microanalysis.

2.3.2.6. Solid state analysis

Physical changes in the samples (microcapsules) during heating were monitored by Hot Stage Microscopy (HSM). A hot plate (FP 52 Mettler, Grefensee, Switzerland) connected to a temperature controller (FP 5 Mettler) was used. A small amount of the lipidic microcapsules was placed on the sample stage and heated in the temperature range of 30-100°C at 2°C/min. The changes in samples were observed via an optical microscope (Nikon Eclipse E400) (magnification 10X).

Temperature and enthalpy measurements of the raw materials of the lipidic microparticles and the agglomerates were performed by means of Differential Scanning

Calorimetry (Mettler DSC 821e STARe, Mettler Toledo, Switzerland). Samples of about 5–10 mg in pierced aluminum crucibles were subjected to a thermal program from 30 to 300°C, at a scan rate of 10°C/min under a dynamic nitrogen atmosphere (100 ml min^{-1}). Instrument calibration was performed with standard indium and zinc samples (purity >99.99%) of known temperatures and enthalpies of melting.

The powder diffraction data were obtained with a Miniflex X-ray Diffractometer (Rigaku, Tokyo, Japan) with a graphite monochromator in the diffracted beam-path (CuKα radiation, Kα1 1.5406 Å; K α2 1.5443 Å). A system of diverging, receiving, and anti-scatter slits of 0.58, 0.58, and 0.2 mm, respectively, was used. The patterns were collected with 30 kV of tube voltage and 15 mA of tube current in the angular range $2 \leq 2\theta \leq 50°$ in a step scan mode (step width, 0.05°; counting time, 2 s/step).

2.3.2.7. In vitro drug release of microcapsules and agglomerates

Dissolution tests of each formulation were performed using the USP XXXI apparatus rotating at 100 rpm at the temperature of 37°C. Initially, preliminary tests were conducted for one or two hours in 900 ml of simulates gastric fluid (SGF) at pH 1.2 to value the gastroresistance of the formulation. In the second time, in order to evaluate if the system release the drug at pH 7.4 the formulations that showed a satisfactory gastroresistant profile were tested at variable pH. A weighted amount of the 5-ASA formulation was put in 300 mL of 0.1M HCl for 1 h. After this period, 600 mL of aqueous solution containing 2.6 g of NaOH and 6.12 g of KH_2PO_4, were added into the medium in order to reach pH 7.4 [39]. During the dissolution test of the formulations containing lipophilic excipient, a 0.01% of Sodium Lauryl Solphate (SLS) was added in each aqueous medium to improve the of the formulation. The samples were collected at pre-determined time intervals from 0 to 480 min and filtered with 0.42 μm filter. 5-ASA concentration was determined by UV at 301 nm and 330 nm in acid and basic medium, respectively. Drug dissolution studies were performed in triplicate for each batch formulation.

2.3.2.8. Similarity factor

The drug release data of the microcapsules and agglomerates made using different ratio of microcapsules and excipient microparticles, were statistically analysed and compared using Fit Factor described by Moore and Flanner [40]. Briefly, Fit Factor is a model independent method that directly compares the difference between percent drug released per unit time for a test and a reference product. The similarity factor (f_2) can be calculated comparing different pairs of dissolution profiles using equation:

$$f_2 = 50 \times \log\left\{\left[1 + (1/n)\sum(R_t - T_t)^2\right]^{-0.5} \times 100\right\} \quad (1)$$

where n is the number of dissolution sample times, R_t and T_t represent the mean percent drug released at each time point (t) for the reference and the test dissolution profiles, respectively.

The similarity factor (f_2) is a logarithmic reciprocal square root transformation of the sum of squared error and is a measurement of the similarity in the percentage released between curves. In simple terms, f_2 values should be close to 100 (between 50 and 100), when $f_2 < 50$ two release profiles are considered different.

2.3.2.9. Water penetration study

The water penetration study was performed on the microcapsules and the agglomerates (2:1, 4:1, 6:1 and 8:1 microcapsules and excipient microparticles).

In fact, the 5-ASA was dispersed within an insoluble matrix composed by the lipophilic excipients, that reduce the wettability of the formulation; while, in the case of agglomerates, the presence of excipient microparticles around the microcapsules increases the wettability of the formulation. Hence, the rate of penetration of the dissolution medium may have an important influence on the rate of drug release.

It was studied the rate of water penetration in the microcapsules and the agglomerates composed by different ratio of excipient microparticles and microcapsules.

Each formulation was packed by tapping into an empty plastic pipette (5ml). The ends of the tube were blocked with HPLC filters.

The packed column was connected to a Jasco PU-980 Intelligent HPLC pump. Distilled water was then pumped through the column at set flow rate of 0.2 ml/min. The time in which the water moves up the rod to wet predetermined lengths of the packed tube was recorded.

When the water moved out of the tube, the experiment was finished and the weight of the tube filled of water was measurement. The experiment was performed in triplicate for each formula.

2.3.2.10. Calculation of the number of excipient microparticles on the lipid particle surface

Since it was possible to obtain agglomerates using different ratio of the lipidic microcapsules and the excipient microparticles, the theoretical number of excipient microparticles necessary to deposit a layer on the lipid microcapsules surface was calculated. First, the particle sizes of lipid and excipient microparticles were evaluated.

The size distribution of lipidic microcapsules was measured by sieve analysis using a vibrating shaker (Amplitude 3; Analysette 3 Fritz model, FritschGMB, Idar-Oberstein, Germany) and eight standard sieves (Scientific Instrument s.r.l. Milan, Italy) of 90, 125, 180, 250, 355, 500, 710 and 1000 μm stacked. A weight frequency distribution was obtained.

The particle size distribution of the excipient microparticles was measured using the laser light scattering apparatus (Mastersizer X, Malvern Instruments Lid, U.K.) suspending the particles in ethyl acetate. The particle size was expressed as a number frequency distribution.

From the distribution data, the plot of cumulative frequencies undersize plotted versus the particles size were obtained: on the number basis in the case of excipient microparticles and on the basis of weight in the case of lipidic microcapsules. From the last plot it was possible to obtain an important parameter, the geometric mean diameter with the symbol of d_g on the number distribution and d'_g on the weight distribution,

respectively. The reference point used was the logarithm of the particle size equivalent to 50% on the probability scale. The geometric standard deviation, σ_g, was extrapolated by the graphic:

$$\sigma_g = \frac{50\%size}{16\%undersize} \quad (2)$$

where 50% size is the particle size at the 50% probability level and 16% undersize the particles size at lower value of 16% probability.

Supposing that a lipid particle was a sphere, it was covered by excipient microparticles as the projection of the circles of excipient microparticles on the lipid sphere. For this reason, the area of the lipid microcapsules and the projection of the circles of excipient microparticles were calculated.

First, it was necessary to obtain the values of surface diameter (d_{sn}) and length diameter (d_{ln}) of the lipid microcapsules and the excipient microparticles, respectively. In fact, it was possible to convert the geometric diameter in other statistical diameter using the Hatch-Choate equations. The surface diameter (d_{sn}) was determined from the geometric diameter of lipid microcapsules, resulting from the weight distribution:

$$\log d_{sn} = \log d'_g - 4.606 \log^2 \sigma_g \quad (3)$$

The following equation allowed to obtain the length-number diameter from a geometric diameter (d_{ln}), resulting from the number distribution of excipient microparticles:

$$\log d_{ln} = \log d_g + 1.515 \log^2 \sigma_g \quad (4)$$

Finally, the number of excipient microparticles that covered one lipid microcapsule was calculated through the ratio between the sphere's area of a lipid microcapsules and the value of the area of the circle (of excipient microparticles) projection. It was considered also the area of square having as a side the length diameter of the excipient microparticles, because in the case of the circle's area, the space between the circles it was not considered.

To interpret this result, it was calculated how many milligrams of excipient microparticles were necessary to cover 1 gram of lipid microcapsules.

First, it was calculated the number of lipid microcapsules per unit weight, N:

$$N = \frac{6}{\pi d_{vn}^{3} \rho} \quad (5)$$

where ρ is the value of true density of the lipid microcapsules and the value of volume-number (d_{vn}) of lipid microcapsules was calculated using the equation of Hatch-Choate:

$$\log d_{vn} = \log d'_{g} - 3.454 \log^{2} \sigma_{g} \quad (6)$$

The value of true density, ρ, was measured using a helium pycnometer (Micromeritics Multivolume Pycnometer 1305, Milan, Italy).

In order to obtain the number (N) of excipient microparticles that was necessary to cover 1 gram of lipid particles, the total number of lipid microcapsules (presented in one gram) was multiply by the number of excipient microparticles used to cover one lipid particle.

Finally, the mass of excipient microparticles was calculated by using the following equation:

$$grams\,(excipient\ microparticles) = \frac{(\pi d_{vn}^{3} \rho)N}{6} \quad (7)$$

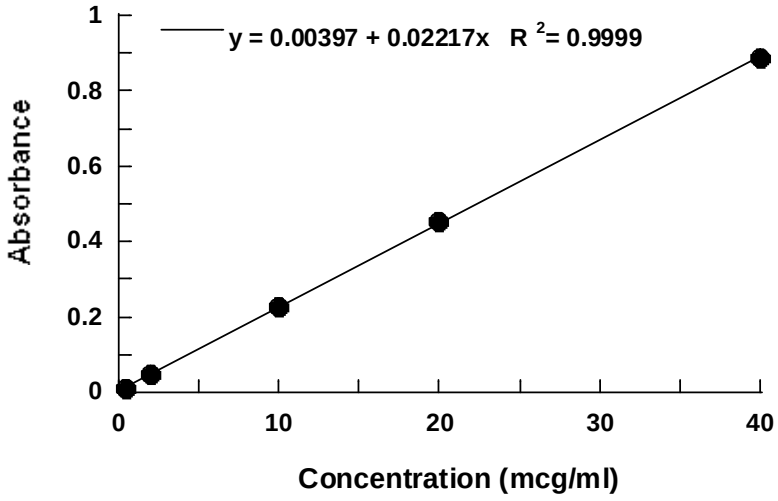
where ρ is the value of true density of the excipient microparticles and d_{vn} is the volume number diameter of the excipient microparticles calculated with the following equation:

$$\log d_{vn} = \log d'_{g} + 3.454 \log^{2} \sigma_{g} \quad (8)$$

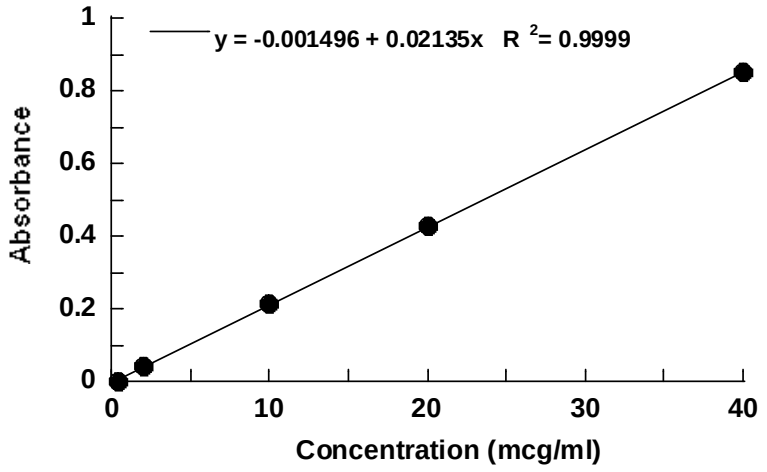
2.4. RESULTS AND DISCUSSION

2.4.1. Validation of the analytical method

The 5-ASA was quantified by UV spectroscopy. A calibration curve was constructed by dissolving the drug either in simulated gastric fluid (SGF) and in simulated intestinal fluid (SIF) following the method reported in Eur. Ph. 6th Ed. (Figure 2.6). The values of λ_{max} for 5-ASA were 301 nm and 330 nm in SGF and SIF, respectively. Each sample was injected six times, the linearity and precision (expressed as relative standard deviation, RDS%) of the assay were determined.



(a)



(b)

Figure 2.6. Absorbance versus the 5-ASA standard solution concentration in (a) SGF and (b) in SIF.

In the drug concentration range of 0.4-40 μg/ml the correlation coefficient (R^2) for the calibration curve was 0.9999, indicating good linearity. The reproducibility of the method was considered acceptable as the RSD% values obtained were for each standard <2% (Tables 2.I and 2.II).

Table 2.I. UV RDS% Values of different concentration samples in SGF.

Concentration (μg/ml)	Mean Abs	Standard Deviation	RDS%
40.0	0.8884	0.011	1.17
20.0	0.4531	0.001	0.23
10.0	0.2257	0.001	0.44
2.0	0.0475	0.001	1.34
0.4	0.0106	0.001	1.58

Table 2.II. UV RDS% Values of different concentration samples in SIF.

Concentration (μg/ml)	Mean Abs	Standard Deviation	RDS%
40.0	0.8884	0.002	1.42
20.0	0.4455	0.002	0.58
10.0	0.2221	0.001	0.34
2.0	0.0455	0.0002	0.52
0.4	0.0071	0.0006	0.26

2.4.2. Formulation of 5-ASA spray-dried (SD) microparticles

In this part of work, the development of a gastroresistant formulation by using several polymers was studied. The developed formulations are summarized in Table 2.III.

The first problem met during the formulation of the gastroresistant microparticles was the crystalline nature of the mesalazine. In fact, the needle shape of drug crystal did not favor its coating (Figure 2.7a). Moreover, the drug was very soluble in acid medium as reported in the monograph of the European Pharmacopea [41], so a very thick coating was necessary to avoid the drug release in the stomach. Another limitation was the fact that drug loading in the formulation had to be very high, i.e. for children was 1-3 g/day [26]. Hence, on the one hand a low amount of excipient has to be employed to avoid a large mass of formulation since the drug loading is high; on the other hand a thick coating is necessary to prevent the release of 5-ASA in acid medium.

In the first group of formulations, it was employed 5-ASA and a high amount of Eudragit®, pH-sensitive polymer, that dissolved at pH above 7.0, commonly used in the coating processes. It was always maintained the ratio between 5-ASA and Eudragit® , 0.45g : 1.8g, respectively. In the case of formulation #1 (see the Table 2.III), as 5-ASA was very soluble also in alkaline solution, 0.45g of 5-ASA was solubilized in 0.3% w/v NaOH solution containing 1.8g Eudragit®S100. Moreover, in the same solution, a different hydrophilic polymer, that could control the drug release through a swelling process in an aqueous medium, was added to the solution.

The hydrophilic used polymer was hydroxylpropylmethylcellulose (HPMC) with a viscosity of 3000-5600 cps, that it could swell quite fast in the aqueous medium. Methocel® 4K was selected on the basis of work published by Raffin et al. [39]. The alkaline solution was sprayed by setting the Spray-Dryer instrument at the condition reported in the Table 2.IV. The inlet temperature was fixed at 140°C to guarantee the total evaporation of the solvent without affecting the drug stability. The latter temperature was widely below the temperature of melting point of 5-ASA (around 280°C). In this case, the yield of the process was low due to the gluing of the HPMC in the drying chamber of the spray drying instrument. The SEM image of the microparticles is shown in Figure 2.7c. The microparticles presented round and collapsed shape with a particle size below 20 μm. However, the dissolution profile in

acid medium of the formulation #1 is reported in the Figure 2.8 and presented a release of about 60% of the drug in the first hour.

In the formulation #2 0.45 g of HPMC (Methocel® 4K) was replaced with 0.45 g of another hydrophilic polymer, the carboxymethylcellulose (CMCNa). The choice of this polymer for the modification of drug release was suggested by Abdelkader [42]. In this case, an homogeneous microparticulate was not obtained; in fact, the SEM image showed the presence of mesalazine crystals surrounded by the excipient microparticles (Figure 2.7d). Probably the 5-ASA, being no well solubilized in the sprayed solution, did not change the shape or was not coated by the excipients. This consideration is supported by the dissolution profile shown in the Figure 2.8 as 5-ASA was immediately released in acid medium. Moreover, the presence of an hydrophilic polymer did not control the release of the drug and consequently it wouldn't improve the gastroresistant of the formulation. On the basis of these unsatisfactory results, the hydrophilic polymer was removed from the formulation.

In the formulation #3, an alkaline solution of only 5-ASA and Eudragit S®100 was sprayed. In absence of other polymers the drug content in the preparation was increased; this fact was favorable due to the high drug dosage in the formulation. The inlet temperature of the spray drying was increased of 10°C to improve the evaporation of the solvent with respect to the other formulations. From the SEM image it was possible to observe that the 5-ASA crystals were absent and the obtained microparticles presented a round shape with the dimensions lower than 5 μm (Figure 2.7e). However, the release of mesalazine in acid medium raised the 80% in one hour, as shown in Figure 2.8.

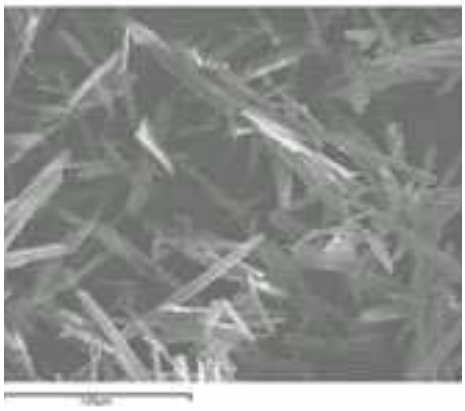
Due to the impossibility to obtain a gastroresistant formulation by spraying an alkaline solution of mesalazine, it was decided to spray drying a 5-ASA suspension (formulation #4): 5-ASA was dispersed in a hydroalcoholic solution of Eudragit®S100. The nozzle of the spray drying was changed with another of bigger size trying to increase the amount of 5-ASA incorporated. However, from the SEM image (Figure 2.7f) it is possible to observe the presence of mesalazine crystals mixed with the excipient microparticles. The dissolution profile confirmed that the formulation #4 was not gastroresistant because of 80% of loaded drug released in 1 hour (Figure 2.8).

Table 2.III. Formulation of 5-ASA SD microparticles.

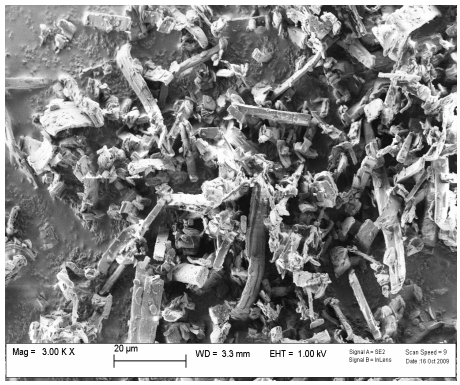
Formulation	5-ASA (g)	Eudragit® S100 (g)	Methocel® K4M (g)	CMCNa (g)	Chitosan (g)	Stearic acid (g)	NaOH (g)	H_20 (mL)	EtOH (mL)	HCl 1N (mL)	Acetic acid2% (mL)	Starch (g)	Pectin (g)
#1	0.45	1.8	0.45	-	-	-	0.3	100	-	-	-	-	-
#2	0.45	1.8	-	0.45	-	-	0.3	100	-	-	-	-	-
#3	0.45	1.8	-	-	-	-	0.3	100	-	-	-	-	-
#4	0.45	1.8	-	-	-	-	-	20	38	-	-	-	-
#5	0.50	0.05	-	-	-	0.45	-	-	100	-	-	-	-
#6	1	0.25	-	-	-	3.75	-	-	500	-	-	-	-
#7	2	0.05	0.6	-	-	0.45	-	80	100	30	-	-	-
#8	1	0.05	-	-	1	0.45	-	50	100	14	30	-	-
#9	3.85	0.15	-	-	-	1.35	-	500	200	-	-	-	0.15
#10	2	0.1	-	-	-	-	-	200	60	-	-	-	-
#11	2	0.4	-	-	-	-	-	175	160	-	-	0.5	-

Table 2.IV. Parameters of spray drying process.

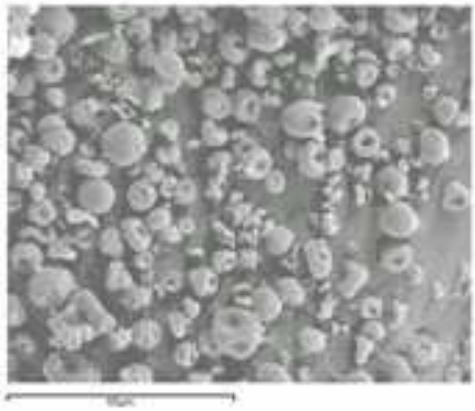
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7** (1°STEP)	**#7** (2°STEP)	**#8** (1°STEP)	**#8** (2°STEP)	**#9** (1°STEP)	**#9** (2°STEP)	**#10** (1°STEP)	**#10** (2°STEP)
Temperature $_{Inlet}$ (°C)	140	140	150	110	60	60	130	60	130	60	130	60	130	60
Temperature $_{Outlet}$ (°C)	60	72	74	56	43	34	72	38	74	42	62	34	62	34
Pump (ml/min)	5	5	5	5	5	7.5	5	5	5	20	30	30	30	30
Flow (l/h)	600	600	600	600	600	600	600	600	600	600	600	600	600	600
Aspiration (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Nozzle diameter (mm)	0.7	0.7	0.7	1	1	1	1	1	1	1	1	1	1	1
Yield process (%)	31.5	64.3	61.3	72.1	54.5	50	35	32.8	42	37	77	30	56.2	30



(a)



(b)



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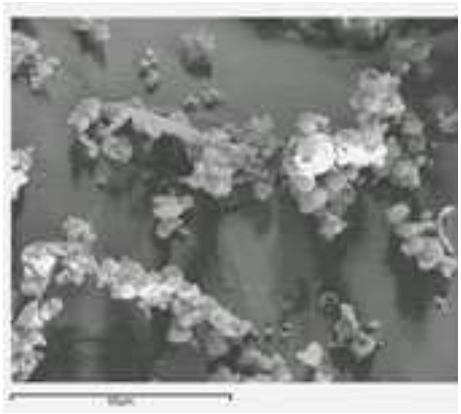

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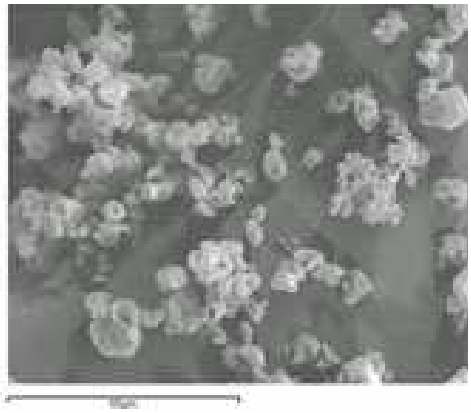
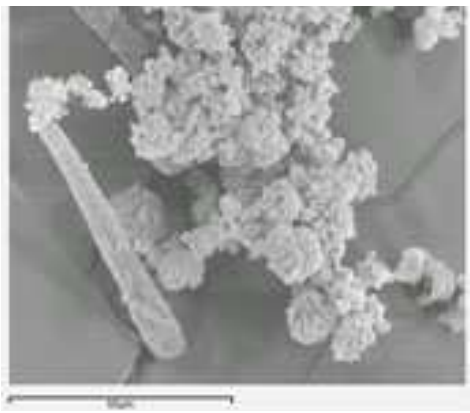


(e)



(f)

Figure 2.7. SEM images of (a) 5-ASA raw material (500X), (b) 5-ASA micronized raw material (3000X) and of the SD microparticles: (c) #1, 1000X, (d) #2, 1000X, (e) #3, 1000X and (f) #4, 1000X.

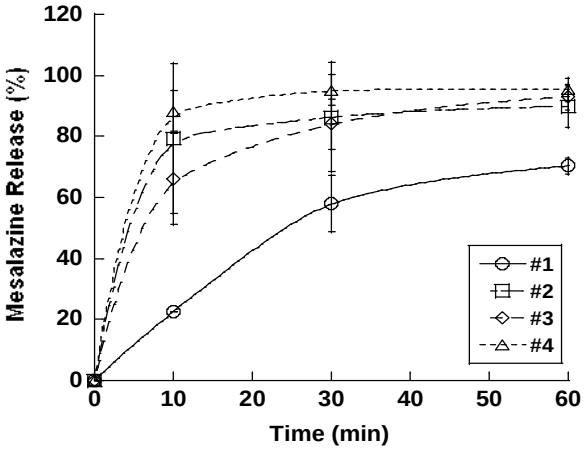


Figure2.8. Dissolution profile in acid medium of the formulations #1, #2, #3 and #4.

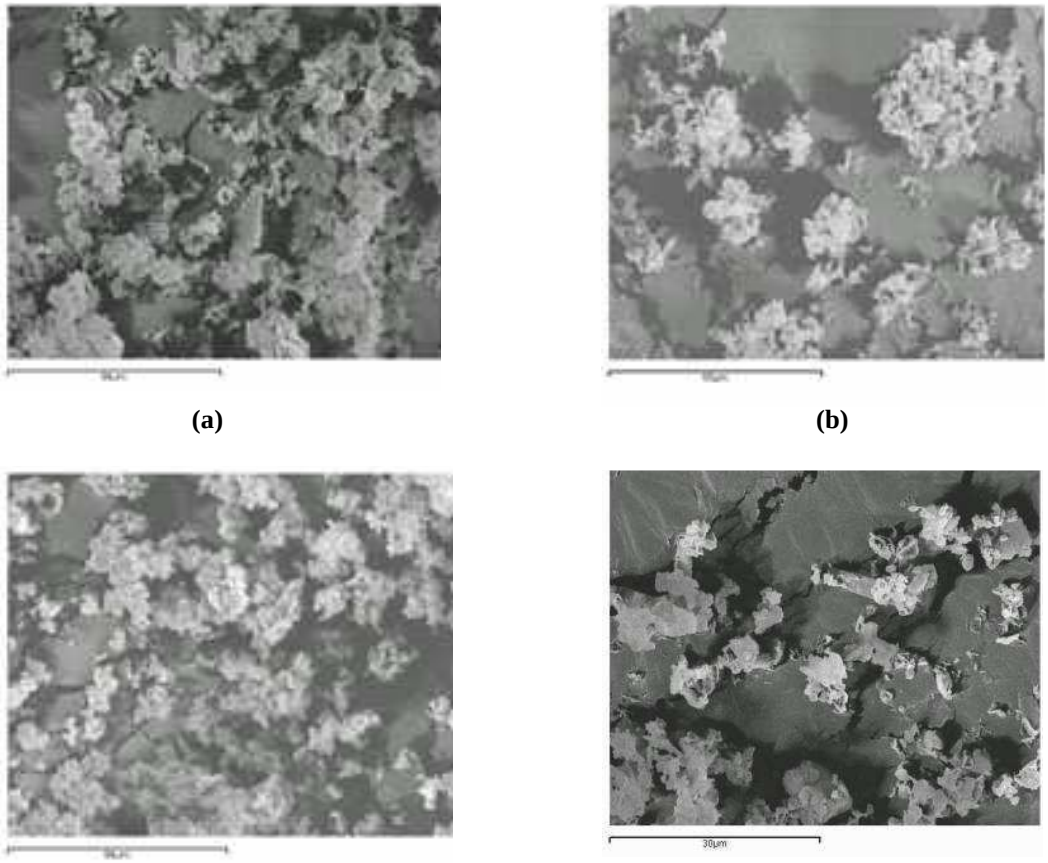
The needle-shape and the dimension of the 5-ASA were unsuitable to be covered with a gastroresistant polymer, so it was decided to micronize the 5-ASA powder (Figure2.7b). Moreover, since the maximum allowed amount of the gastroresistant polymer Eudragit® is 2mg/kg bw [43], the quantity that can be used to produce microparticles has to be lower. Furthermore, if a formulation for paediatric target is considered, the amount of gastroresistance polymer is even lower.

In the next formulations, the amount of Eudragit® S100 was decreased and instead of it a proper amount of stearic acid was added. The stearic acid is a liphophilic excipient that it could be used to gain the gastroresistant property due to its capacity to form a soluble salt only in the basic medium [44].

In the formulations #5 and #6, the 5-ASA was suspended in ethanolic solution of Eudragit®S100 and stearic acid in different ratio, then it was sprayed. Due to the drying process of only an ethanolic sospension, the inlet temperature was decrease to 60°C. In the formulation #6 the speed flow was fixed at 7.5 ml/min instead of 5 ml/min to improve the coating process of the excipients around the 5-ASA crystals. However, the formulations #5 and #6 were still no gastroresistant due to the release of 80% of mesalazine in one hour in the acid medium (data no shown). The 5-ASA was not totally incorporated in the lipophilic excipient, this fact was confirmed also from the presence of 5-ASA crystals in the SEM images (Figures 2.9a and 2.9b) in both formulations.

At this point it was decided to modify the spray-drying process through a “double spray”. In the first step, a solution of 5-ASA and a hydrophilic polymer, that could control the release, was sprayed. The obtained microparticles were subsequently suspended in ethanol containing Eudragit®S100 and stearic acid, then sprayed again. It was tried to achieve a gastroresistant formulation through both the combination of pH-dependent drug release by the coating of the microparticles with stearic acid and Eudragit®S100 and time-dependent drug release by using hydrophilic polymer, that swells in aqueous medium. In the case of formulation #7, in the first step the HPMC (Methocel® 4K) was used as hydrophilic polymer. An aqueous solution of 5-ASA and HPMC was sprayed. In the second step, the SD powder was dispersed in ethanol of Eudragit®S100/ stearic acid and sprayed again. From the in vitro dissolution reported in Figure 2.10, it was evident that the 5-ASA release from the formulation #7 was rapid, demonstrating the lack of gastroresistance of this formulation.

In the first step of the next preparations, the hydrophilic polymer was substituted either with chitosan, a natural polymer that it could be degraded by colonic enzymes (formulation #8) [45, 46], pectin (formulation #9), a natural polymer with gastroresistant properties [47] and modified starch (formulation #10) [48]. In the second step the obtained microparticles so far were dispersed again in an alcoholic solution of gastroresistant polymers (Eudragit®S100 and stearic acid) and sprayed again. From the SEM images reported in the Figures 2.9c-e, it is still possible to observe the presence of 5-ASA crystals in all the formulations, especially in the formulation #9 and formulation #10. The dissolution profiles in the Figure 2.10 show that the 5-ASA was released in an hour in acid medium.



(a) **(b)**

(c) **(d)**

(e)

Figure 2.9. SEM images at different magnification of 5-ASA spray-dried microparticles formulations: (a) #5, 1000X; (b) #6, 1000X; (c) #8, 1000X; (d) #9, 2000X, and (e) #10, 2000X.

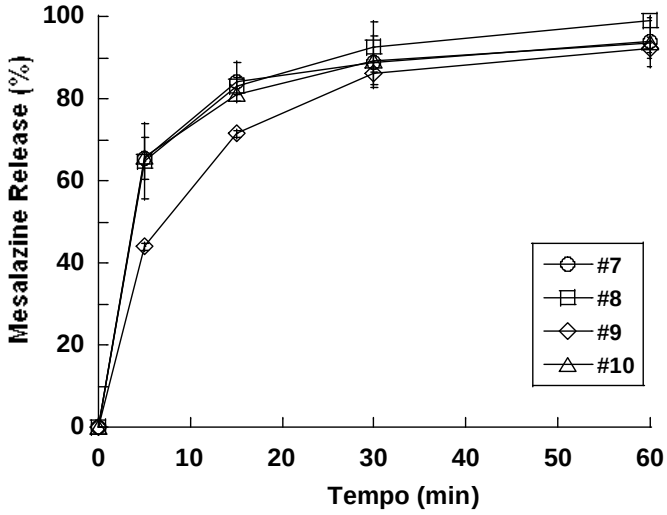


Figure 2.10. Dissolution profile of the formulations #7, #8, #9, #10 in acid medium for 1 hour.

Due to the impossibility to obtain 5-ASA gastroresistant SD microparticles, it was studied the possibility to obtain a gastroresistant agglomerate using SD microparticles containing 5-ASA and pectin (a natural polymer that can be employed without toxicity problems), calcium chloride to improve the gelation of the pectin and the SD excipient microparticles (mannitol-lecithin) acting as a binder for the production of the agglomerates. The formulations of the SD microparticles (both the microparticles gastroresistant containing 5-ASA and the excipient microparticles) are reported in the Table 2.V, instead the manufacturing parameters are shown in Table 2.VI.

As suggested in many published works, pectin and derivates can be widely used as specific drug carriers for the drug targeting to the colon, exhibiting gelling and enzyme-decomposing properties. Initially, the swelling process creates a diffusion barrier which is degraded by colonic enzymes or bacteria at a later stage [47, 49].

Low methoxy amidated pectin (degree of esterification <50%) was used as this kind of pectin readily forms gels in aqueous solution in the presence of free calcium ions. The latter ions cross-link the galacturonic acid chains of the pectin in a manner described by the ‘egg-box’ model [50].

First, the 5-ASA was suspended in aqueous solution of pectin and then sprayed. Instead, the excipient microparticles were obtained spraying an hydroalcholic solution of

mannitol and lecithin. The drug content of 5-ASA in the SD. microparticles was 50.1±0.4%. The different population of SD microparticles with the addition of calcium chloride were mixed in Turbula® for 15 minutes. Successively, the SD microparticles were placed on two piled sieves (850 μm and 106 μm) to be agglomerated through the vibration process. The agglomeration process was repeated twice, each run lasted 5 minutes. The agglomerate formulations are reported in Table 2.VII. The agglomerates, coded AMPMLC#1, were collected on the 106 μm sieve and used for further studies. The agglomerates presented irregular shape and no uniform dimensions (Figure not shown). The dissolution profile of the agglomerates AMPMLC#1 is reported in Figure 2.11: the 5-ASA was immediately released, outlining that the system was not gastroresistant.

Table 2.V. Formulations of the 5-ASA and pectin SD microparticles and the SD excipient microparticles.

Sample	5-ASA (g)	Pectin (g)	Mannitol (g)	Lecithin (g)	H_20 (ml)	EtOH (ml)	NaOH 1M (ml)
5-ASA- pectin (1)	3	3	-	-	300	-	-
5-ASA- pectin (2)	3	3	-	-	300	-	25
Mannitol-Lecithn	-	-	5.1	0.9	270	30	-
Mannitol-Pectin-Lecithin	-	1.2	3.9	0.9	270	30	

(1) A suspension of 5-ASA was sprayed.

(2) A solution of 5-ASA was sprayed.

Table 2.VI. Spray-drying process parameters.

	5-ASA-pectin (1)	5-ASA-pectin (2)	Mann- lecit SD micropart	Mann-pect-lecit SD micropart
Temperature $_{Inlet}$ (°C)	140	130	95	95
Temperature $_{Outlet}$ (°C)	71	64	52	40
Pump (ml/min)	5	4.5	4	5
Flow (L/h)	600	600	600	600
Aspiration (%)	100	100	100	100
Nozzle diameter (mm)	1	1	0.7	1
Yield process (%)	61	-	42	33

(1) A suspension of 5-ASA was sprayed.

(2) A solution of 5-ASA was sprayed.

Table 2.VII. Formulations of agglomerates based on different populations of microparticles (5-ASA-pectin SD microparticles, SD excipient microparticles and chloride salt) in different ratio.

	5-ASA-pectin sd micropart(1)	5-ASA-pectin sd micropart(2)	Mann-Lec sd micropart (85:15)	Mann-Pec-Lec sd micropart (65:20:15)	$CaCl_2$	Calcium acetate	Drug loading (%)
AMPMLC #1	1 g	-	1g	-	0.05g	-	24.2±1
AMPMLC #2	-	0.7g	0.7g	-	-	0.1g	19.5±0.4
AMPMLC #3	-	0.7g	-	0.7 g	-	0.1 g	26.7±0.8

(1) A suspension of 5-ASA was sprayed.

(2) A solution of 5-ASA was sprayed.

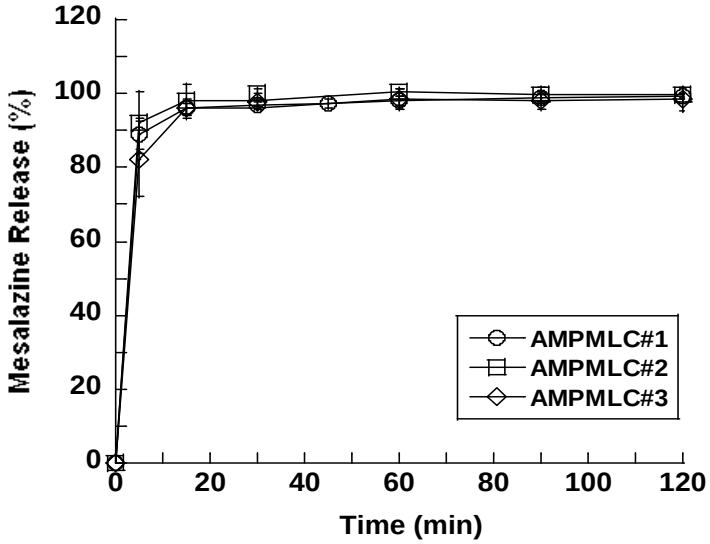


Figure 2.11. Dissolution profiles of the AMPMLC#1, AMPMLC#2 and AMPMLC#3 agglomerates.

Even if the gastroresistance of the agglomerates was not achieved, this approach was not abandoned, trying to modify at least 5-ASA crystals shape through the spray drying of 5-ASA and pectin solution. For this reason, an alkaline solution of 5-ASA and pectin was sprayed. The obtained SD microparticles were mixed in Turbula® together with the SD microparticles of mannitol and lecithin and the powder of calcium acetate. The agglomerates of this formulation were coded AMPMLC#2 and the dissolution profile is showed in Figure 2.11. The 5-ASA was immediately released from the agglomerates in acid medium, then it was decided to increase the amount of pectin to faster reach the gelation of the system.

Moreover, the excipient microparticles of mannitol and lecithin were replaced with SD microparticles of pectin, mannitol and lecithin. For this reason, an ethanolic solution of lecithin was added to an aqueous solution of mannitol and pectin and then it was sprayed. The SD excipient microparticles were mixed in Turbula® with the 5-ASA-pectin SD microparticles and the calcium acetate powder. The dissolution profile of this formulation (AMPMLC#3) is reported in Figure 2.11. Again, the agglomerates were not gastroresistant and the 5-ASA was immediately released.

The mesalazine was immediately released due to the slow pectin gelation process. In fact, due to the high solubility of the 5-ASA in the acid medium the swelling process of

the agglomerates should be instantaneous, allowing the formation of a gastroresistant network. Moreover, as suggest by the Chourasia and Jain [11], the loaded drug in the microparticles has to be lower than 10% to achieve the gelation of the pectin. In this study it was not possible to use a low concentration of 5ASA due to the high drug dosage.

Due to the impossibility to obtain gastroresistant agglomerates, formulations of 5-ASA and pectin as granules were also studied. First, 5-ASA and amidated pectin were granulated with either a binder solution of 1M calcium chloride (granulate #1) or 2M calcium chloride solution (granulate #2). The granulates were obtained using the oscillating granulator (ERWEKA AR400 D, Düsseldorf, Germany) equipped with a mesh screen of 0.5 mm diameter. The granules were dried in the oven at the temperature of 50°C for 4 hours. The formulations of the granulates were reported in Table 2.VIII. The dissolution profiles, reported in Figure 2.12, shown an immediate release of 5-ASA from the granules of pectin. As a consequence, it was decided to decrease the amount of mesalazine and to increase the strength of the gelation process, introducing an amount of calcium acetate in the granulate (granulate #3). Instead, in the granulate #4 the pectin was replaced with sodium alginate, another polymer which in presence of calcium ion could form a gel [51]. However, both the granulates #3 and #4 were not gastroresistant and the 5-ASA was immediately released as reported in Figure 2.12.

In the granulates #5 and #6 the natural polysaccharides (pectin or sodium alginate) was replaced with a semisynthetic hydrophilic polymer as HMPC (Methocel®4K); moreover, a lipophilic excipient as stearic acid was added in different ratio in the two granulates. The goal was to obtain a gastroresistant granulate through the insolubility of the stearic acid in acid medium and the possibility to control the drug release due to the swelling of HPMC. In these cases, it was used a solution of 10% w/v PVP K30 as a binder solution. However, these systems did not swell fast and, as a result, to limit the release of the 5-ASA. From the dissolution profile shown in Figure 2.12, it was observed that the 5-ASA was immediately released.

Table 2.VIII. Formulations of 5-ASA granules.

Gran	5-ASA	Pectin (Unipectine)	Na Alginate	Stearic Acid	HPMC (Metochel®4K)	CaCl2	Calcium Acetate	PVP	Drug loading (%)
#1	5g	5g	-	-	-	5 ml sol 1M (0.5g)	-	-	46.0±1.2
#2	5g	5 g	-	-	-	6 ml sol 2M (1.33 g)	-	-	42.4±0.2
#3	2.5 g	5 g	-	-	-	5 ml sol 2M (1.108g)	1g	-	26.1±1.0
#4	2.5 g	-	5 g	-	-	5 ml sol 2M (1.108g)	1 g	-	27.6±0.6
#5	2 g	-		4 g	4 g	-	-	5,5 ml (0.55g)	19.2±0.5
#6	2 g	-	-	2 g	6 g	-	-	7 ml (0.7g)	19.5±0.6

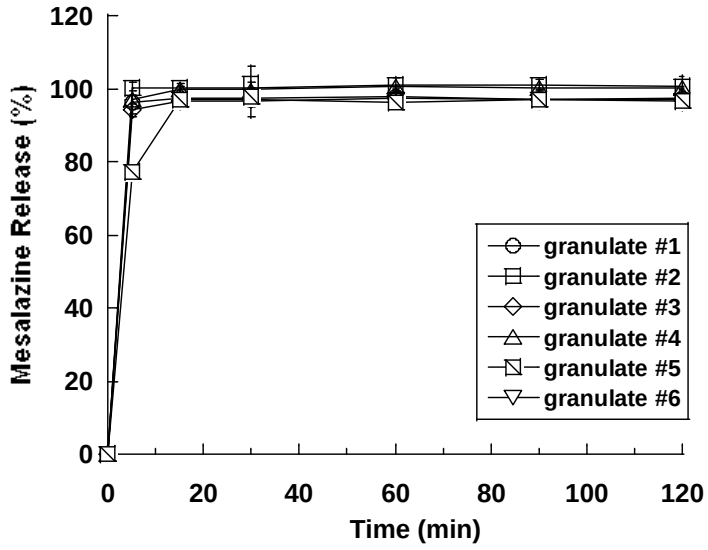


Figure 2.12. Dissolution profiles of the 5-ASA granulates #1, #2, #3, #4#, #5 and #6.

Since the agglomerates and the granulates were not gastroresistant, it was decided to obtain a gastroresistant formulation through two different steps: first the formulation of the granulate and, then, a lipophilic matrix containing the granules.

A granulate of only mesalazine raw material was prepared using a binder solution of 2% w/v Eudragit® S100 solubilized in a 50%v/v hydroalcoholic solution. The granulate was

dried in the oven for 5 hours at 50°C. Then, the granulate was added to the melted stearic acid in a water-bath and mixed until the matrix was cooled. The matrix systems were sieved through a 1mm sieve and collected for further studies.

The 5-ASA loading in the granules was 33.4±0.6%. For the first time, from the dissolution profile in the acid medium (Figure 2.13a) it was observed that the granules high loaded of 5-ASA showed a prolonged release: in fact 40% of the drug loaded was released after two hours. A dissolution test was also conducted in phosphate buffer at the pH of 7.4 for estimating if the system could release the drug in the intestinal fluid. As shown in Figure 2.13b, the system released completely the mesalazine in three hours. Finally, the 5-ASA matrix was also tested in pH variable medium: the dissolution was conducted for the first hour in acid medium, subsequently the pH of the dissolution medium was raised up to 7.4 by adding phosphate buffer solution to the medium (Figure 2.13c). The dissolution profile reported in Figure 2.13c showed that the matrixes were partially gastroresistant because of the release of 30% of 5-ASA after an hour in acid medium. Moreover, when the pH of the medium raised up the value of 7.4, the formulation released completely the drug. However, the manufacture of this system did not allow the repeatability of the process due to an uncontrolled cooling rate and to a manual stirring during the cooling process.

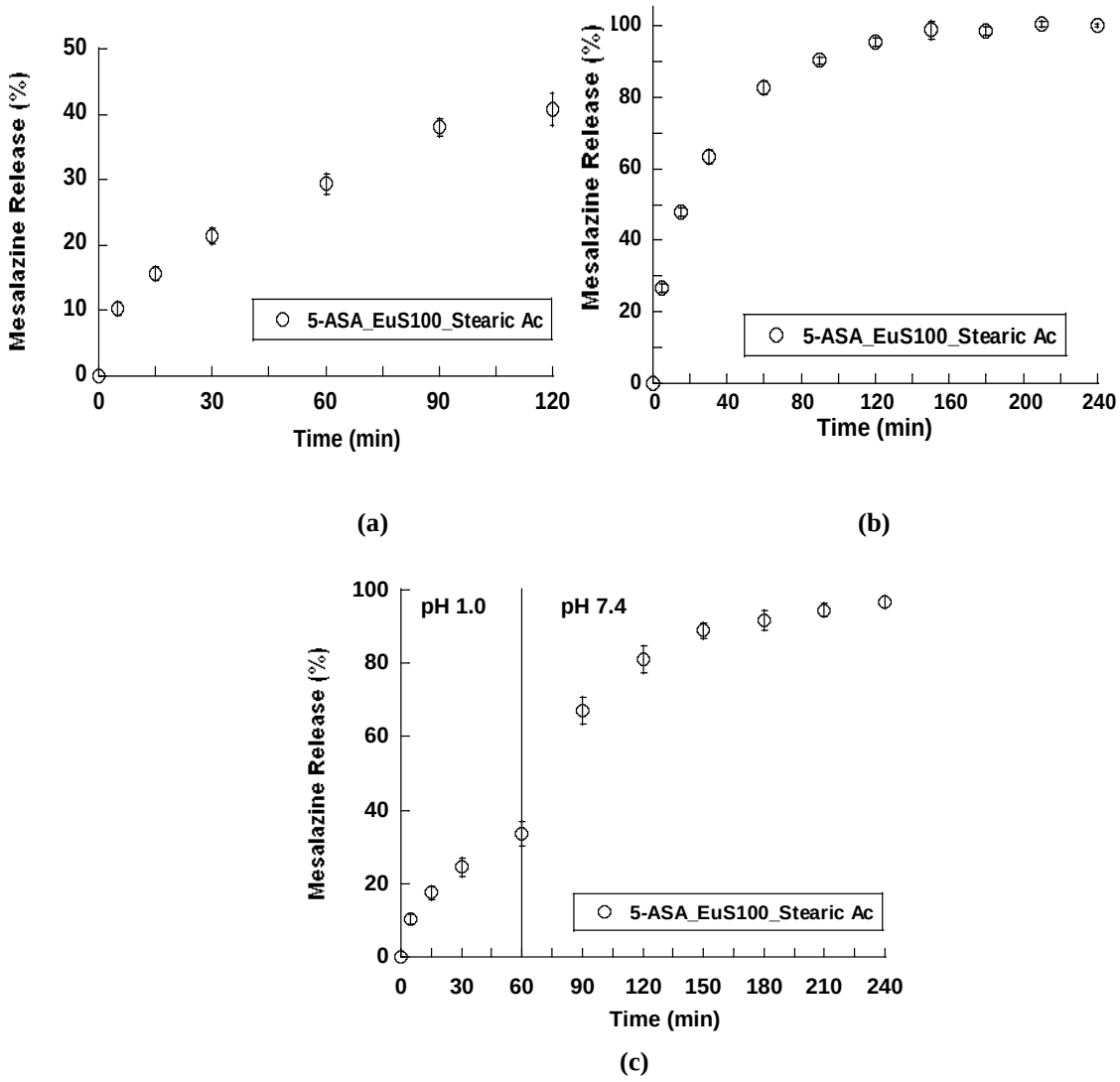


Figure 2.13. Dissolution profiles in (a) simulated gastric fluid, (b) simulated intestinal fluid and (c) variable medium for the 5-ASA_EuS100_Stearic AC matrix.

Taking stock of the situation, since the high drug loading of 5-ASA in the formulation, it was not possible to obtain directly gastroresistant microparticles using the gastroresistant polymer as Eudragit®.

In order to create a gastroresistant network, other approaches were employed, for example the formulation of agglomerates composed by SD microparticles of 5-ASA and pectin, SD excipient microparticles in the presence of ions calcium.. 5-ASA was again released from the formulation, since 5-ASA is very soluble in acid medium and the gelling process was too slow.

Moreover, the manufacturing of gastroresistant granules in one step was studied. Unfortunately, the granules faster released the 5-ASA, so it was decided to produce granules through two steps to increase the gastroresistant. Finally, a partial gastroresistant due to the presence of lipophilic excipients was obtained. However, repeatability process problems occurred.

At this point another technique, as the spray congealing, was used. In fact, this technique is optimal for atomizing a solution or a dispersion of the drug into a molten carrier.

2.4.3. Production of microcapsules

The production of gastroresistant multiparticulate systems were attempted by meaning of spray congealing technique in order to manufacture microcapsules.

The manufacture of microcapsules was divided in two steps. In the first step, the cores were manufactured according to the formulations reported in the Table 2.IX. The first formulation contained 5-ASA, carnauba wax and a low amount of Eudragit®L, while the second one had the same formulation of the first one without the polymethacrylate polymer.

Table 2.IX. Compositions of 5-ASA cores.

	5-ASA	**Carnauba wax**	**Eudragit® L**	**Pressure of atomization**
Core	31%	68%	1%	3 bar
Core without Eudragit®L	32%	68%	0%	3 bar

In the second step the cores were dispersed in the melted stearic acid and sprayed again using the spray-congealing technique. The final compositions were resumed in the Table 2.X.

Table 2.X. Formulations of the microcapsules.

	5-ASA	Carnauba Wax	Eudragit®L	Stearic Acid	Pressure of atomization
Formulation	17.05%	37.40%	0.55%	45.00%	1.2
Formulation without Eudragit®L	17.60%	37.40%	-	45.00%	1.2

2.4.3.1. Characterization of the microcapsules

A preliminary dissolution test of the microcapsule formulations was conducted in pH variable medium (Figure2.14) to verify the microcapsules gastroresistance and the 5-ASA release from the microcapsules.

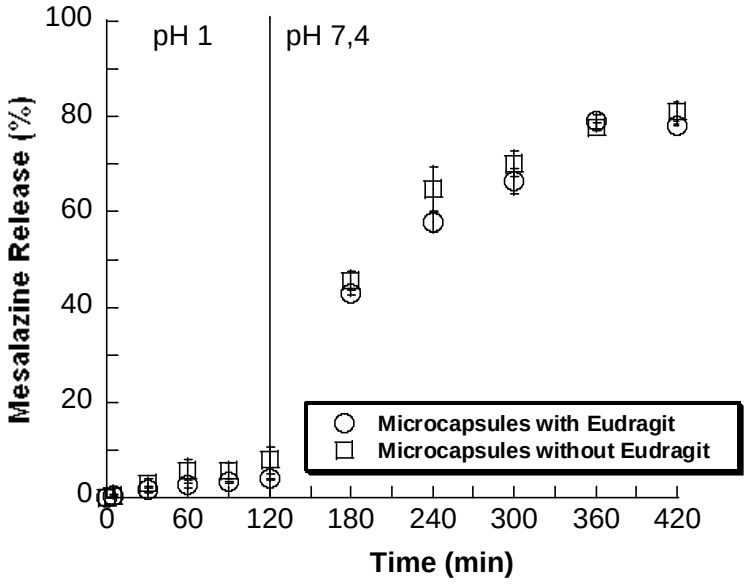
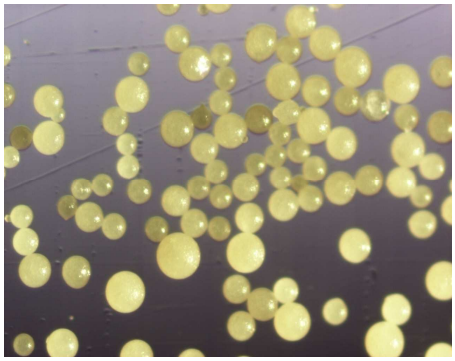


Figure 2.14. Dissolution profiles of microcapsules with and without Eudragit®L in variable medium: two hours in acid medium and then in phosphate buffer at pH 7.4.

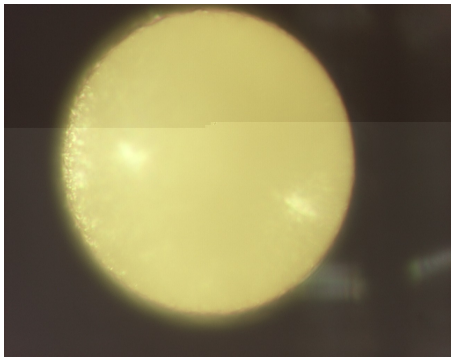
In both formulations less than 10% of 5-ASA was released in acid medium after 2 hours, while the drug release rose the 80% after 5 hour in medium pH 7.4.

For the further studies the formulation with Eudragit®L was selected as microcapsules, even if the similarity factor f_2 of the two formulations was 74, highlighting the same dissolution profile.

The cores and the microcapsules were analyzed by optical and scanning electron microscopy (SEM). From the image 2.15a, it is possible to observe that the cores had round shape with a homogeneous distribution size around 50 - 75 µm. The surface was smooth and without irregularity (Figure 2.15b). The microcapsules, as shown in Figure 2.15c, had less round shape, due to the incorporation of the cores inside the lipophilic matrix. It is important to notice that the cores inside the matrix of the microcapsule are visible, as reported in the Figure 2.15d. The surface of the lipid microcapsules was smooth and needles of 5-ASA raw material were not observed (Figure 2.15e). Some rifts were present on the surface of the lipid microcapsules (Figure 2.15f) due to the spray congealing process [52], but they did not affect the gastroresistant characteristics of the lipid microcapsules.

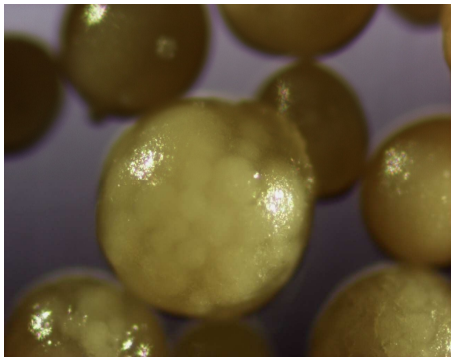


(a)

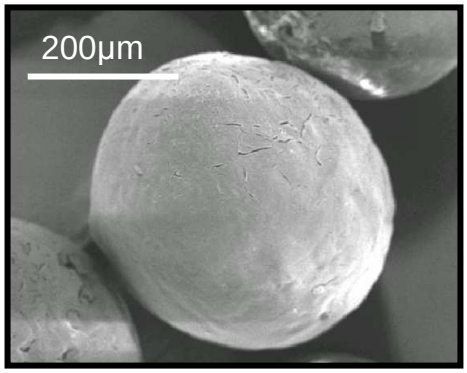


(b)

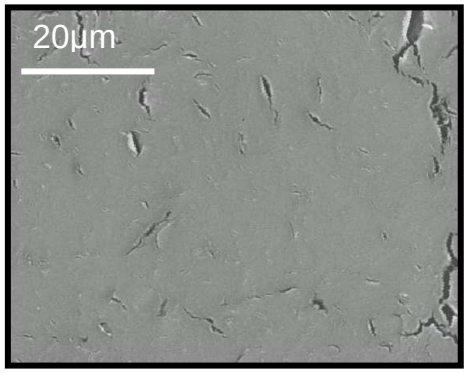
(c)



(d)



(e)



(f)

Figure 2.15. Optical images (a) 4X and (b) 20X of the microspheres and (c) and (d) 4X of microcapsules. SEM images of (e) 20X the microcapsules and (f) the detail of the microcapsule surface.

A possible drawback of the spray congealing technique is that the solidification from the melt is very fast, thus there is the possibility that this technique brings along modifications of the carrier and/or the drug solid state (e.g. transition from the original crystalline form into a different polymorph or an amorphous state). Therefore HSM (Hot Stage Microscopy), DSC (Differential Scanning Calorimetry) and PXRD (Powder X-Ray Diffraction) were used to detect possible modifications of the physicochemical characteristics of the drug and/or the carriers and possible interactions between 5-ASA and the lipids or waxes. The HSM confirmed the encapsulation of 5-ASA cores inside the matrix of stearic acid. The images showed that the microcapsules were intact until 56°C when the stearic acid started to melt (Figure 2.16). While heating, the carnauba wax started to melt at about 79°C in correspondence with the endothermal event registered with the DSC instrument (see later). After the fusion of the wax, floating of 5-ASA crystals in the molten carrier were still detected.

The Figure 2.17 reports the DSC curves of pure 5-ASA, stearic acid, carnauba wax, Eudragit®L and 5-ASA microcapsules. The endotherm peak of 5-ASA was shown at 290°C in agreement with the melting point of the drug. The stearic acid showed a broad melting point at about 57 °C, while the carnauba wax exhibited an endotherm at 83°C with a shoulder at lower temperature, which may indicate the presence of more than one crystalline form. The Eudragit®L showed a glass transition at about 220°C. The thermogram of the microcapsules showed endotherm peaks at about 59°C and 77°C, due to the melting of the stearic acid and the carnauba wax respectively, and a very small peak at 288°C corresponding to the melting point of the drug. The PXRD patterns of microcapsules (blue) compared to the raw materials are depicted in Figure 2.18. The diffractogram of 5-ASA raw material (brown) showed high intensity reflections at 2θ angle of 7.85°, 14.15°, 15.10°, 27.05° and 28.15° and these diffraction peaks were still present in the microcapsule diffraction pattern, demonstrating that the 5-ASA crystal structure remained unmodified. The carnauba wax (red) showed some very intense signals (2θ 21.45°, 23.75° and 36.20°). Stearic acid (black) also showed intense peaks at 2θ 21.7°, 24° and 36.5° and further peaks of minor intensity. The diffraction pattern of microcapsules displayed the intense signals of microcrystalline wax and stearic acid while the main signals of the drug were still present but less intense because of a dilution effect due to the small amount of active ingredient in the system.

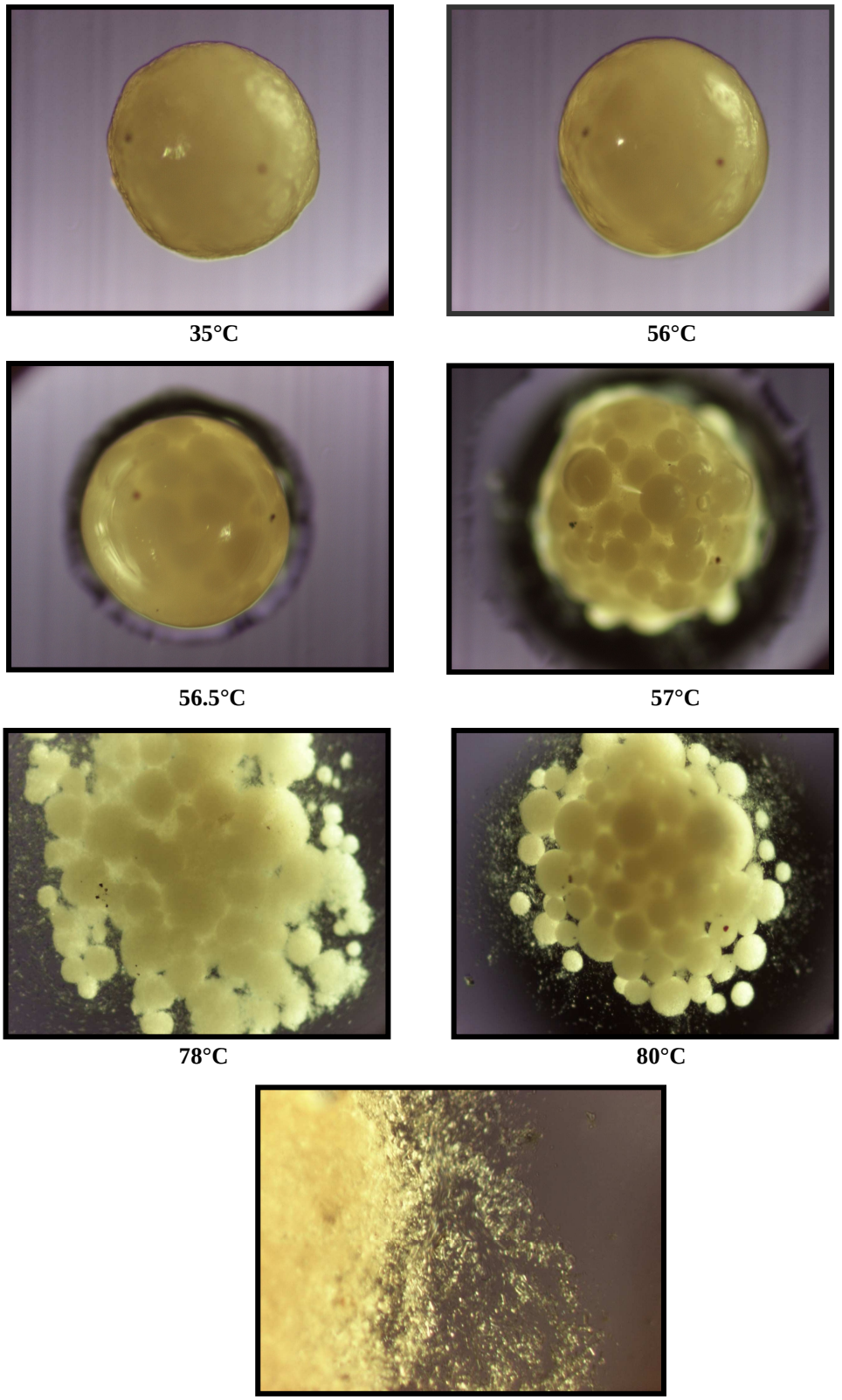


Figure 2.16. HSM micrographs of the microcapsule at the different temperatures.

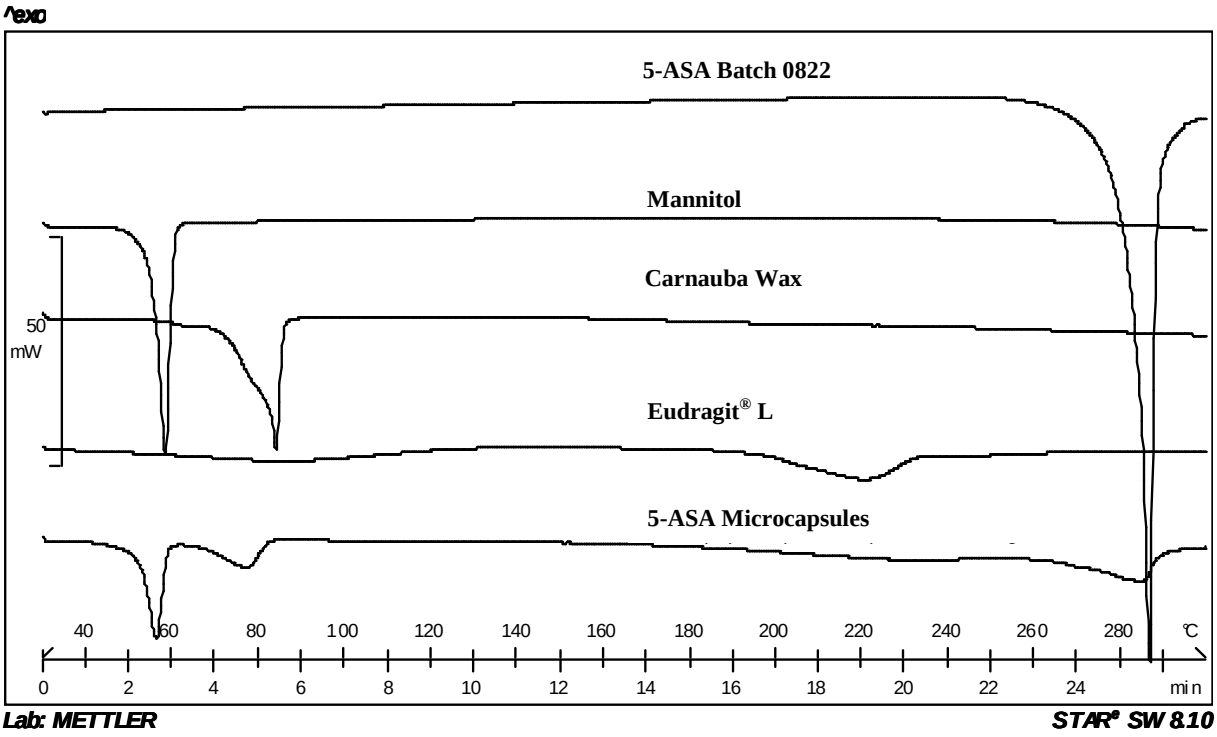


Figure 2.17. Thermograms of the 5-ASA raw material, the stearic acid, the carnauba wax, the Eudragit®L and the 5-ASA microcapsules.

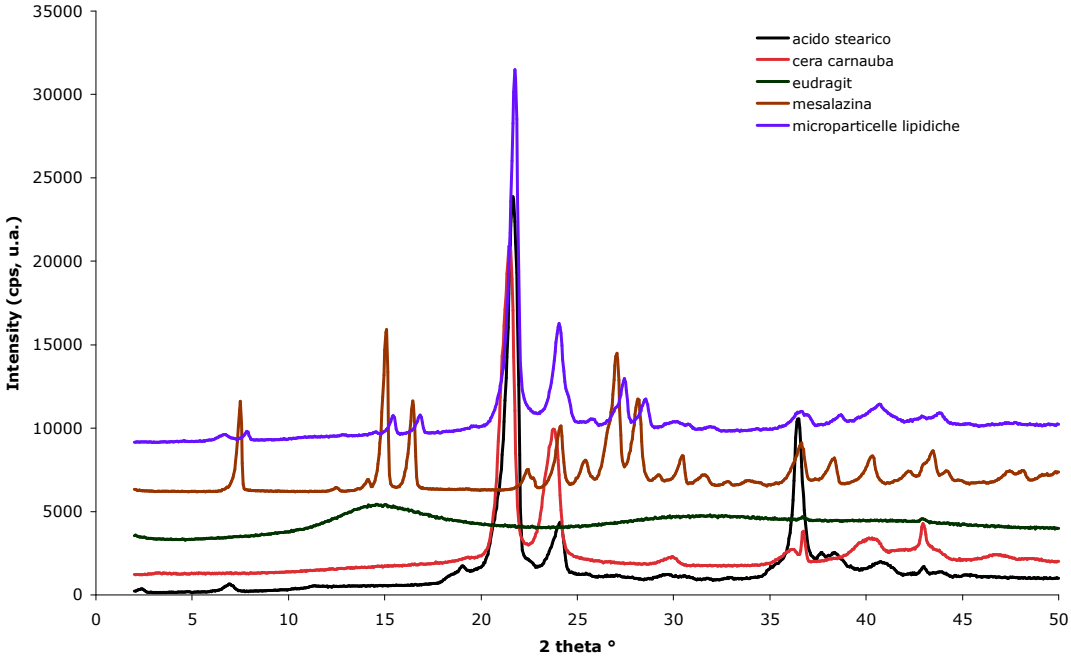


Figure 2.18. X-ray pattern of (blue) microcapsules, (brown) 5-ASA, (green) Eudragit®L, (red) carnauba wax and (black) stearic acid.

2.4.4. Formulation of the agglomerates

In despite of the gastroresistant property of the microcapsules, they were not well dispersed in water or soup due to their lipidic nature. The administration to children could be problematic due the floating of the microcapsules in the liquid where they are dispersed.

For this reason, the microcapsules were agglomerated with spray dried excipient microparticles as mannitol ad lecithin. In fact, the lecithin, acting as a surnatant, could improve the wettability of the microcapsules increasing the dispersion of the microparticulate.

The microcapsules were agglomerated in different ratio (see Table 2.XI) with the SD mannitol/lecithin excipient microparticles. The two different populations of particles were introduced in a bakelite holder with two inox spheres; then the system was mixed in the Turbula®. The presence of the two spheres was essential for the mixing process and afterwards to produce the agglomerates because increased the friction between the lipid surface and the lecithin present in the SD excipient microparticles. This mixing technique improved the adhesion of the SD excipient microparticles on the lipid microcapsules surface. It was tried to manufacture the agglomerates without the presence of the two sphere balls, however the two populations of particles were not well mixed and remained separated after mixing. The drug loading of 5-ASA of the agglomerates are reported in the Table 2.XI.

Table 2.XI. Agglomerates made by different ratio of the lipid microcapsules and the SD excipient microparticles.

Code	5–ASA lipidic microparticles: excipient microparticles ratio	5-ASA theoretical drug loading (%)	5-ASA experimental drug loading (%)
Agglomerates 2:1	2:1	11.4	11.9±0.4
Agglomerates 4.1	4:1	13.4	13.8±0.2
Agglomerates 6.1	6:1	14.6	14.8±0.1
Agglomerates 8.1	8:1	15.2	15.9±0.2

2.4.4.1. Characterization of the agglomerates

The optical microscopy images showed that the agglomerates presented a less shiny surface, but always the rounded shape of the lipidic microcapsules (Figures 2.19a-d). When the ratio of SD excipient microparticles increased, the agglomerates appeared more covered with SD excipient microparticles due to the increasing of mannitol and lecithin microparticles layer deposition. In fact, in the case of 8:1 agglomerates, where the amount of excipient microparticles was less, some surface of lipid microparticles seemed not completely covered (Figure 2.19a).

The SEM images showed the surface of the agglomerates (Figures 2.19 e-h). As already observed from the optical microscopy image, the surface of 8:1 agglomerates seemed not completely covered, due to the limited amount of excipient microparticles (Figure 2.19f). Instead, in the case of 2:1 agglomerates the SEM image showed an uniformity layer of excipient microparticles on the lipid microcapsule surface (Figure 2.19g).

However, the x-ray microanalysis confirmed that the lecithin present in the SD excipient microparticles was detected all around the surface of the agglomerates. The phosphorous, element present only in the lecithin, was detected on the surface of all the agglomerates. As known, the lecithin is placed outside in the SD microparticles [53, 54]; then not the excipient microparticles but the lecithin molecules could coat the surface of the agglomerates. In fact, in the picture of the 8:1 agglomerate surface, some area were not coated with the excipient microparticles (Figure 2.19.f). Probably, the lecithin form weak bond with the stearic acid of the lipid microparticles due to the friction induced from the impact of the two inox spheres during the blending process. In fact, the agglomerates were not produced without the inox spheres.

From the images reported in the Figure 2.19, it possible to deduce that from the blending process no agglomerates were obtained but a dry coating of the lipidic microcapsules. As the SD excipient microparticles increase more layers are deposited on the lipid microcapsule surface..Anyway the term “agglomerate” was used for the classification of the products obtained from the blending process.

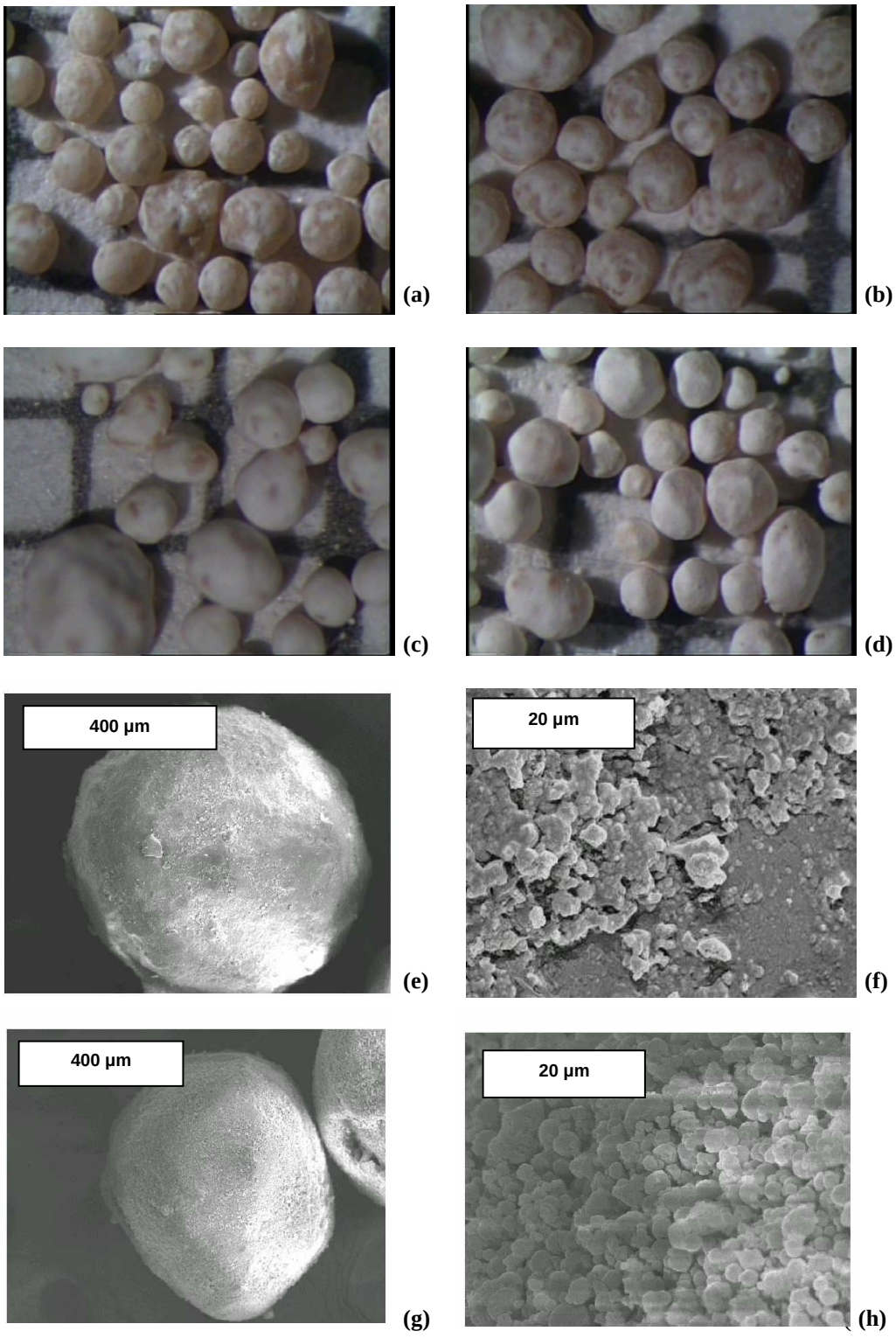


Figure 2.19. Optical microscopy pictures of agglomerates based on the lipid microparticles and lipid microparticles: excipient microparticles in different ratio 8:1 (a), 6:1 (b), 4.1 (c) and 2:1 (d) (magnification 40x); SEM images of (e) agglomerates 8:1 and (f) a detail of the surface agglomerates; (g) 2:1 agglomerates and (h) a detail of the surface.

In order to confirm the hypothesis that more layers of the excipient microparticles were settled on the surface of the lipid microcapsules as the quantity of the excipient microparticles increased, the samples were analysed by meaning of Differential Scanning Calorimetry and Powder X-ray Diffractometry.

The thermograms of the 5-ASA, of the microcapsules and of the agglomerates 2:1, 4:1, 6:1 and 8:1 of the microcapsules and the SD excipient microparticles are shown in the Figure 2.20. The thermogram of 5-ASA showed a sharp endotherm around 290°C (Figure 2.20, curve a). This peak was present also in the trace relating to the lipid microcapsules (Figure 2.20, curve b), although less pronounced, due to the low drug loading (17%). The DSC curve of mannitol/lecithin microparticles (Figure 2.20, curve c) showed an endothermic peak at 175°C, corresponding to the fusion of mannitol. In the case of the DSC thermograms of 2:1 (Figure 2.20, curve d), 4:1 (Figure 2.20, curve e), 6:1 (Figure 2.20, curve f) and 8:1 (Figure 2.20, curve g) agglomerates, the melting peak of 5-ASA shifted to around 262°C. Moreover, as the ratio of the “excipient microparticles” decreased, the area of the peak at around 165°C is reduced. These results were further confirmed by PXRD studies (Figure 2.21). The intensity of the diffraction peaks at around 2θ angle 18.7 and 36.7 was reduced as the amount of the excipient microparticles in the agglomerates decreases. This effect strengthened the result of a lower deposition on the lipid microcapsule surface.

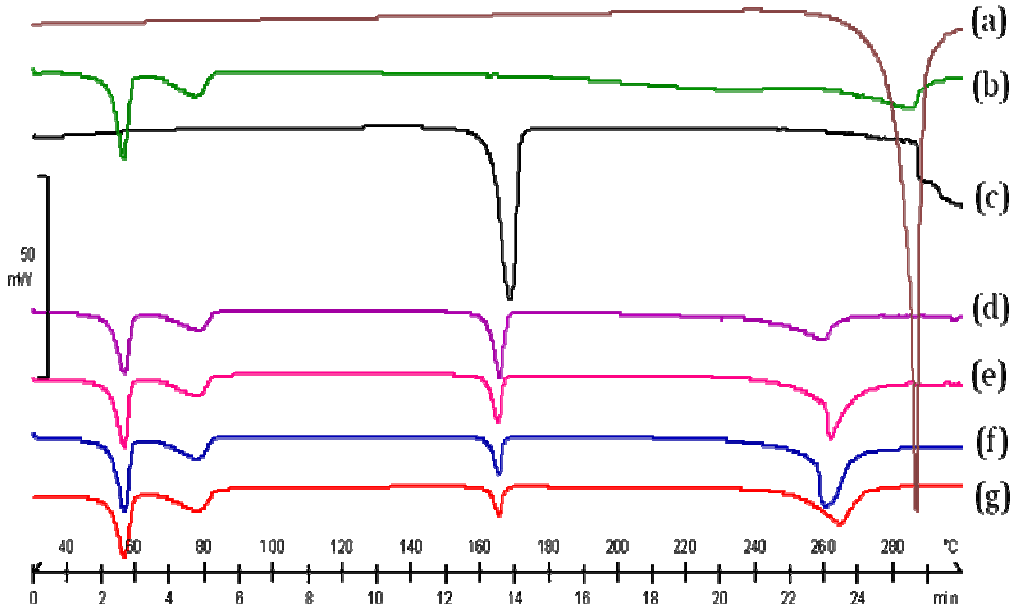


Figure 2.20. DSC thermograms of (a) 5-ASA, (b) the lipid microcapsules, (c) the SD excipient microparticles and the agglomerates (d) 8:1, (e) 6:1, (f) 4:1 and (g) 8:1 of the microcapsules and mannitol/ lecithin microparticles.

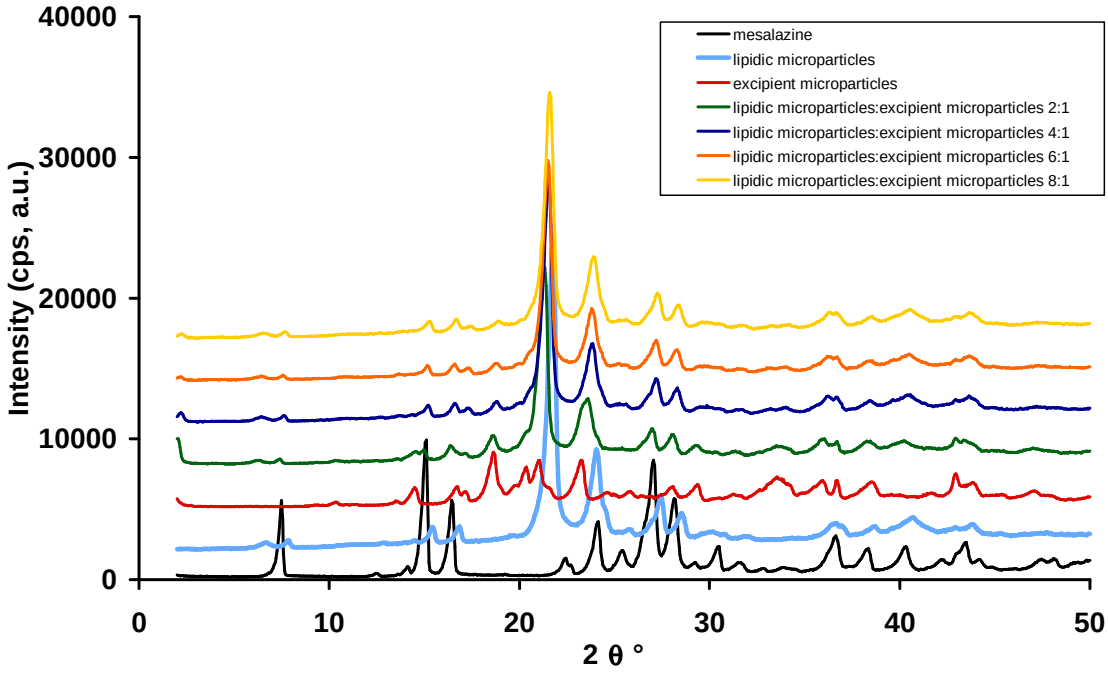


Figure 2.21. PXRD patterns of mesalazine, lipidic microparticles and agglomerates at different lipidic microparticles/ excipient microparticles ratio.

In order to evaluate the gastroresistant property of the formulations, a dissolution test at different pH (in acid medium for the first two hours and in the simulated intestinal medium for the remaining six hours) was carried out both on the lipid microcapsules and the agglomerates (Figure 2.22). The lipid microcapsules released less than 10% of drug loaded in the first two hours. When buffer pH changed, the 5-ASA release rose rapidly to 80% in three hours due to the dissolution of stearic acid and Eudragit L®. The agglomeration process did not affect the gastroresistance of the system in acid medium and the drug release in the phosphate buffer medium. 5-ASA was released less than 10% at pH 1 in the case of 8:1, 6:1, 4:1 agglomerates. Instead, the 2:1 agglomerates showed a release of about 15% after the two hours in acid medium. In fact, since a higher amount of mannitol and lecithin microparticles deposited on the lipid microcapsule surface and as the lecithin acting as surfactant, the wettability of the system increased, releasing more drug in acid environment. When the pH of the buffer changed, the 5-ASA release in the agglomerates was completed.

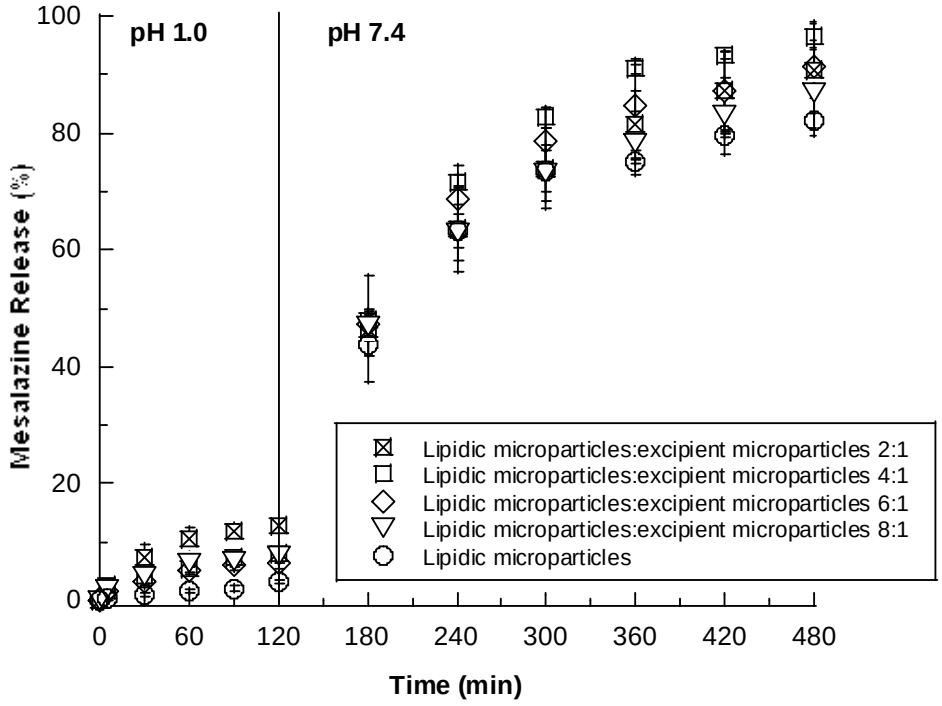


Figure 2.22. Dissolution profiles of 2:1, 4:1, 6:1, 8:1 lipid microparticles: "excipient microparticles" agglomerates and lipidic microcapsules (mean± s.d., n=3).

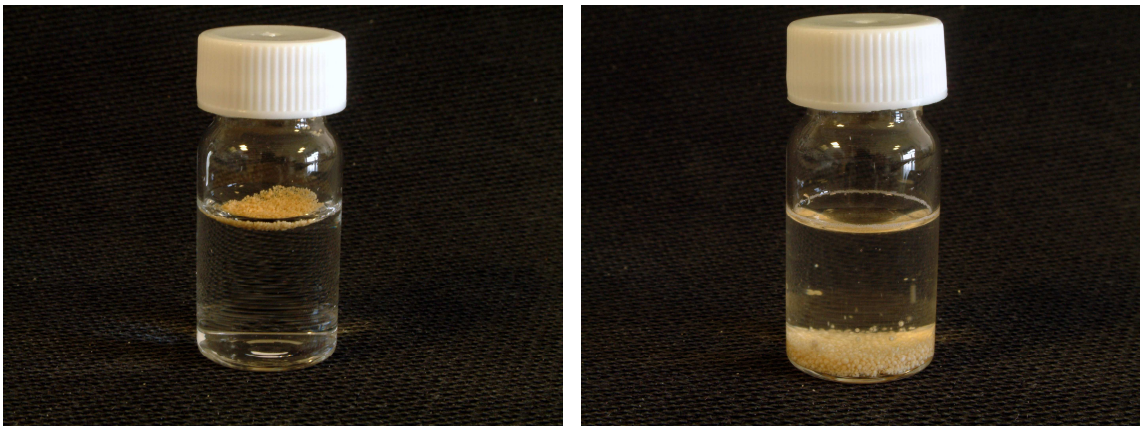
Analysis of similarity factor confirmed that the agglomerates 8:1, 6:1, 4:1 and the microcapsules had similar dissolution profile (Table 2.XII). Instead, the 2:1 agglomerates had a similarity factor < 50. This result confirmed the data of dissolution profile.

Table 2.XII.Similarity factor (f_2) for the microcapsules and the agglomerate formulations.

Reference formulation	Test formulation	F_2
Microcapsules	Agglomerates 8:1	69.6
Microcapsules	Agglomerates 6:1	63.7
Microcapsules	Agglomerates 4:1	73.2
Microcapsules	Agglomerates 2:1	45.9

2.4.5. Study of water penetration on agglomerates and microcapsules

This study was performed in order to demonstrate that with the agglomerates the wettability of the formulation was increased, as shown in Figure 2.23.



(a) **(b)**

Figure 2.23. Picture of (a) microcapsules and (b) 4:1 agglomerates after shaking.

Every formulation was packed inside the rod by tapping. Then, the rod was connected to a low constant flow of water. Figure 2.24 shows the time for the various formulations vs certain fixed length values.

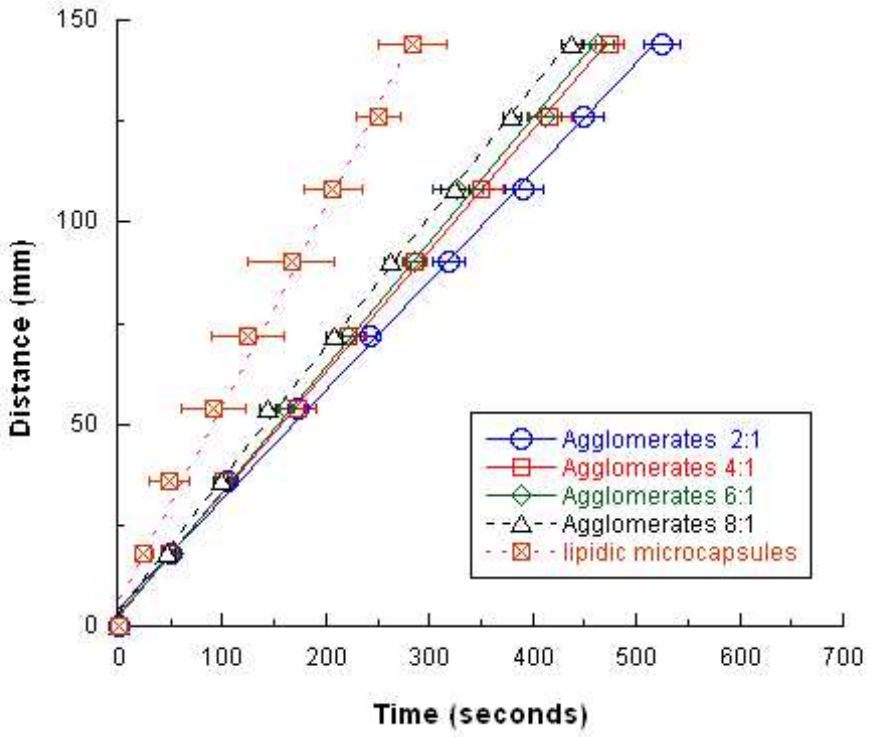


Figure 2.24. Time (seconds) as a function of length (mm) for the various formulations (mean± standard deviation, n=3).

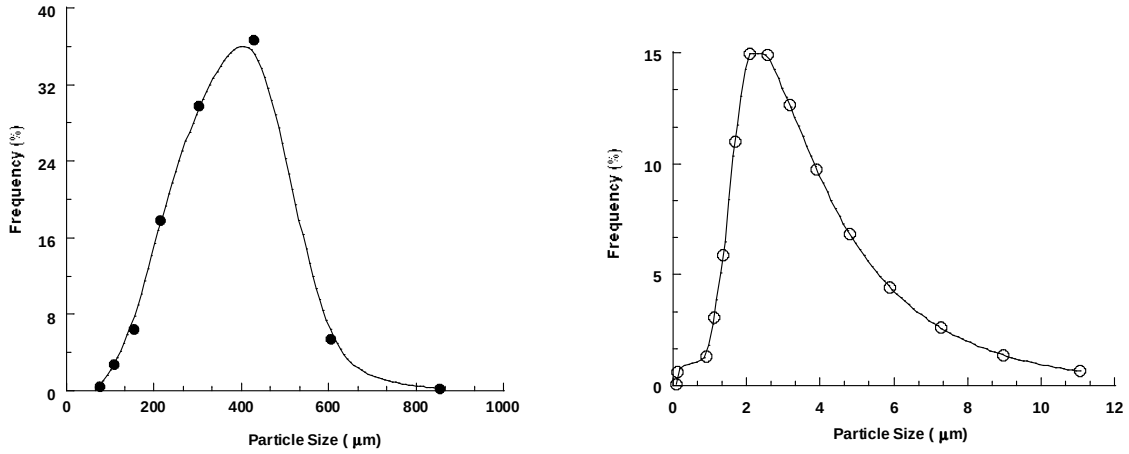
Theoretically, for capillarity the water had a higher transit time in the case of microcapsules due to the resistance of the lipidic excipient. Surprisingly, a minor time of transit was observed in the case of the lipid microcapsules, while in the case of agglomerates the water transit time increased significantly. The presence of a pumping of water, even if low, was the cause of this result. In fact, the microcapsules were not soluble in water and did not interfere with the water movement across the rod. In the case of agglomerates, the mannitol present in the excipient microparticles dissolved in the medium increasing the viscosity of the fluid. Thus, this experiment did not explain why the wettability increased in the agglomerates, but it provided a relationship of the rate of water penetration through the column filled of agglomerates: the water penetration rate was found inversely proportional to the amount of the excipient microparticles.

2.4.6. Calculation of excipient microparticle number needed to cover the lipid microcapsules

In order to optimize the ratio between excipient microparticles and lipidic microcapsule, it was calculated the number of excipient microparticles that can cover the lipid microcapsule. First, it was determinated size distribution of lipid microcapsules versus the weight frequency percentage through the sieve analysis (Figure 2.25a). The plot of the size distribution versus the cumulative undersize percentage was drawn (Figure 2.26a). The geometric diameter (d'_g = 244.74 μm) as the particle size at the 50% probability level on a weight basis was obtained. Moreover, the relative standard deviation was calculated (σ_g =1.53 μm). Finally, the value of mean diameter surface of a lipidic microcapsule (d_{sn} = 171.04 μm) was calculated by the equation of Hatch-Choate (equation 3). Then the superficial area of a sphere was calculated (91906.3 μm^2).

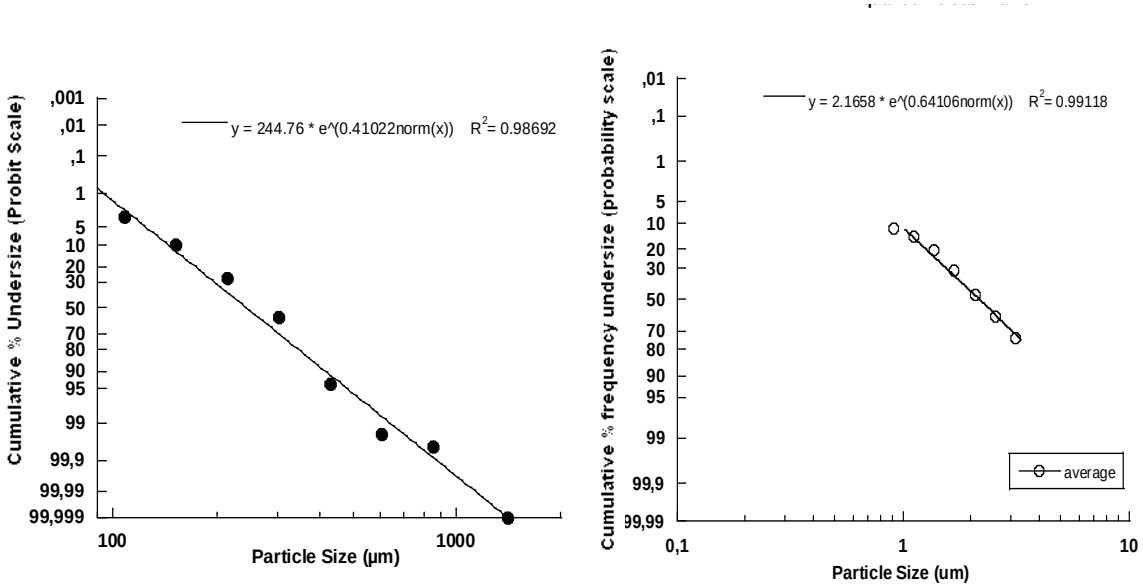
The number distribution of SD excipient microparticles was measured and reported in the Figure 2.25b. Thus, from the plot of the cumulative frequency undersize was obtained (Figure 2.26b), the geometric diameter on number basis was found d_g = 2.17 μm and the relative geometric standard deviation was calculated, σ_g =1.91μm. Through the equation of Hatch-Choate (equation 4) the value mean of diameter length-number of a excipient microparticle (d_{ln}= 2.67 μm) was calculated. Then the project area of an excipient microparticle was calculated (5.6 μm^2). To minimize the estimation error due to the no consideration of the space between the particles, also a projection of a shadow of a square with length diameter of the excipient microparticle as the side was calculated. In this case, the value of the area was 7.13 μm^2.

To calculate the number of excipient microparticles necessary to coat with a monolayer one lipid microcapsule, the value of surface area of a lipid microcapsule was divided by the projection area of the excipient microparticles..The result was that 16412 excipient microparticles were required for making a monolayer on a lipid microcapsule. This value was overestimated since the ratio between the lipid pmicrocapsule surface area and the excipient microparticles projected area does not take into account the surface area of lipidic microcapsule that cannot be occupied by the deposition layer of excipient microparticles.



(a) **(b)**

Figure2.25. Frequency distribution plot of (a) lipidic microcapsules and (b) frequency distribution plot of SD excipient microparticles.



(a) **(b)**

Figure2.26. Log- probability plots of (a) lipid microcapsules and of (b) SD excipient microparticles.

Now, the amount of excipient microparticles used to cover one gram of lipidic microcapsules was calculated.

First, it has to be calculated the number (*N*) of lipid microcapsules that were present in one gram of lipid microcapsules. Assuming that the lipid particles were spheres, through the equation of Hatch-Choate (equation 5) the value of diameter volume-number of a lipid microcapsule was as well calculated (d_{vn} = 187.2 μm). Using the value of the true density (1.139 g/cm^3), the value *N* of lipidic microcapsules in one gram was calculated as 255645. Since the number of excipient microparticles that can cover one lipidic microparticle was 16412, the number of excipient microparticles necessary to cover one gram of lipid microcapsules was 4.95 x 10^9.

Finally, using the d_{vn} = 4.06 μm of the excipient microparticles and the value of true density of the excipient microparticles (ρ = 1.510 g/cm^3) the mass of excipient microparticles per gram of microcapsules was calculated. The mass of excipient microparticles that was necessary to coat one gram of lipidic microcapsules was 0.22 g.

The results indicated that for a single layer of the excipient microparticles on the surface of the lipid microcapsules a ratio around 4.5:1 of lipid microcapsules and excipient microparticles was necessary. As previously shown, the value is overestimated so the ratio of lipid microcapsules:excipient microparticles for monolayer coated microcapsules is close to 6:1.

In order to evaluate if the results obtained from the calculation of the excipient microparticle number necessary for covering one lipidic microcapsule were exact, the data extrapolated from the DSC thermograms of the agglomerates were reworked. In particular, the variation of melting peak of mannitol was estimated.

In Table 2.XIII the values of fusion entalphy (ΔH_f) of mannitol in the mannitol/lecithin microparticles and in the agglomerates are summarized. In the case of the excipient microparticles, a value of ΔH_f for mannitol of -289 J/g was observed. In the case of the agglomerates a decrease of this value is noted (about 15% for the 8:1, 6:1 and 4:1 agglomerates and about 27% for the 2:1 agglomerates). As a rule, the reduction of the enthalpy value of a crystalline product is associated to a decrease of crystalline degree.

The blending process occurs for physical mixing of the two powders in Turbula: due to the frictions induced by the spheres inside the container an interaction between lecithin

present in the excipient microparticles and the wax surface of the lipidic microcapsules occurs. As the blending process shouldn't significantly influence on the crystalline state of the mannitol, it was excluded that the lowering of crystalline degree of this excipient was related to the process.

Table 2.XIII. Values of fusion entalphy (ΔH_f) of mannitol in the mannitol/lecithin microparticles, degree of cristallinity and mannitol peak area.

Agglomerates	ΔH_f average (J/g)	RDS (%)	Degree of Crystallinity (%)	Mannitol peak area (mJ)
Mannitol/lecithin microparticles	-289.2	0.29	100	
8:1	-242.6	1.40	83.9	-126.6
6:1	-246.1	1.81	85.1	-172.8
4:1	-243.4	1.86	84.1	-235.2
2:1	-211.5	1.83	73.1	-325.9

In order to support this hypothesis, the physical mixtures of lipidic microcapsules/excipient microparticles with the same ratios used for the agglomerate manufacturing and the samples were subjected to DSC analyses were prepared. From the examination of the DSC curves, variation on the value of ΔH_f of mannitol with respect to the value in the mannitol raw material was not observed.

In order to find out an explanation for the decrease of value of ΔH_f, the peak areas of mannitol melting in the agglomerates were compared (see Table 2.XIII). According to the results obtained in the particle calculation (see paragraph 2.4.6), for the formation of a single coating layer on the surface of the lipidic microcapsules about a 6:1 lipidic microcapsules/excipient microparticles ratio was necessary. The melting peak area of mannitol in the 6:1 agglomerates ((-172,8 mJ) was taken as reference for the calculation of the mannitol peak area in the other agglomerates by using the following equation:

$$\Delta H_{f\ calc} = \frac{Mannnitol\,Peak\,Area(mJ)}{\%\,excipient\,microparticles\,in\,6:1\,agglom} \times \%\,excipient\,microparticles\,in\,the\,agglom$$

In the case of 8:1 Agglomerates

$$\Delta H_{f\ calc} = \frac{-172.8}{14.28} \times 11.1 = -134.4\, mJ$$

while ΔH_f measured was - 126.6 mJ;

In the case of 4:1 Agglomerates

$$\Delta H_{f\ calc} = \frac{-172.8}{14.28} \times 20.0 = -241.0\, mJ$$

while ΔH_f measured was - 235.2 mJ;

In the case of 2:1 Agglomerates

$$\Delta H_{f\ calc} = \frac{-172.8}{14.28} \times 33.3 = -403.3\, mJ$$

while ΔH_f measured was – 325.9 mJ.

In the case of the 8:1 and 4:1 agglomerates, the values of $\Delta H_{f\ calc}$ are in agreement with the experimental data extrapolated from the DSC curves. On the contrary, in the case of the 2:1 agglomerate the values of $\Delta H_{f\ calc}$ is significantly higher than the experimental value.

According to SEM and DSC analyses and to the calculation of number of excipient microparticles it is possible deduce that the minimum quantity of excipient microparticles, necessary to make a single coating layer onto the lipidic microcapsule surface, was corresponding to the 6:1 lipidic microparticles/excipient microparticles ratio. On the contrary, the amount of excipient microparticles in the ratio 8:1 is detected from the DSC analysis, but it is not sufficient to cover the lipid microcapsules.

On the other hand, in the case of 4:1 agglomerates, more layers were deposited. In the case of 2:1 agglomerates the system is saturated so part of the excipient microparticles remained as powder in the bottom of the container without agglomerating. This phenomenon could explain the reduction of the values of ΔH_f of mannitol.

2.5. CONCLUSIONS

In this part of the thesis an oral gastroresistant multiparticulate system for the 5-ASA delivery to the colon for the treatment of the inflammatory bowel diseases was studied.

Initially, it was tried to formulate gastroresistant microparticles by using a spray-drying technique. Since 5-ASA raw material was characterized by the presence of needle-shape crystals and its therapeutic dose was very high, the amount of gastroresistant polymers necessary to guarantee the gastroresistance was too excessive reaching toxic level, especially for a paediatric administration.

An alternative utilized approach was the manufacture of microcapsules based on lipid excipients through two steps using the spray congealing technique. The gastroresistant property was guarantee as demonstrated by the in-vitro dissolution profiles. However, due to the lipidic nature of these excipients, the microcapsules were not well dispersed in a liquid and presented a bitter taste unwise if the formulation must be administered to children.

These problems could be resolved through the agglomeration process of the lipid microcapsules with SD mannitol/lecithin microparticles in different ratio.

In reality, agglomerates are lipidic microparticles coated with excipient microparticles. It was demonstrated that the excipient microparticles could create a single or multilayer deposition on the lipidic surface. The dissolution profiles showed that the agglomerate systems were still gastroresistant. In addition, the agglomerates improved the wettability of the microcapsules due to the presence of the lecithin acting as a surfactant. Moreover, the taste of system was improved through the presence of mannitol that acts as sweetening masking the bitter taste of lipidic microcapsules.

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CHAPTER 3

ARTEMISININ-CLINDAMYCIN COMBINATION FOR THE TREATMENT OF UNCOMPLICATED FALCIPARUM MALARIA

3.1. INTRODUCTION

3.1.1. Malaria

Malaria is still one of the major health problems in many tropical and subtropical countries. Although, in the 1950s, with the use of residual insecticides and effective drugs there was the hope of eradication, this hope vanished by the 1960s with the appearance of dichlorodiphenyltrichloroethane (DDT) resistance. The situation worsened with the emergence of chloroquine-resistant strains of Plasmodium falciparum. In fact, the malaria parasite is responsible for two million deaths every year. The victims are mostly children under the age of five living in sub-Saharan Africa. Generally, the 80-90% of the world malaria burden is carried by this continent [1].

Malaria is a mosquito-borne infection caused by a protozoa of the genus Plasmodium. There are four species of Plasmodium that infect human beings- P. vivax, P. malaria, P. ovale and P. falciparum. The last one, P. falciparum, is the most pathogenic, accounting for the highest mortality rate especially among non-immune children.

3.1.1.1. Plasmodium life cycle

The life cycle of Plasmodium is adequately described by Majori [2] and Ejigiri [3]. Infection in humans begins with the bite of an infected female anopheles mosquito Plasmodium falciparum (Figure 3.1).

Sporozoites released from the salivary glands of the mosquito enter into the bloodstream during feeding and quickly invade parenchymal cells of the liver, where during the next 14 days in the case of P. falciparum, the liver-stage parasites differentiate and undergo asexual multiplication, known as exoerythrocytic schizogony, resulting in tens of thousands of merozoites which burst from the hepatocyte. The duration varies 5- 11 days according to the species. During this phase, the patients are symptom-free. On reaching maturity, the merozoites are released from the liver cells into the bloodstream and invade erythrocytes undergoing a trophic period in which the parasite enlarges.

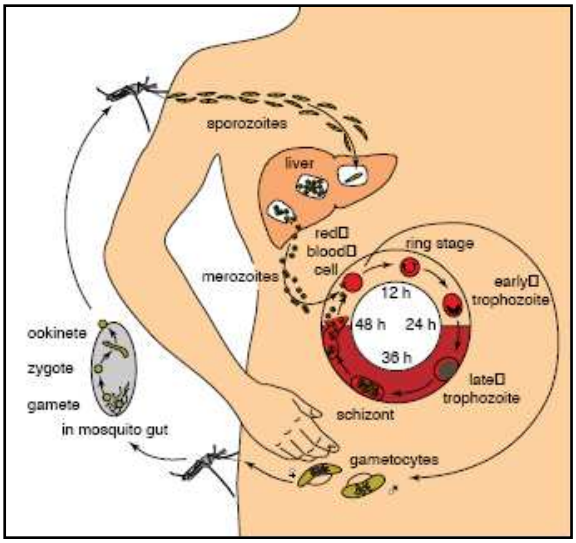


Figure 3.1. Life cycle of the malaria parasite Plasmodium falciparum in humans [1].

Merozoites bud from the mature schizont and are released following rupture of the infected erythrocyte. Invasion of erythrocytes reinitiates another round of the blood-stage replicative cycle. The length of this erythrocytic stage of the parasite life cycle depends on the parasite species: 48 hours for P. falciparum and 72 hours for the other types of plasmodium. This cycle is characteristically synchronous and periodic.

After many asexual replicative cycles, the parasite can differentiate into sexual forms known as macro- or microgametocytes. The gametocytes are large parasites which fill up the erythrocyte, but only contain one nucleus. Ingestion of gametocytes by the mosquito vector induces gametogenesis (i.e., the production of gametes) and the gametes escape from the host erythrocyte. Microgametes, formed by a process known as exflagellation, are flagellated forms which will fertilize the macrogamete leading to a zygote. The zygote develops into a motile ookinete which penetrates the gut epithelial cells and develops into an oocyst. The oocyst undergoes multiple rounds of asexual replication resulting in the production of sporozoites. Rupture of the mature oocyst releases the sporozoites into the hemocoel (i.e., body cavity) of the mosquito. The sporozoites migrate to and invade the salivary glands, thus completing the life cycle.

3.1.1.2. The clinical disease and diagnosis

The first symptoms of malaria are non-specific and similar to the symptoms of minor systemic viral illnesses. An initial chill, lasting one hour, occurs when a generation of merozoites is released into the blood from the ruptured erythrocytes. The other symptoms comprise headache, lassitude, abdominal discomfort, nausea, anorexia. Moreover a febrile stage, characterised by spiking fever reaching 41°C can last several hours: during this stage, the parasites invade new erythrocytes. Another complication is the anemia caused by the hemolysis of the erythrocytes and bone marrow depression [4]. Organ dysfunctions are also observed: liver necrosis, accumulation of pigments in the spleen causing splenomegaly and complication in the tubules causes severe kidney complications.

3.1.2. Artemisinin

Artemisinin (Figure 3.2) is the active principle isolated from the Chinese medical plant "Artemisia annua L." (Quinhao). The herb has been used in the treatment of fever for centuries in China and only in the XIX century it was known that the antipyretic propriety was confined to the treatment of malaria [5].

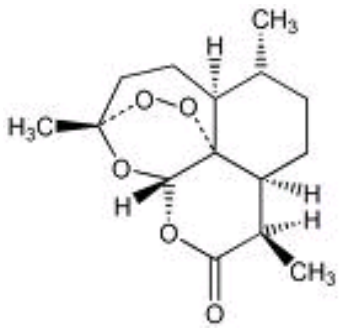


Figure 3.2. Chemical structure of artemisinin.

In 1972, artemisinin was isolated by Chinese scientists and structurally characterised as a sesquiterpene lactone with a peroxide bridge [6]. Artemisinin is a parent of an important class of antimalarials, possessing a 1,2,4-trioxan ring and lacking of a nitrogen-containing heterocyclic ring system, seen in existing quinoline antimalarials. The endoperoxide moiety (C-O-O-C) confers artemisinin its antimalarial propriety. Deoxyarteimisinin as well as other nonperoxy metabolites and other sesquiterpenes lacking the peroxide bridge are devoid of antimalarial activity.

Artemisinin is poorly soluble in water and in oil, with an octanol-water partition coefficient of 160. Artemisinin shows good solubility in most aprotic solvent and remains stable up to 150 °C, suggesting remarkable thermal stability [4]. It decomposes in other protic solvents, likely through opening of the lactone ring.

Artemisinin exhibits high jenunal permeability though the gastrointestinal mucosa and crosses the membrane easily though passive diffusion without the involvement of any saturable carrier-mediated transport mechanism. Thus, decreased membrane transport at the intestinal barrier is not a rate limiting step towards its absorption. Artemisinin is neither a substrate nor an inducer of P-glycoprotein, thus implying that low and variable bioavailability is not the result of increased efflux of artemisinin mediated by P-glycopreotein. The CYP3 A4 is the only intestinal phase I enzyme involved in the metabolism of artemisinin. Thus, the low and variable bioavailability of artemisinin is probably a result of low solubility in the gastrointestinal tract and significant hepatic first pass metabolism.

The lactone of artemisinin can be easily reduced with sodium borohydride, resulting in the formation of dihydroartemisinin, which has even higher antimalarial activity in vitro than artemisinin itself. Many derivatives have been synthesized from dihydroartemisinin, among which artemether, arteether, artesunic and artelinic acid are either currently in use or being evaluated for use. The rationale behind the use of these semisynthetic derivatives of artemisinin is related to their physical properties: artemisinin has poor solubility in water and in oil, it can thus only be administered orally. However, in patients with severe malaria, oral treatment is often impossible and an injectable formulation of the drug is required. Therefore, water-soluble artesunate, the hemisuccinate of dihydroartemisinin, and the oil-soluble artemether have been developed by Chinese scientists for intravenous and intramuscular administration,

respectively. Development of oil-soluble arteether has been promoted by the WHO [7] and the water soluble artelinic acid by the Walter Reed Army Institute of Research. Artemether and artesunate are now also used as oral formulations and are also available in the form of suppositories. It is WHO policy to promote the use of these drugs intra-rectally as an emergency treatment in primary healthcare situations in developing countries. However, these artemisin derivatives also shows some problems: artemeher and arteether have higher acute toxicities as demonstrated in laboratory animals [8], sodium artesunate is extremely instable in aqueous solution due to the hydrolysis of the ester linkage [9] and artelinic acid displays low oral bioavailability and high elimination rate in rabbits [10]. In any case, they all have more higher antimalarial activity than the parent compound [5]. The action of artemisinin and its derivatives is different from the other antimalarial drugs, although both the artemisinin drugs and the 4-aminoquinolines interact with heme. Artemisinin has a very fast action and its parasite clearance time is much shorter than with other malarial drugs [11].

Artemisinin and its derivates show a high recrudescence rate, likely due, at least in part, to the rapid elimination of artemisinin. Consequently, it is necessary to prolong the treatment in monotherapy or preferably to combine it with some other longer-acting agent [12]. Combination chemotherapy tends to delay the onset of resistance [1].

Artemisinin has several advantages over the existing antimalarials. It gives shorter parasite clearance time than chloroquine, leading to more rapid symptomatic relief. A characteristic of the artemisinin drugs is the rapid onset of action with clearance of parasites from the blood within 48h in most cases [4]. In the first clinical study conducted in China, P. falciparum and P. vivax malarial patients were successfully treated with artemisinin. Artemisinin has been shown to be effective against chloroquine-resistant P. falciparum malaria in a clinical study on patients infected with cerebral malaria. It produced more rapide recovery than treatment with chloroquine [13]. Additionally, there is no report of artemisinin resistance from all parts of the world despite wide clinical usage. In Africa, there is widespread resistance to chloroquine, and resistance to the second-line drug pyrimethamine-sulphadoxine is increasing. The problem of drug resistance is greater in South- East Asia, where there is evidence for resistance or reduced sensitivity against all antimalarial drugs including mefloquine, halofantrine and even quinine [4]. In fact, the mefloquine resistant parasite is now

common in Thailand, Myanmar as well as Cambodia and is emerging as a serious threat in Africa [1]. The increasingly failure of the above mentioned drugs has highlighted the important role of artemisinin in the current antimalarial armamentarium.

Oral formulations of artemisinin and its derivatives are absorbed rapidly but incompletely with considerable inter-individual variability [14]. Peak plasma concentrations is reached in 1-2 h and artemisinin and derivates have a short elimination half-life of 1-3 h following oral intake. The oral clearance of artemisinin is high and variable, approximating 200 to 400 litres/h after a single dose of 10 mg/kg. Four artemisinin metabolites have been isolated, namely deoxyartemisinin, dihydrodeoxyartemisinin, 9-10 dihydroxyhydroartemisinin and indenefuran derivate [15]. All metabolites are devoid of the peroxide group and thus do no exhibit any antimalarial activity. Artesunate acts like a prodrug with fast transformation into dihydroartemisinin and has an elimination half-life of less than half an hour. Intramuscular and rectal dosing exhibit slower and more variable absorption and elimination. For arteether, an elimination half-life of 23 h has been reported in healthy subjects after a single intramuscular dose. Time-dependent pharmacokinetics have also been observed in studies with artemether [16].

Adverse effects are rare in patients treated with artemisinin derivatives. In a prospective study of over 3,500 patients in Thailand, there was no evidence for serious adverse effects [17]. Artemisinin derivatives also appear to be safe in pregnant women [4]. The benefits compared with the potential risks suggests that artemisinin derivatives should be used to treat uncomplicated falciparum malaria in the second and third trimesters of pregnancy, but should not be used un the first trimester until more information becomes available [18].

Acute toxicity studies in animals have shown that artemisinin and its derivates have higher LD_{50} values and better chemotherapeutic indexes than chloroquine. However, transit first-degree heart block has sporadically been observed in patients receiving artemisinin derivates such as artesunate and artemether [19].

3.1.2.1. Cyclodextrin

Since the artemisinin is poor-soluble drug, it is possible to increase the solubility by complexation with cyclodextrins [20, 21]. The cyclodextrins are able to generate aqueous drug solution without the use of organic co-solvents, surfactants or lipids. The Ph.D. thesis of Dr. Wong was based on the development of complex artemisinin-cyclodextrin, ratio molar 1:1, by slurry method. According to Wong, α-, β- and γ-cyclodextrin were capable to interact with artemisinin to form complexes. The degree of complexation in solution increased in the order of α- cyclodextrin, γ-cyclodextrin and β-cyclodextrin, while in the solid state it was α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin.

The first reference to cyclodextrins was made in a publication of Villiers in 1891. From a culture of Bacillus amylobacter, grown on a medium containing starch, he isolated a small amount of a crystalline substance. Villiers named his crystalline product "cellulosine" owing to its alleged similarity to cellulose [22]. The later characterization as a cyclodextrin was made by Schardinger and Cramer in 1935 [23]. Recent biotechnological advancements have resulted in dramatic improvements in cyclodextrin production, which has lowered their production costs. This has led to the availability of highly purified cyclodextrins and cyclodextrin derivatives which are well suited as pharmaceutical excipients [21].

The cyclodextrin are formed through the degradation of starch by the glucosyltrasferase enzyme (CGT), then the primary product of chain splitting undergoes an intramolecular reaction without the participation of water, forming 1-4 linked cyclic products known as cyclodextrin [24].

The most common cyclodextrins are α-cyclodextrin (αCD), β-cyclodextrin (βCD) and γ-cyclodextrin (γCD), which consist of six, seven, and eight glucopyranose units, respectively. While it is thought that, due to steric factors, cyclodextrins having fewer than six glucopyranose units cannot exist, cyclodextrins containing from 9 to 35 glucopyranose units (large-ring cyclodextrin, LR-CD), have been purified and characterized. Furthermore, it is still difficult to produce purified LR-CDs in aqueous solutions since LR-CDs are less chemically stable than αCD, βCD or γCD [23].

CDs are cyclic (α-1,4)-linked oligosaccharides of α- D-glucopyranose containing a relatively hydrophobic central cavity and hydrophilic outer surface (Figure 3.3).

Owing to lack of free rotation about the bonds connecting the glucopyranose units, the cyclodextrins are not perfectly cylindrical molecules, but are toroidal or cone-shaped. Based on this architecture, the primary hydroxyl groups are located on the narrow side of the torus while the secondary hydroxyl groups are located on the wider edge [23].

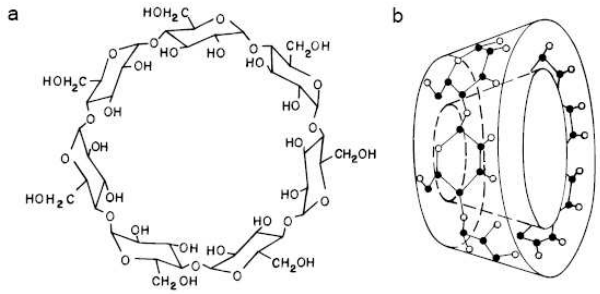


Figure 3.3. The chemical structure (a) and the toroidal shape of the β-cyclodextrin molecule (b).

The central cavity of the CD molecule is lined with skeletal carbons and ethereal oxygens of the glucose residue, which gives it a relatively lipophilic character [24].

Free rotation of the primary hydroxyls will reduce the effective diameter of the cavity on the side on the primary hydroxyls [25, 26].

In aqueous solutions, the hydroxyl groups form hydrogen bonds with the surrounding water molecules resulting in a hydration shell around the dissolved CD molecule [21].

The C2-OH group of one glucopyranoside unit can form hydrogen bond with the C3-OH group of the adjacent glucopyranose unit. In the cyclodextrin molecule a complete secondary belt is formed by these hydrogen bends, making a rigid structure. This is the probable explanation for the observation that β-cyclodextrin has the lowest solubility of all the cyclodextrin. The hydrogen bond belt is incomplete in the α-CD molecule, as one glucopyranose unit is in a distorted position, so only four of the six possible hydrogen-bonds can be established. While the γ-CD has a noncoplanar and more flexible structure, that allows the higher solubility [27]. Generally, the aqueous solubility of the natural CDs is much lower than that of the comparable acyclic dextrins [23]. Substitution of any of the hydrogen bond forming hydroxyl groups, even by

hydrophobic moieties such as methoxy and ethoxy functions, increase the water solubility of the cyclodextrin [23].

The internal diameter of various cyclodextrin is different, depending on the number of glucopyranose units forming the cyclodextrins. The α, β and γ- CDs have an internal diameter of approximately 4.7-5.3,6.0-6.5, 7.5-8.3 Å, respectively [22]. Only substrates having a compatible size with the dimensions of three cyclodextrin cavities are capable of forming inclusion complexes.

The chemical structure of CDs (i.e., the large number of hydrogen donors and acceptors), their molecular weight (i.e., >972 Da) and their very low octanol/water partition coefficient (approximately log Po/w between less than −3 and 0) are all typical characteristics of compounds that do not readily permeate biological membranes [28]. Insignificant amount of intact βCD is detected in the blood after oral administration to rats [24]. Hence, CDs represent a true drug carrier by keeping the drug molecules in solution for delivery to the surface of the absorption site. CDs are not hydrolysed during the upper part of the gastrointestinal tract. They are resistant towards the usual starch hydrolyzing enzymes, like β amylase, however CDs could be degraded by α –amylase at low rate [29]. When CDs reach the colon, they are metabolized by the microflora: the transit time through the colon could be 40 hourlong to allow the induction of cyclodextrinase and subsequent partial or complete hydrolysis of CDs by enzymes.

Toxicity studies have demonstrated that upon oral administration CDs are practically non-toxic due to the lack of absorption from the gastrointestinal tract . After oral administration, the non-toxic effect level of βCD was determined in rats and in dogs [30]. Toxicological considerations proved that β-CD cannot be used in parenteral formulations, thus the introduction of α-CD into parenteral formulations is severely limited. In animal studies, γ-CD has been found to be virtually non-toxic when given intravenously [24].

After oral administration no definite acute toxicity values can be determined; it was possible to demonstrate that in mice and rats the LD_{50} value is higher than 10 g/kg [31].

However, the hemolytic effect of CDs has been reported in several in vitro studies, even though toxicological implication in vivo is considered negligible. The in vitro haemolytic activity of CDs is reported in the order β-CD > α-CD > HP-β-CD> γ-CD >>

HP-γ-CD ≥ HP-α-CD in erythrocytes freshly collected from human blood [32]. The haemolytic activity of CDs correlates well with their ability to solubilize cellular membrane lipids rather than with their intrinsic solubility or surface activity. This is supported by the fact that a positive correlation exists between the haemolytic activity of several CDs and their capacity to solubilize cholesterol, a main component of lipid bilayers, irrespective of their quite different physicochemical properties [24].

Inclusion complexes are entities comprising two or more molecules, in which one of molecules, the "host", includes a "guest" molecule, totally or in part, only by physical forces, without no covalent bond.

The various CDs can be considered as empty capsules of molecular size and represent typical "host" molecules. CDs are capable of forming inclusion complexes with compounds having a size compatible with the dimensions of the cavity. CDs are useful in pharmaceutics because they can interact with drug molecules to form inclusion complexes. Then, in aqueous solution, the inclusion complex dissociates and there is dynamic equilibrium with the guest and the host molecules. This formation of an inclusion complex, often a 1:1 interaction, is usually described as an equilibrium:

$$Drug_{free} + CD_{free} \underset{}{\overset{K}{\rightleftharpoons}} Drug/CD_{complex}$$

The inclusion of a guest in the CD cavity essentially results from the substitution of the included water molecules by the less polar guest molecules. This process is an energetically favoured interaction of the relatively non-polar guest molecules with a solvated hydrophobic cavity. The guest molecule is likely inserted in the larger base of the conical cyclodextrin, because it sheds its hydrate shell and assumes the state of ideal gas. This empty hydrate shell collapses and rearranges. The guest molecule enters the empty CD cavity and the complex is stabilized by van der Waals interactions and sometimes by hydrogen bonding. The guest molecule retains one-dimensional rotational freedom. The displaced water molecules condense from the gaseous state to the liquid state. Moreover, the structure of water is restored around the exposed part of the guest molecule and integrated with the CD ring hydrate shell.

The complex formation is characterized by the stability constant K, which reflects the good adjustment of guest molecule to the host molecule cavity.

$$K = \frac{[Drug/CD_{complex}]}{[Drug_{free}][CD_{free}]}$$

The equilibrium constant can also be defined as the ratio of the forward and reverse rate constants for this process. The formation and dissociation rates of drug/CD complexes are very close to the diffusion controlled limits and drug/CD complexes are continuously being formed and broken apart. A high stability constant indicates that a reasonably strong complex is formed while a small value reflects minimal association between the guest molecule and the cavity of the cyclodextrin. The value of stability constant has to be between 50 and 2000 M^{-1} for most pharmaceutical utilizations with the aim of enhancing drug release and bioavailability.

3.1.3. Clindamycin

Clindamycin (Figure 3.4) is the 7(S)-chloro-7-deoxy derivate of lincomycin, first synthesised in 1966 by Margerlein and coworkers [33]. Clindamycin is available in three forms: clindamycin hydrochloride capsules, clindamycin palmitate which is used orally as a suspension and clindamycin phosphate, another water soluble ester with phosphoric acid used, in general, parenterally. Both the palmitic acid and phosphoric acid esters of clindamycin are inactive against microorganisms in vitro, but the esters are hydrolysed to active clindamycin again in vivo [34].

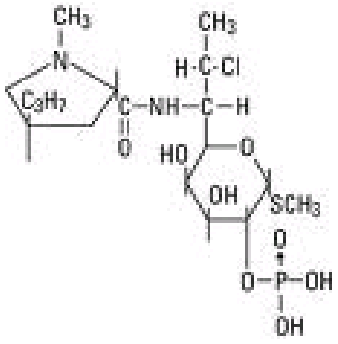


Figure 3.4. Chemical structure of clindamycin phosphate.

When given orally, clindamycin is well absorbed and peak concentration is found about 45 min after ingestion. It is metabolized into three major, biologically active derivatives and is mainly excreted into the bile, with about 20% excreted by the kidneys [35]. The normal elimination half-life of clindamycin is about two to four hours and also is not altered in patients with severe renal disease, but impaired liver function leads to a prolongation of elimination [36].

The activity of clindamycin against anaerobic bacteria makes it an important agent in clinical practice. Furthermore, it is active against organisms such as Plasmodium, Toxoplasma and Babesia. According to Georgiev, clindamycin is the drug of choice for prophylaxis of Toxoplasma chorioretinitis in newborn infants [37]. Clindamycin is also recommended in regimens against both Babesia microti and Babesia divergens [38].

In 1983, Geary and Jensen observed that antibiotics that in vitro inhibit proteins possessed antimalarial properties [39]. The activities of antibiotics could be ascribed to action against the plasmodial mitochondrion or an unusual organelle called the apicoplast, which is similar to plant chloroplasts and unique to plasmodia and other apicomplexan parasites [40]. The apicoplast is a four-membrane-bound relict plastid of endosymbiotic, eukaryotic algal ancestry and in other apicomplexans. Several hundred nuclear encoded proteins are localized to the apicoplast. These proteins are essential for apicoplast metabolic pathways, including those for type II fatty acid synthesis, non-mevalonate isoprenoid synthesis, and a portion of heme biosynthesis. The apicoplast also contains an independent genome, encoding prokaryote-like RNA polymerase subunits, 70S ribosomal subunits, tRNAs, and a small number of proteins. The presence of multiple putative antibiotic targets in the apicoplast suggests that clindamycin and other antibiotics like ciprofloxacin and chloramphenicol, may act on this organelle, inhibiting the transpeptidation of prokaryotic protein synthesis, abrogate Plasmodium growth.

Clindamycin dose-response curves with P. falciparum in vitro demonstrated a plateau region from 0.01 to 10mg/l; similar curves were obtained with the three major metabolites: clindamycin-sulphoxide, N-demethyl clindamycin and N-demethyl clindamycin-sulphoxide. Furthermore, it was shown that the resistance to the antiplasmodium effects of clindamycin does not emerge readily in vitro. Moderate to slow accumulation of the drug in P. falciparum in vitro may reflect the slow onset of

action seen in vivo [41]. Since 1940s the antimalarial activities of antibiotics were observed, even if in the 1952 Coatney and Greenberg suggested that these drugs were too slow to be useful clinically [42]. However, the worldwide spread of chloroquine-resistant Plasmodium falciparum led to a re-evaluation of the use of antibiotics to treat malaria in the 1970s [43, 44]. At the end of 60s Lewis and Powers demonstrated the antiplasmodial activity of oral clindamycin in mice and monkeys, respectively [34]. Moreover, for the first time, chloroquine-resistant P. falciparum was cured by clindamycin administration. No cross resistance was observed with many conventionally used antimalarial compounds, including tetracyclines [45]. The resistance to clindamycin in mice developed very slowly. However, the mechanism of resistance is not clear but it is not due to an impaired uptake of drug by infected erythrocytes, as it has been shown for chloroquine.

In 1975 the first moderate effectiveness of clindamycin administered in monotherapy resulted from three days courses against P. falciparum [34]. However, further studies showed that, in order to be effective, clindamycin must be given for at least 5 days on a twice-a-day regimen. Analysis of all published studies that have used this regimen shows that clindamycin monotherapy has an average efficacy of 98% [46].

In fact, being clindamycin a slowly acting drug, the parasite clearance time was in the range of 4 to 6 days. This is unfavourable in comparison with the parasite clearance times of other antimalarials, which lie between 2 and 3 days for chloroquine and quinine or which are even about 1 day in the case of artesunate [34].

Clindamycin is well tolerated and adverse events are mild and self-limiting. The adverse events encountered in all published trials correspond to the adverse event profile of clindamycin when it is used for the treatment of bacterial infections. Thus, clindamycin may be used as an efficient and safe alternative treatment for uncomplicated resistant falciparum malaria in semi-immune patients in the tropics alongside other drugs.

However, clindamycin monotherapy for falciparum malaria cannot be recommended. The slow onset of action makes it potentially dangerous in cases fast parasite clearance is necessary, such as in children and non-immune adults [47].

Quinine, with its fast action and short elimination half-life, makes a good partner for clindamycin. In addition, in vitro studies have shown a synergistic or additive effect when the two drugs are used in combination and also the levels of both drugs in plasma

are unchanged by coadministration [48]. In a study conducted in Gabon it was demonstrated that quinine-clindamycin was favourable compared with the standard quinine treatment [49].

In another study, always conducted in Gabon, chloroquine was administered with clindamycin for 3 days. The treatment was effective for semi-immune adults in areas with high rates of chloroquine resistance. However, in non-immune individuals, a 3-day treatment is effective only together with a dosage of chloroquine that is too high for common use.

Recently, in a trial study conducted by Ramharter and Kremsner a combination of artesunate and clindamycin was evaluated. The drugs were administered twice a day for 3 days (artesunate 2 mg/kg, and clindamycin 7 mg/kg, per dose) compared with a standard quinine-clindamycin regimen given twice daily for 3 days (quinine, 15 mg/kg, and clindamycin, 7 mg/kg, per dose) for the treatment of uncomplicated falciparum malaria in 100 Gabonese children aged 3–12 years [27]. Times to fever clearance and parasite clearance were shorter in the artesunate-clindamycin group and also the frequency of adverse events was comparable in the 2 groups. Hence, artemisinin administrated with a short plasma half-life, as the clindamycin, merits further attention for use in regions in which the rate of transmission of malaria is high.

3.1.4. Combinations of antimalarial drugs

Malaria is an important cause of death and illness for children and adults in tropical countries. Mortality, currently estimated at over a million people per year, has risen in recent years, probably due to the increase of resistance to antimalarial drugs [4, 50]. Early and effective chemotherapy for malaria has a pivotal role in reducing morbidity and mortality especially since a vaccine will be available the next decade [4].

Multidrug resistance has been reported from most parts of the world and as a result, the monotherapy or some of the combination chemotherapies available for malaria are either ineffective or poorly effective.

The concept of combination therapy is based on the synergistic or additive potential of two or more drugs. Conditions treated with combination therapy include tuberculosis,

leprosy, cancer, malaria and HIV/AIDS [51-53]. Combination therapy with antimalarial drugs consists in the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and/or different biochemical targets in the parasite [4]. The rationale of combining antimalarials with different modes of action is twofold: the combination improves the therapeutic efficacy and also delays the development of resistance to the individual components of the combination.

Chloroquine has been the mainstay of antimalarial drug treatment for the past 40 years [54], but chloroquine resistance is now virtually universal in Plasmodium falciparum and the efficacy of sulfadoxine-pyrimethamine has become increasingly compromised [55]. The treatments now recommended by the World Health Organization (WHO) for uncomplicated falciparum malaria are ACTs, artemisinin-based combination treatments [4]. ACT is superior to the standard monotherapy in most settings, where it is evaluated for reducing parasitaemia and gametocytaemia [56]. Artemisinin-based combinations are known to improve cure rates, reduce the development of resistance and they could decrease transmission of drug-resistant parasites [54]. The ACTs seem to be tolerated as well or even better in children than in adults [55].

The possible disadvantages of combination treatments are the potential of increasing the risk of adverse effects and the higher costs. In fact, the affordability of the therapy remains an issue, as ACTs are nearly 10 times more expensive than the first-line choices. In spite of this, since 2001, 41 out of 54 African countries in which malaria is endemic, have changed their malaria treatment protocols to make ACTs first-line. The impact of these changes on morbidity and mortality has yet to be documented [57].

In northwest Thailand, the use of a combination of mefloquine and artesunate since 1994 as a standard treatment of uncomplicated falciparum malaria has completed the decline in the efficacy of mefloquine and also reduced the incidence of malaria .

For the treatment of uncomplicated falciparum malaria in the United States artemisinin derivates are partnered with longer-acting drugs, like mefloquine, doxycycline or clindamycin to ensure a high likelihood of cure [58].

The use of clindamycin is not restricted in children or pregnant women, and similar evidence emerges for the use of artemisinins [59]. This is an invaluable advantage of this antimalarial combination, because children and pregnant women have the highest risk for malaria-associated morbidity and mortality [60].

The combination of two drugs does not mean only the administration of two different formulations at the same time. In fact, two drugs could be formulated in the same dosage form to modify the release in the term of space and/or time. An example of this approach is the use of the Dome Matrix® technology developed in the laboratory of University of Parma. It is a particular platform for oral controlled release of substances, based on a technology defined module assemblage. In the typical execution, this technology was based on modules or release units, such as swelling matrices possessing their own delivery program. This dosage form is suitable for adult administration.

On the other hand, the soft agglomerates can be administrated to elders and children. The agglomeration is a process in which the powder size is enlarged by constructing weak clusters of microparticles, namely primary. In fact, the agglomerates are defined "soft" because when they are cluster of microparticles held together with weak bonds and when they enter in contact with the water release immediately the primary microparticles. The technology of the agglomerates could be employed for the combination of two formulations. In this case, artemisinin agglomerates could be administered together with clindamycin agglomerates. This dosage form is useful for children. The administration of a single system is a possible simplification of the posology, especially for the particular class of patients as elders and children that have problems to swallow oral dosage form as tablets or capsules.

3.2. AIM OF THIS SECTION

As previously introduced, the treatments for uncomplicated falciparum malaria recommended by the WHO are artemisinin-based combination treatments [27], due to the increased chloroquine resistance in the last years.

The aim of this part of the thesis was the use of the agglomerate technology for formulating a combination-system of the two drugs, artemisinin and clindamycin. The agglomerates could be administered to particular patients such as children and the elderly, that could have some problems to swallow solid dosage forms due to large size of products and swelling of some excipients.

Soft agglomerates can be administered previous dispersion in water in order to make a liquid dosage form. In alternative, the soft agglomerates can be administered directly in month, since they are able to disintegrate immediately when they enter in contact with water. It was observed that in children the dose administration can be more efficiently performed with a powder directly introduced in month, since a liquid could be easy spit out as reaction to bitter taste.

The development of both artemisinin and clindamycin formulations were studied. In the case of artemisinin, the complexation behaviour of the drug with β-cyclodextrin was investigated. In order to optimise the complexation between artemisinin and cyclodextrin three different methods namely, slurry, kneading method and spray drying technique were used. Subsequently, agglomerates of artemisinin-β-cyclodextrin were manufactured by sieve-vibration. In the case of clindamycin, well tolerated antibiotic against anaerobic bacteria with also anti-malarial properties, spray dried microparticles containing clindamycin were investigated. Therfore, the primary microparticles of clindamycin were agglomerates by vibration on sieves.

3.3. MATERIALS AND METHODS

3.3.1. Materials

Artemisinin standard (batch #H23624, purity 100.52%) and artemisinin powder (batch #H56679) were supplied by Aldrich® (St. Louise, USA) and by Kunminig Pharmaceutical Corporation (Kunming, China), respectively. Clindamycin (batch #990158) was donated by ACS DOBFAR S.p.A. (Milan, Italy). β-cyclodextrin (batch# 416502 Kleptose R, Roquette, Alessandria Italy) was employed as drug carrier. Mannitol (batch #H61489, USM, Penang Malaysia) and lecithin (soybean lecithin, Epikuron 135 F®, Degussa, Düsseldorf, Germany) were employed to manufacture the excipient microparticles. All other excipients (sodium hydroxide, sodium lauryl sulphate, di-sodium-hydrogen phosphate, potassium dihydrogen phosphate) and solvents (ethyl alcohol, methanol, acetic acid, hydrochloric acid, ortophosphoric acid) were reagent-grade, while acetonitrile was HPLC grade.

3.3.2. Methods

3.3.2.1.Preparation of artemisinin - β-cyclodextrin inclusion complex

The complex artemisinin - β-cyclodextrin (ARTβCD) was prepared using three different techniques, namely slurry and kneading methods and spray drying technique. For each type of complex, two batches were manufactured. In the first case, the ART-βCD complex was prepared using the slurry method (ARTβCDslu) at molar ratio of 1:1 (artemisinin: cyclodextrin). β-cyclodextrin (24.0 g) was mixed with 25 ml of water until a homogenous suspension was obtained. Artemisinin, which was previously ground and passed through a sieve size of 300 μm (Endecotts Ltd, England), was then added to the suspension. The mixture was stirred for 24 hours at room temperature (25-27°C) before drying by an extraction fan. The resulting product was ground in a glass mortar for size reduction. Particles passing through the sieve size of 300 μm were selected and kept for further studies. For sake of comparison, the physical mixture was freshly prepared just

before use, because complexes with cyclodextrins have been reported to be formed during storage of a dry mixture of cyclodextrin and its guest molecule [61].

For the complex prepared by the kneading method (ARTβCDknead), the same molar ratio of 1:1 ART-βCD was maintained. β-cyclodextrin and artemisinin were separately ground and passed through a sieve size of 300 μm. Then, β-cyclodextrin (2.0 g) was wet in a mortar with 0.5 ml of water to improve its swelling properties [62-64]. Artemisinin (0.5 g) was dissolved in 20 ml of methanol to obtain a clear solution. Few drops of the artemisinin solution were dripped onto the β-cyclodextrin powder and kneaded until the material was dried. This procedure was conducted until the end of the alcoholic solution. Finally, the complex was passed through sieve size of 300 μm and kept for further studies.

In addition to the previous procedures, ART-βCD complex was obtained by spray drying. The preparation was carried out spraying a solution (ART-βCDsd) of the ART-βCD complex. Both β-cyclodextrin and artemisinin were ground and passed through a sieve size of 300 μm, individually. Then, β-cyclodextrin (2.0 g) was dispersed in 100 ml of water at a temperature of 40°C to obtain a solution, while artemisinin (0.5 g) was dissolved in 100 ml of methanol. Before spray drying, the alcoholic solution was added dropwise to the aqueous solution. The solution of artemisinin and β-cyclodextrin, maintained under continuous stirring and at the temperature of 40°C, was spray dried. The total solid concentration in the solution was 1.25% (w/v).

The spray dryer equipment employed to spray the solution was the Büchi Mini Spray Dryer B-191 (Büchi Laboratoriums-Tecnik, Flawil, Switzerland). The solution was spray dried under the following conditions: inlet Temperature 130°C, outlet Temperature 49-52°C, feed rate 4ml/min, nozzle diameter 0.7 mm and drying air flow 600L/h.

3.3.2.2. Preparation of spray dried excipient (SD) microparticles

Different batches of SD microparticles were produced by spray drying technique. The formulation of these excipient microparticles was based on different mannitol: lecithin ratio, namely 95:5, 90:10 and 80:20. A 10% (v/v) ethanolic solution was the solvent system. First, lecithin was dissolved in ethanol at 45°C to obtain a solution, while

mannitol was dissolved in water. Before spraying, the ethanolic solution was added dropwise to the aqueous solution. In Table 3.I the spray drying conditions were summarised.

3.3.2.3. Artemisinin - β-cyclodextrin agglomerate production

All ART-βCD complexes (manufactured through different methods) were agglomerated in presence of excipient microparticles. For manufacturing, the two particle populations (complex plus excipient microparticles) were introduced into a bakelite cylindrical jar (diameter 5.0 cm, length 4.4 cm), then fixed with an orientation of 45° to the axis of a friabilometer (Erweka TA3R, GmbH Germany). In order to mix the two different powders and obtain the mixture for subsequent agglomeration, the apparatus was rotated for 30 minutes at 45 rpm. Moreover, two glass balls were inserted in the jar to improve the mixing process. Mixture homogeneity was assessed based on drug content by HPLC analysis.

Then, the obtained mixture between each ART-βCD complex and excipient microparticles was put on the top of two stacked sieves with nominal apertures of 800 and 100 μm, respectively (20 cm diameter sieves, Endecotts Ltd, London, UK) located on a laboratory sieve shaker (amplitude 5-6; Analysette 3 Fritz model, Fritsch GMBH, Idar-Oberstein, Germany). The system was vibrated for 5 minutes. Agglomerates deposited on the 100 μm sieve were collected for further studies. Reprocessing of non-agglomerated powder was repeated five times.

3.3.2.4. Solubility studies

The solubility of artemisinin (raw material) and of the ART-βCD complex obtained by spray-drying process were tested in distilled water.

An excess of either artemisinin or complex was added to a 5 ml solvent flask. The samples were left under stirring for 24 hours at room temperature (25-27°C). At different time points (4, 8 and 24 hours) 1 ml was collected from every flask and centrifuged at 13000 rpm for 10 minutes (Labofuge 200, Heraeus, Germany). After

filtration with a 0.45 μm PTFE membrane (ReZist Syringe Filter, Whatman), filtered samples were analyzed by HPLC for artemisinin concentration. Each sample was performed in triplicate.

3.3.2.5. Artemisinin HPLC assay

Artemisinin concentration was determined using high performance liquid chromatography (HPLC) with UV detector. For the analysis performed at the USM, the HPLC apparatus used was a Jasco PU-980 solvent delivery system (Jasco Corporation, Tokyo, Japan), equipped with a Jasco UV-975 UV/Vis detector, a D-2500 Chromato-Integrator (Hitachi, Tokyo, Japan) and a Rheodyne 7725i injector fitted with a 50 ml sample loop (Rheodyne, Rohnert Park, CA). The stationary phase was a Genesis C18 column (150 x 4.6 mm), equipped with a refillable guard column (Upchurch Scientific, Oak Harbor, WA) packed with Perisorb RP-18 (30–40 mm, pellicular). The mobile phase consisted of 25% acetonitrile and 75% of 0.01 M di-sodium-hydrogen phosphate, adjusted to pH 6.5 with glacial acetic acid. The retention time of artemisinin was 7.5 minutes with flow rate of 0.8 ml/min. The detector was operated at a wavelength of 260 nm with sensitivity being set at 0.005. Standard curve was performed to demonstrate linearity of the method.

Samples were pre-treated prior to injection into the HPLC system. One millilitre of the sample was added to 200 μL of 10M sodium hydroxide and the mixture was heated at 45°C for 25 minutes, after which it was left cooling till room temperature. Finally, 150 μL of glacial acetic acid were added to this mixture and the sample injected.

The artemisinin standard was dissolved in methanol: water 50:50 (v/v) to produce standard solutions at concentrations of 0.16, 0.4, 2 and 100 μg/ml. The artemisinin loading in the complex powder was determined by dissolving a known amount (40 mg of product) in a 50% of a methanolic solution, then it was diluted 100 times with the same solvent. Finally, the sample was injected into the HPLC, after pre-treatment. Each determination was performed in triplicate.

Further HPLC analyses were then performed at the University of Ferrara, Italy. In this case, sample needed no pre-treatment before analysis. The HPLC apparatus was Agilent

series 1100 comprising an autosampler, a UV-Vis detector and a chromatograph integrator. The stationar phase was a Phenomenex Luna C18 3U column (150 x 4.6mm). The mobile phase consisted of 65% acetonitrile and 35% of 0.02 M sodium dihydrogen phosphate, adjusted to pH 2.7 with phosphoric acid. The retention time of artemisinin was 6.5 minutes with flow rate at 0.6 ml/min. The detector was operated at a wavelength of 216 nm with sensitivity being set at 0.005. Standard curve was performed to demonstrate linearity of the method.

3.3.2.6. Production of clindamycin spray dried microparticles

Microparticles of clindamycin, mannitol and lecithin were manufactured by spray drying technique. Different drug loadings were selected, either 20% or 33%. Separately, excipient microparticles (i.e., without drug) were also manufactured.

In all cases, the solvent system was an aqueous solution containing 8% (v/v) ethanol and the sprayed liquid system containing a total solid concentration of 2% (w/w). Mannitol and clindamycin were dissolved in water, whereas lecithin was dissolved in ethanol. Before spray drying, the ethanol solution was added to the water solution dropwise under stirring. The same parameters were used to produce excipient microparticles without drug.

3.3.2.7. Clindamycin HPLC assay

Microparticle drug loading was determined by HPLC with UV-Vis detector. The HPLC apparatus comprised a Jasco PU-980 solvent delivery system (Jasco Corporation, Tokyo, Japan), a Jasco UV-975 UV/Vis detector, a D-2500 Chromato-Integrator (Hitachi, Tokyo, Japan) and a Rheodyne 7725i injector fitted with a 50 ml sample loop (Rheodyne, Rohnert Park, CA). The stationary phase was a Zorbax Eclipse XDB C8 column (250 x 4.6 mm), equipped with a refillable guard column (Upchurch Scientific, Oak Harbor, WA) packed with Spherisorb RP-18 (30–40 mm, pellicular). At the beginning, the mobile phase consisted of 80% of 0.1 M dipotassium hydrogen phosphate and 20% acetonitrile (v/v); then, the mobile phase was adjusted to pH 2.5

with phosphoric acid 85%. In these conditions clindamycin retention time was 12 minutes. In order to shorten the retention time, the organic phase was increased to 25% (v/v). This change resulted in a retention time of about 5.2 minutes. Flow rate was set at 1 ml/min. The detector was operated at a wavelength of 210 nm with sensitivity being set at 0.005.

3.3.2.8. Clindamycin agglomerates manufacturing

An amount of SD microparticles was put on top of two pile sieves with nominal apertures of 800 and 100 μm, respectively (20 cm diameter sieves, Endecotts Ltd, London, UK) located on a laboratory sieve shaker (Retsch® Test Sieve, Germany). The system was vibrated for 5 minutes at the amplitude of 4/5. The agglomerates retained on the 100 μm sieve were collected and kept for further studies. Reprocessing of non-agglomerated powders was repeated five times.

In case of agglomerates made using different population of microparticles, the different powders were mixed before agglomeration in bakelite cylindrical jar (diameter 5.0 cm, length 4.4 cm), fixed to the axis of a friabilometer (Erweka TA3R, GmbH Germany). The jar was maintained tilted at 45° and twirled at 45 rpm for 30 minutes. Two glass balls (1 cm diameter) were present inside the jar to improve mixing. Production yields (%) were measured by weighing the amount of powder before and after both mixing and vibration processes.

3.3.2.9. Solid State Characterization

3.3.2.9.1. SEM analysis

The morphological features of the artemisinin and the ART-βCD complexes were examined by meaning of electron scanning microscopy (SEM, (Leica Cambridge S-360, Leica, UK). The samples were fixed onto an aluminium stub with double-sided tape before being coated with aurum to a thickness of approximately 20 nm to make them electrically conductive (Polaron SSC-515, VG Microtech, UK). For the agglomerates images, the SEM used was Zeiss SUPRA 40, Carl Zeiss (Oberkochen, Germany) at the

University of Parma. It is a particular technique was developed by which it was possible to dip the agglomerates in silver glue to fix the sample for SEM analysis, otherwise it was not possible to stick them onto the bioadhesive tape without breaking them. Briefly, the agglomerates were deposited on metallic support, previously cover with particular paint, namely Agar Silver Paint. This paint allowed the formation of thin film highly conductive. In all cases, the magnifications selected were 500-4000x, being adequate to appreciate in detail the general morphology of the powder under study.

3.3.2.9.2. Differential Scanning Calorimetry (DSC)

DSC studies were performed on individual components (pure compounds), on complexes (prepared by slurry, kneading methods and spray drying) and on the physical mixture of ART-βCD. Moreover, the thermal behaviour of clindamycin raw material and agglomerate formulations thereof were investigated. The instrument employed was a Perkin-Elmer Pyris 6 (Beaconsfield, UK). Samples were heated from 0 to 250 °C at a rate of 10 °C/min. Helium was used as purge gas with at a flow rate of 40 ml/min. A non-hermetic aluminium pan (Perkin-Elmer, Beaconsfield, UK) was filled with an accurately weighed sample. Each pan was non-hermetically sealed by crimping with its lid. The net weights of the samples analyzed ranged between 4.5-6.5 mg. The mass of the sample and the reference pan were matched to ± 0.1 mg. Each sample was run in triplicate and the results expressed as mean ± standard deviation.

The parameters considered in these thermal analyses were the melting temperature and the heat or enthalpy of fusion (ΔH_f) of artemisinin and clindamycin. Both values were obtained through calculations using the Pyris 6 software (Perkin-Elmer, Beaconsfield, UK).

In order to calculate the percentage of artemisinin complexed by β-cyclodextrin for each preparation method, the thermograms of the complexes were compared with that of artemisinin raw material after normalization for the sample weight. This was based on the assumption that the area under the melting peak of artemisinin was directly proportional to the amount of artemisinin in the sample, and that the artemisinin peak observed in the thermograms from the inclusion complexes was due to the free drug

(i.e. not complexed). Thus, the percentage of artemisinin complex was calculated using the following relationship:

$$\frac{\Delta H_{f\ art}\ of\ the\ ART-\beta-cyclodextr\ incomplex}{\Delta H_{f\ art}\ of\ artemi\ \sin\ inrawmaterial} x100$$

3.3.2.9.3. Fourier-Transformed Infrared (FTIR) analysis

Fourier-Transformed Infrared (FTIR) analyses were performed using a Nicolet Nexus 7 FTIR (ThermoNicolet, Madison, WI, USA). The scanning range was 4000 - 400 cm^{-1} and the resolution was 1 cm^{-1}.

The methodology for sample preparation employed potassium bromide (KBr). KBr crystals were homogenised and reduced to fine powder using an agate mortar and a pestle. The fine powder was then put in a disc mould and placed under pressure using an 8-ton press. Once the disc formed, it was placed inside the measuring chamber and the baseline reading was obtained and used as background. Every sample was mixed with KBr at the ratio of 1:99 and then moulded into a disc. Each analysis was replicated twice.

3.3.2.9.4. Powder X-Ray diffraction (PXRD)

PXRD of artemisinin raw material, β-cyclodextrin and the complexes were carried out. Diffraction data were obtained with a Miniflex X-ray Diffractometer (Rigaku, Tokyo, Japan) with a graphite monochromator in the diffracted beam-path (CuKα radiation, Kα1 1.5406 Å; Kα2 1.5443 Å). A system of diverging, receiving and anti-scatter slits of 0.58, 0.58, and 0.2 mm, respectively, was used. The patterns were collected with 30 kV of tube voltage and 15 mA of tube current in the angular range $2 \leq 2\theta \leq 50°$ in a step scan mode (step width, 0.05°; counting time, 2 s/step).

3.3.2.10. Dissolution tests

The in vitro dissolution tests of ART-βCD complexes made by different methods (slurry method, kneading method and spray drying technique) as well as of the physical mixture were determined using the USP 31 paddle method dissolution test apparatus (Type PTWSIII/S12/d6-PTWS3C, Pharma Test Dissolution Tester, Hamburg, Germany). Due to the solubility issues of artemisinin, experiments were conducted in different mediums (water, water + ethanol and simulated gastric fluid without enzymes). Different amounts (0.005%, 0.01% and 0.1% w/v) of surfactant, namely sodium lauryl sulphate (SLS), were added. The dissolution was conducted over a 2 h period in 900 ml of medium maintained at 37 ± 0.5 °C with a paddles rotating at 100 rpm. 4 ml samples were withdrawn at various predetermined time points (0, 5, 15, 30, 45, 60, 90 and 120 minutes). Samples were filtered through nylon membrane with polypropylene housing and pore size of 0.2 μm (Puradisc, Whatman, USA). The initial portion of the filtrate was discarded, while the subsequent portion was collected for determining drug concentration by the HPLC method described above.

After a first series of experiments it was not possible to dissolve 100% of complexed drug. Assuming that incomplete drug release from the complexes was due to an analytical error, the dissolution tests were repeated at the University of Ferrara. This second series of dissolution test was conducted in water:ethanol (90:10) and in simulated gastric fluid (SGF). Samples were analyzed using the HPLC method developed at the University of Ferrara. Each formulation was dissolved in replicates of three.

Dissolution test of clindamycin agglomerates was conducted over a one hour period using the USP 31 Apparatus 2 (Paddle Method) in 900 ml of simulated gastric fluid without enzymes (SGF, 2 g of NaCl and 7 ml of HCl 37% in 1000 ml of water, pH 1.2) maintained at 37 ± 0.5°C with paddle rotating at 100 rpm. 5 ml samples were collected at various predetermined time points (0, 2, 5, 10, 20, 30 and 60 minutes). Samples were filtered through nylon membrane with polypropylene housing and pore size of 0.2 μm (Puradisc, Whatman, USA). After discharging the initial portion of the filtrate, the rest was collected to determine clindamycin concentration by the HPLC method described above. The test was run in replicates of three.

3.4. RESULTS AND DISCUSSION

3.4.1. ARTEMISININ

3.4.1.1. Validation of artemisinin HPLC methods

For the method developed at USM linearity was assessed. As shown in Figure 3.5, the linearity was optimal in the concentration range 0.16-100 mg/ml (correlation coefficient =1).

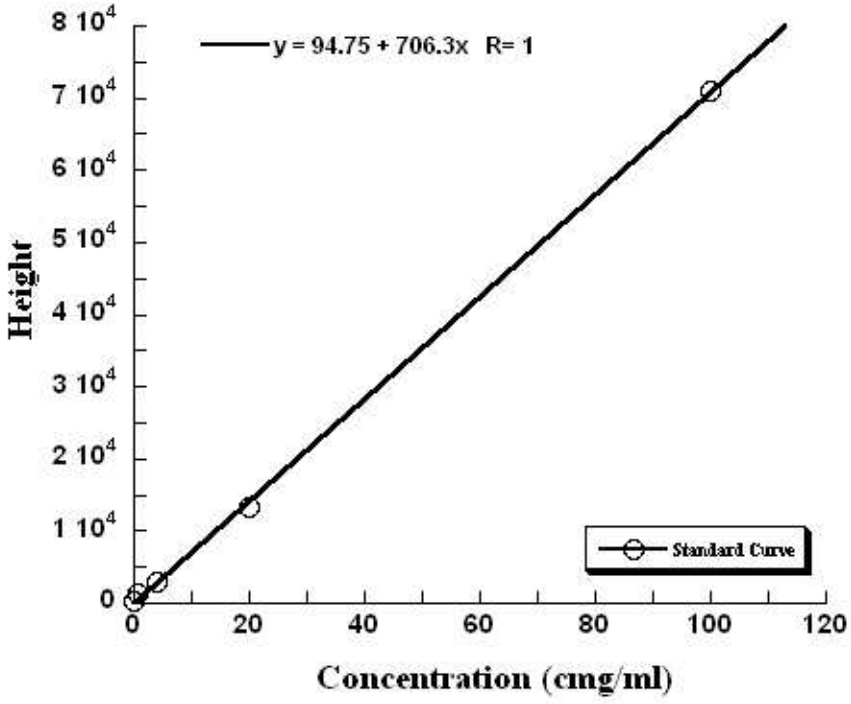


Figure 3.5. Standard curve of artemisinin in methanol:water 50:50% (v/v).

As for the analytical method developed at the University of Ferrara, linearity was confirmed as shown in Figure 3.6. This second method had also optimal linearity (correlation coefficient $R^2 = 0.9999$).

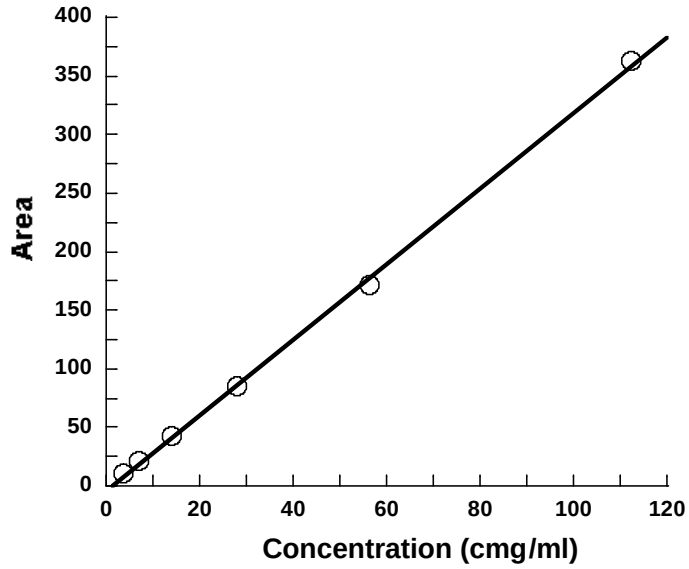


Figure 3.6. Standard curve of artemisinin in methanol:water 50:50% (v/v).

3.4.1.2. Production of complex between artemisinin and β-cyclodextrin

The artemisinin raw material presented a value of solubility in water very low due to its strong hydrophobic nature. The solubility value of ART in distilled water at room temperature (25-27 °C) was 44.7 μg/ml (n=3) after 24 hours. Similar values for ART solubility in water are reported in literature [63]. In order to improve its solubility the possibility to include the ART in the β-cyclodextrin was studied.

ART was complexed by the β-cyclodextrin through three different methods: slurry, kneading and spray drying. The amount of ART included in the complexes was determined by HPLC. In all cases, the theoretical drug loading was 0.2 mg of ART per mg of powder.

In the case of ARTβCDslu, the amount of loaded artemisinin ranged between 0.18 and 0.20 mg/mg microparticles for all batches, corresponding to 90-100% of the theoretical value. In the case of the ARTβCDknead, for both batches manufactured the value of artemisinin were 0.18 mg per mg of microparticles. The drug loading for the ART-βCD complex obtained spraying a solution was 0.21 ± 0.01 mg/mg sd microparticles and the

yield was 56%. The results of drug loading were in agreement with the expected theoretical values.

However, from this analysis it was not possible to determinate if the artemisinin was complexed by the β-cyclodextrin or if only a physical mixture of the two powders was obtained. In fact to establish the amount of ART in the product, the powder was dissolved in an organic solution. DSC and X-Ray analysis have been performed to confirm the complexation of the arteminisinin with the β-cyclodextrin.

3.4.1.3. Spray dried excipient microparticles

Excipient microparticles containing mannitol and lecithin in different ratio were manufactured. In Table 3.I the spray drying conditions were summarised. A first batch of mannitol and lecithin SD microparticles was produced at a ratio of 80:20, respectively. The yield of production was very low (20.2%), due to problems correlated with the evaporation of the hydroalcoholic solvent (10 %v/v ethanol in water) and, likely, to the excessive amount of sticky lecithin. Hence, the amount of lecithin was decreased and some parameters of the spray-drying process were changed: the solution feed was decreased from 7 to 5 ml/min whereas the inlet temperature was increased. Both batches of mannitol:lecithin SD microparticles, at ratio of 95:5 and 90:10 respectively, still showed low production yields. Nevertheless, the 90:10 mannitol/lecithin ratio was selected for the production of agglomerates with suitable mechanical strength. In previous studies it has been shown that lecithin acts as the binder needed for agglomerate formation [65].

Table 3.I. Experimental conditions of spray drying.

Mannitol:Lecithin Ratio	T inlet (°C)	T outlet (°C)	Pump (ml/min)	Aspiration	Yield (%)
80:20	90	56-58	7	100	20.2
90:10	98	58	7	50	21
95:5	98	58-60	5	50	15.7

However, in the case of the ratio 80:20, the microparticles were too sticky and prone to self-agglomerate in an uncontrolled manner. On the other hand, with the ratio 95:5 the

amount of lecithin in the microparticles was too little and led to the formation of very weak agglomerates. For this reason, the 90:10 mannitol/lecithin SD microparticles were used in further studies.

3.4.1.4. Particle Morphology

The SEM microphotographs of artemisinin, raw material and the complexes made by different techniques are shown in Figures 3.7a-h. ART (raw material) exhibited particles lacking of size uniformity; these particles were larger than ART-βCD complex ones and had different morphology (Figures 3.7a-b).

The pictures of ARTβCDslu complex showed irregular aggregated particles (Figures 3.7c-d). This phenomenon occurs naturally in powders, because of the adhesion forces that always exist between fine particles obtained by kneading. The ARTβCDknead complex presented also irregularly-shaped particles prone to aggregation, but smaller in size (Figures 3.7e-f). The drug particles were mechanically covered with a solid layer of β-CD during their co-grinding, thus some particles appeared like a granule [63, 66]. However, in both cases some crystalline particles (most probably those of artemisinin) were observed in the pictures, providing evidence that artemisinin remained not complexed. For the ARTβCDsd complex significant changes in particle size and shape were noticed (Figures 3.7g-h). The spray-dried particles were more homogeneous, some of which almost fused together. In fact, when the inner water phase is evaporated, the skin of particles is destroyed and the outer surface collapses [63, 67].

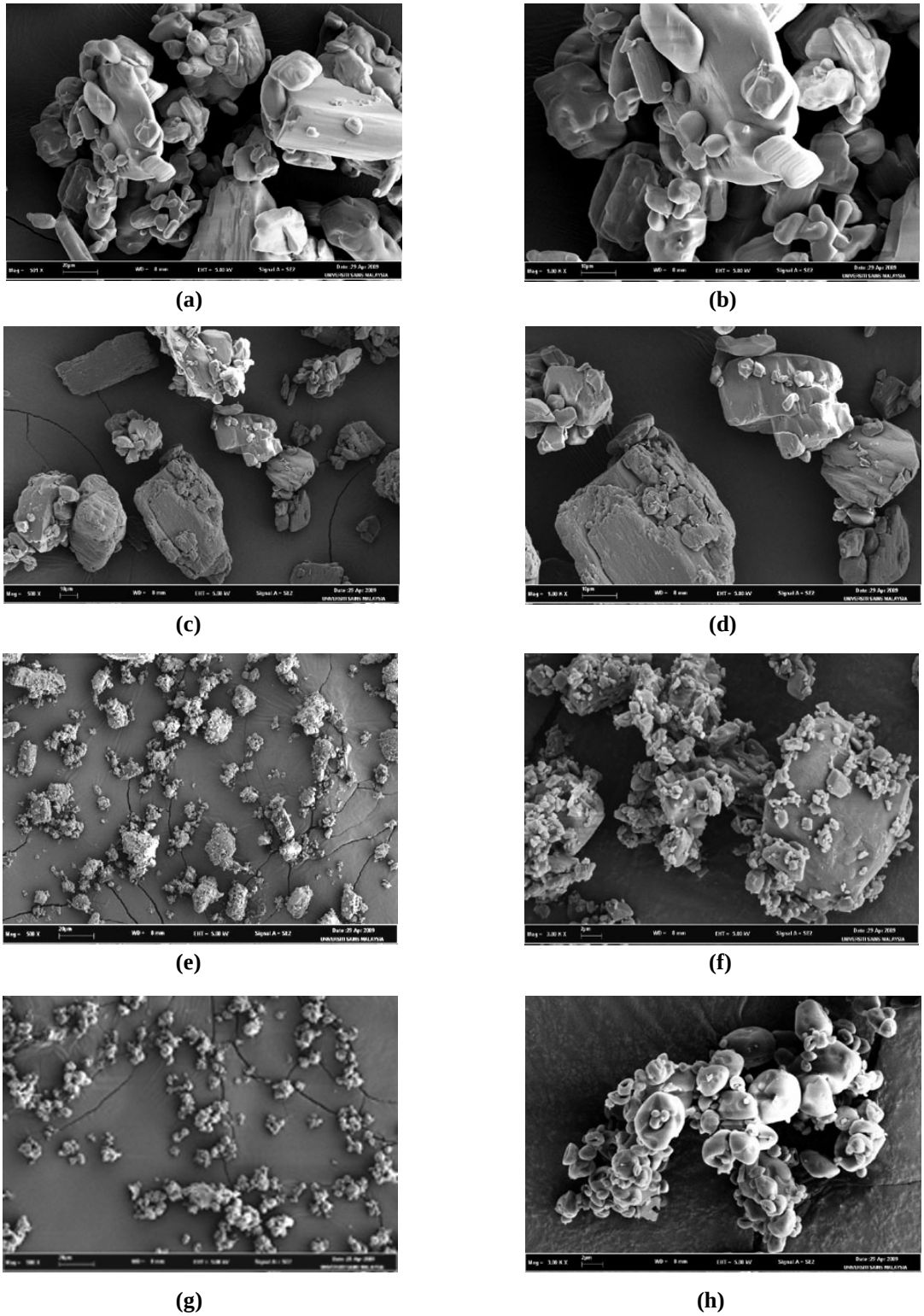


Figure 3.7. SEM pictures of artemisinin raw material ((a) magnification 500 x and (b) magnification 1000x), ARTβCDslu ((c) at 500x and (d) at 1000x)) , ARTβCDknead ((e)at 500x and (f) at 3000x), ARTβCDsd ((g) at 500x and (h) at 3000x).

3.4.1.5. Differential Scanning Calorimetry (DSC) Study

The thermograms of artemisinin raw material, β-cyclodextrin and the physical mixture thereof are shown in Figure 3.8. Artemisinin raw material showed a sharp melting peak at a temperature of 153.40 ± 0.09 °C (n=3), similar to that reported by Chan for the orthorhombic polymorphic form of the drug [68]. In fact, two different form of artemisinin crystals have been reported in the literature, namely triclinic and orthohombic forms [68]. The first form has a melting peak temperature of 157.3°C, while the second one melts at 154°C. An exothermic peak at approximately 180-200 °C was also observed upon further heating, which could be attributed to artemisinin degradation (Figure 3.8a).

The thermogram of β-cyclodextrin (Figure 3.8b) showed a single broad endothermic peak in the temperature range between 50-110 °C, which could be probably ascribed to dehydration or evaporation of water molecules from the cyclodextrin molecule surface or those residing within the cyclodextrin cavity [69].

The thermogram of the physical mixture was essentially superimposed to the DSC profiles of the individual components (artemisinin and β-cyclodextrin (Figure 3.8c)). No change in the melting temperature of artemisinin was recorded.

Table 3.II. DSC data of the examined samples.

Sample	T peak (°C)	ΔH_f (J/g)	Crystalline degree (%)
Artemisinin API	153.40±0.09	14.88±0.90	100
ART/β-CD (physical mixture)	153.14±0.21	14.73±0.50	98.9
ARTβCDknead	152.75±0.60	9.78±5.54	65.6
ARTβCDslu	152.52±0.08	13.28±0.22	89.0
ARTβCDsd	155.18±0.21	4.55±0.06	30.6

The Figure 3.9 reports the DSC thermograms of the ART-βCD complexes made by slurry, kneading and spray-drying methods. The artemisinin melting point in the complexes was substantially unchanged with respect to the value observed for artemisinin raw material (Table 3.II). The DSC thermograms of the complexes were

similar to that of the physical mixture, but were accompanied by a reduction of the $\Delta H_{f,}$ of artemisinin, suggesting that the complexation between β-cyclodextrin and artemisinin was partial (Table 3.II). In particular it was envisaged that not all the artemisinin molecules were involved in the complexation process with β-CD. In fact, the smaller endothermic peak observed was due to the fraction of non-complexed artemisinin. Such incomplete complexation has also been reported by other worker with drugs such as nimesulide [70].

However, referring to Figure 3.9, it is evident that the degree of complexation was also different depending on the production method used, as evidenced by the differences in the ΔH_f reduction for artemisinin. The percentage of artemisinin complexed by β-cyclodextrin for each preparation method was calculated comparing the thermograms of the complexes with that of artemisinin raw material after normalization for the sample weight. This was based on the assumption that the area under the melting peak of artemisinin was directly proportional to the amount of artemisinin in the sample, and that the artemisinin peak observed in the thermograms from the inclusion complexes was due to the free drug (i.e. not complexed).

The spray-dried complex showed approximately 70% of ΔH_f reduction, suggesting that this percentage of artemisinin was complexed (Table 3.II). For the complexes made by slurry method and kneading method the values of complexation were about 11% and 34%, respectively. Therefore, the spray-drying technique resulted the most efficient method for the manufacturing of ART-β-CD complex probably due to the rapid solvent evaporation, which could improve the complex formation.

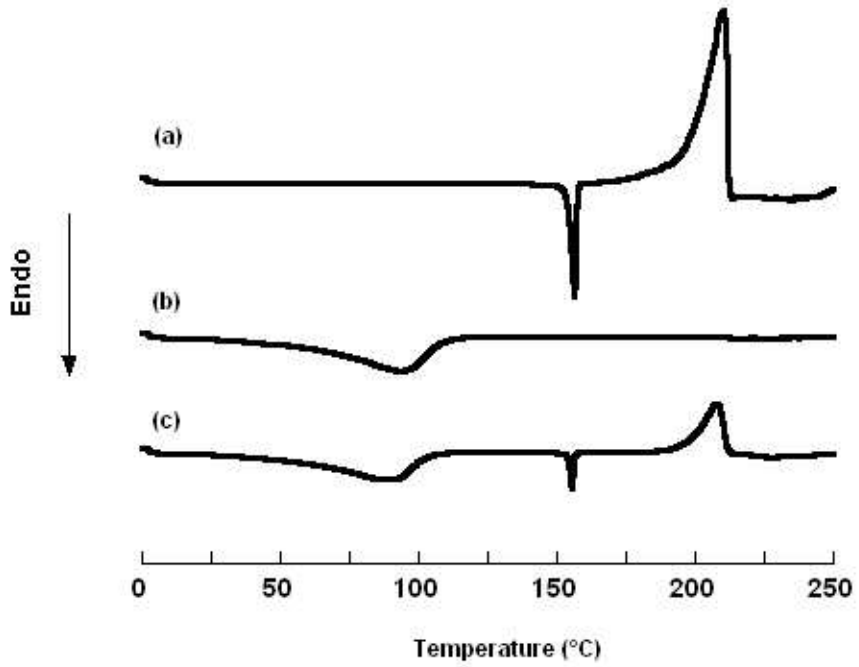


Figure 3.8. DSC thermograms of (a) artemisinin raw material, (b) β-cyclodextrin, (c) physical mixture of ART- βCD.

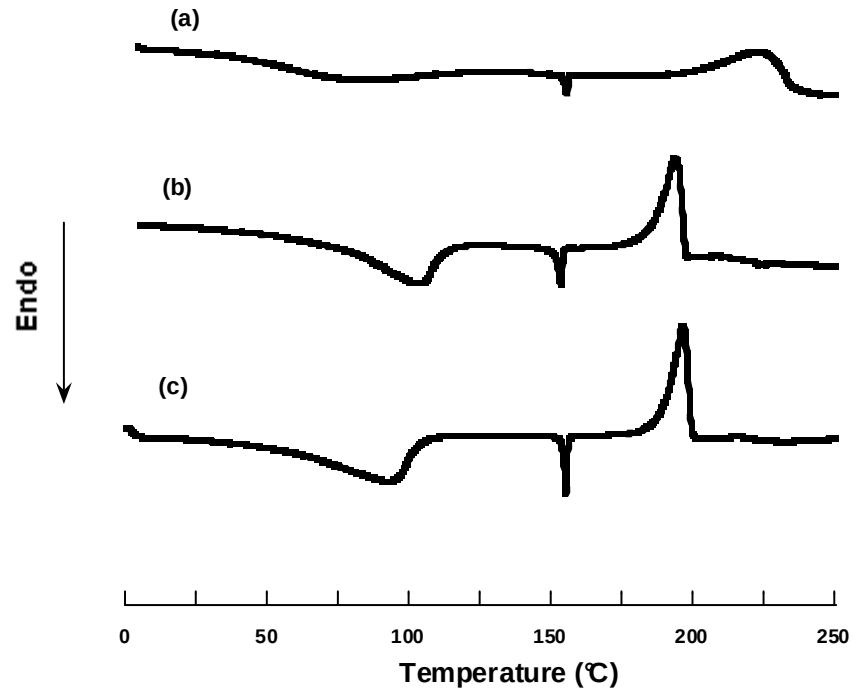


Figure 3.9. DSC thermograms of ART- βCD complexes:(a) ARTβCDsd; (b) ARTβCDslu and (c) ARTβCDknead.

3.4.1.6. PXRD Analysis

The representative X-ray diffraction patterns of ART raw material, of β-cyclodextrin, of the physical mixture and of the complexes made by the different methods were shown in Figures 3.10 and 3.11. The diffractograms indicated changes in the drug crystal structure when the drug was complexed by βCD. The X-ray patterns of ART raw material presented several distinct peaks at 2θ angle of 7.3, 11.79, 14.69, 15.64, 16.67, 18.24, 20.01 and 22.18, all suggesting the crystalline form of the drug. In the case of the ART–βCD physical mixture, all the peaks related to ART were present, even if at lower intensity because the amount of ART in the sample was lower. No new peaks were observed, suggesting the absence of interaction between the drug and the β-CD in the physical mixture. In comparison with the physical mixture, the ARTβCDsd complex showed a similar diffraction pattern (Figure 3.11), but significant lower peak intensities. This confirmed the DSC results, i.e. that in the complex ART was mostly included inside the cyclodextrin cavity and only the non complexed fraction was visible. The complexes obtained by slurry and kneading methods had similar profiles and not significantly different from that of the physical mixture.

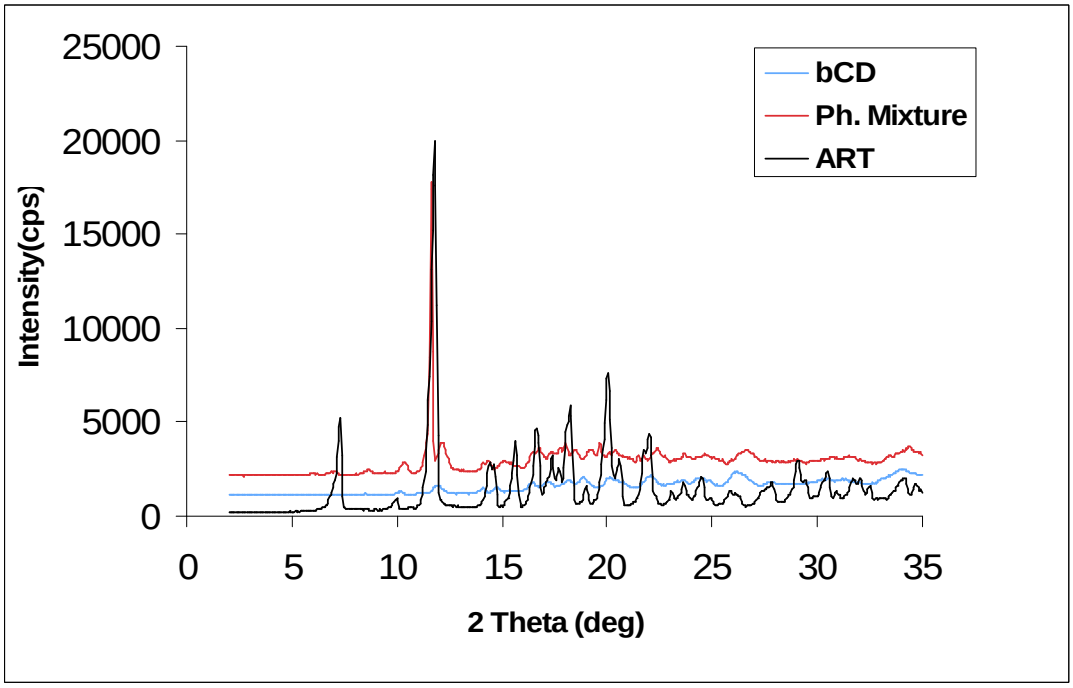


Figure 3.10. X-ray diffraction profiles: (black) artemisinin raw material, (blue) β-cyclodextrin, (red) ART-βCD physical mixture.

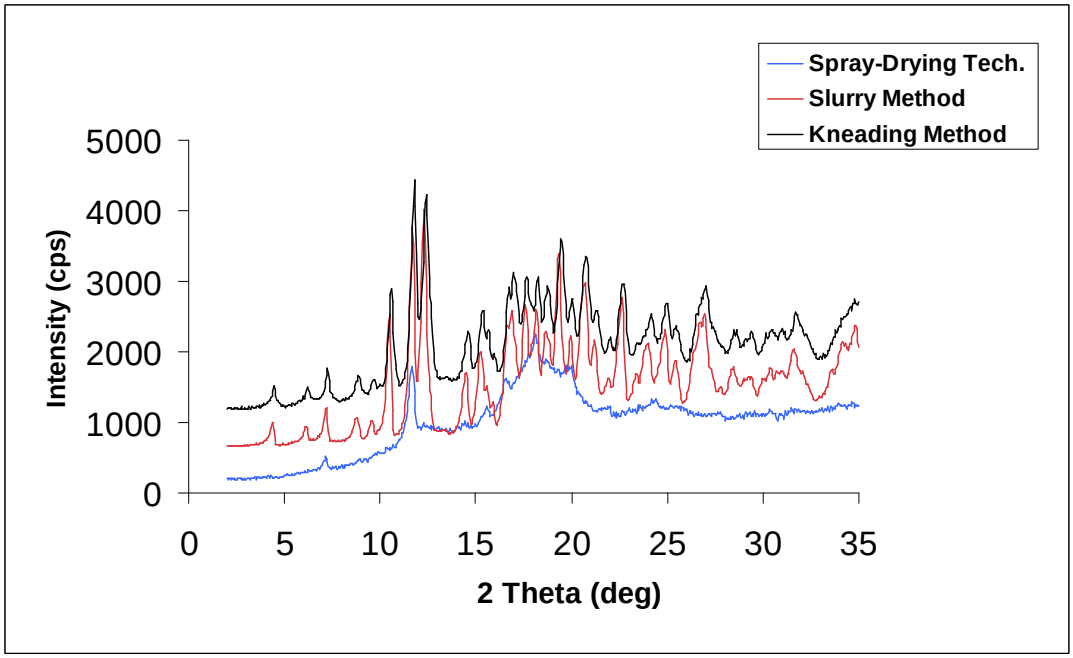


Figure 3.11. X-ray diffraction of ART-βCD profiles prepared through different method: (blue) spray drying technique, (red) slurry method and (black) kneading method.

3.4.1.7. FTIR Analysis

The FTIR spectra of artemisinin raw material and β-cyclodextrin are shown in Figure 3.12. The spectrum of artemisinin raw material showed an absorption peak at 1736 cm^{-1}, corresponding to the stretching vibrations of the δ-lactone carbonyl (C=O) mode (Figure 3.12a). From 1200 to 800 cm^{-1} there were absorption peaks due to the peroxide, O-O and C-O modes of the O-O-C unit [20]. Moreover, ART exhibited a sharp peak typical of C-H stretching vibrations at 2950 cm^{-1}.

The spectrum of β-cyclodextrin showed a very broad band at 3379 cm^{-1}, which was attributed to the presence of C–OH stretching and a broad peak at 2917 cm^{-1} referred to the C-H stretching vibrations (Figure 3.12b). In the spectrum of the ART-βCD physical mixture (Figure 3.12c) no differences in the position of the absorption peaks were observed. The ART–βCD complexes did not show any new peak in the range between from 2500 to 500 cm^{-1}, indicating that no covalent bonds were created in the complexes. The characteristic peaks of C-H stretching of artemisinin appeared also in the physical mixture and in the complexes made by kneading and slurry methods, without variation in the wavenumber position with respect to the pure drug. The absence of shifts in the wavenumber of the FTIR peaks of the composites indicated the lack of significant interaction between the artemisinin and the β-cyclodextrin in the complexes made by slurry and kneading methods (Figure 3.13b-c). However, in the ARTβCDsd complex the peak of C-H stretching of artemisinin was shifted from 2950 to 2980 cm^{-1}, suggesting the formation of hydrogen bonds between artemisinin and β-cyclodextrin (Figure 3.13d) [71].

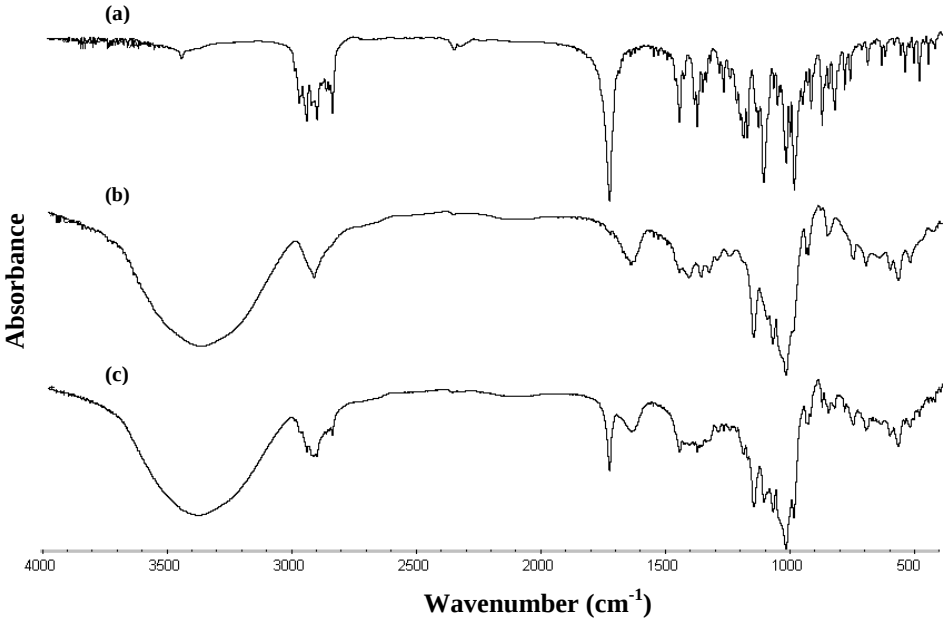


Figure 3.12. FTIR spectra of (a) artemisinin raw material, (b) β-cyclodextrin (c) and physical mixture between artemisinin and β-cyclodextrin.

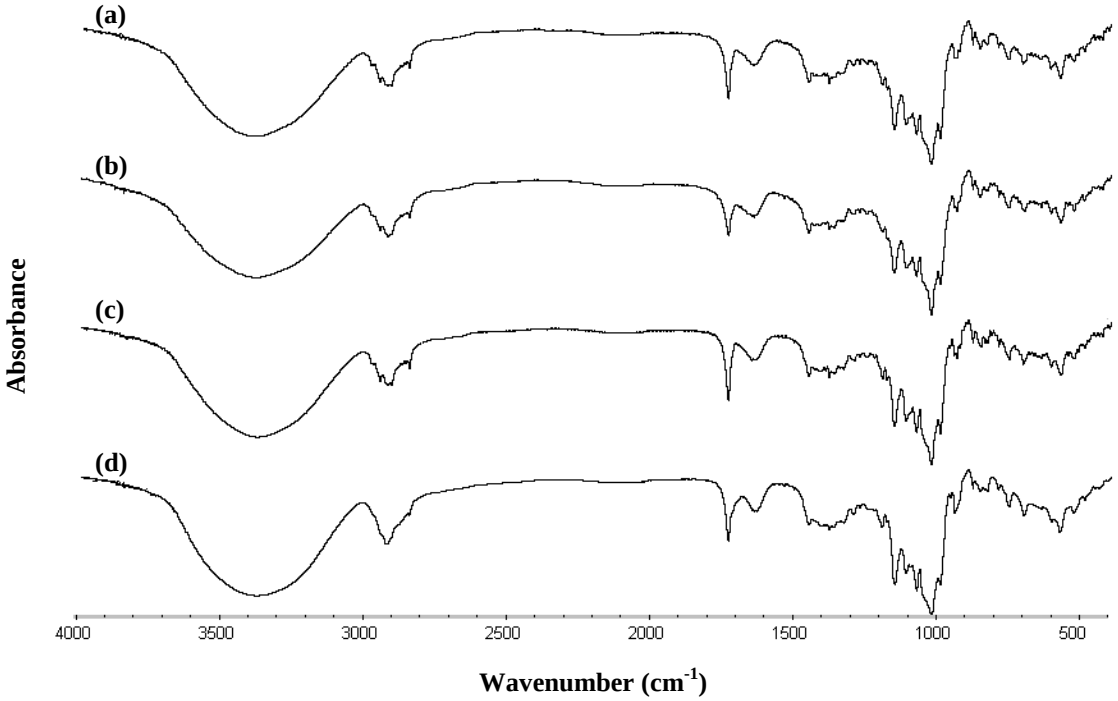


Figure 3.13. FTIR spectra of (a) physical mixture of artemisinin and β-cyclodextrin and complexes: (b) ARTβCDslu; (c) ARTβCDknead and (d) ARTβCDsd.

3.4.1.8. Solubility of artemisinin and ARTβCDsd complex

Table 3.III summarizes the solubility values of artemisinin API and artemisinin in the ARTβCDsd in distilled water (the value is the average of three determinations).

Table 3.III. Values of solubility of the samples at different times at room temperature (25-27 °C).

Time / Sample	4 hours	8 hours	24 hours
Artemisinin API	40.2 μg/ml	39.3 μg/ml	44.7 μg/ml
ARTβCDsd	223.5 μg/ml	207.7 μg /ml	246.6 μg/ml

It is possible to notice that the values of ART solubility were very low in water due to its strong hydrophobic nature. Similar values for artemisinin solubility in water are reported in the literature [63]. The saturation solubility of artemisinin in the complexed form was found to increase approximately six-fold the solubility of the raw material.

3.4.1.9. Dissolution Test

In vitro dissolution is an useful tool that can provide valuable information about the bioavailability of a drug product. One of the most important requirement for properly carrying out in vitro dissolution test is the maintenance of sink conditions throughout the entire experiment duration. Sink conditions means that the drug concentration in the dissolution medium must not exceed a maximum value of 10 - 20% of the drug solubility. For this reason drugs that are slightly and very slightly soluble give rise to difficulties, particularly if they are used at high doses. Several studies reported different ways for maintaining sink conditions, like the use of large fixed fluid volume [64], multiple phase (partition method) [71, 72] and continuous flow (through flow cell).

Assuming the uniformity of loaded artemisinin in the complex, the amount of product dissolved in vitro was about 6 mg of artemisinin. The dissolution medium volume used

was 900 ml. In this condition complete dissolution of artemisinin would result in a concentration equivalent to about 15-16% of ART solubility. The choice of the medium was also determinant to obtain complete release of the drug. In published paper the solvents selected for artemisinin dissolution in vitro were different [64, 71, 73].

The dissolution tests conducted at USM gave no satisfactory results and this despite having tried different dissolution media such as simulated gastric fluid (SGF), water, 90:10 water:ethanol, 90:10 water:ethanol with different percentages of SLS added (0.01 and 0.1% w/v). Complete release of artemisinin was never attained. Initially, it was supposed that this could be due to a solubility problem, even if the test was conducted in sink conditions. Then, it was focused on the fact that the analytical method used at USM required the derivatization of the sample prior to analysis. Probably the derivatization step was not much reproducible from sample to sample, thus leading to highly variable and unpredictable data.

In contrast, with the analytical method developed at the University of Ferrara it was possible to directly analyze the samples with no pre-treatment, eliminating a certain number of variables of the analytical process. First choice of the medium was the SGF at pH 1.2, as it simulates the human ambient of the stomach. However, artemisinin degradation was observed in acidic environment (data not shown). Then, according to the work published by Van Nijlen, a 90:10 water:ethanol solution was used as dissolution medium [73]. In this case, the drug reached the 100% release. Figure 3.14 showed the dissolution profiles of the different ART–βCD complexes in this medium. It was possible to observe that the complex obtained by spray-drying technique released the drug more slowly due to relatively good level of complexation between ART and β-cyclodextrin. This effect could possibly be attributed to the hydrophilic character of the cyclodextrin, which can reduce both hydrophobicity of artemisinin as well as the interfacial tension between artemisinin and the dissolution medium. The dissolution profiles of the complexes made by slurry and kneading methods showed an immediately release of the artemisinin. This proves that in these products most of the artemisinin was not complexed, but simply mixed with β-cyclodextrin.

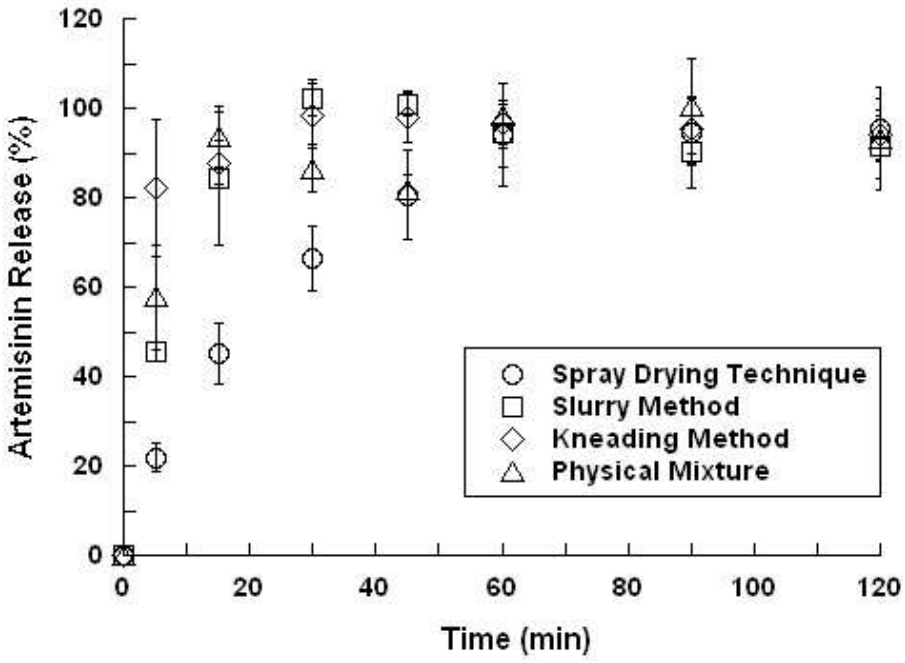


Figure 3.14. Dissolution profile of ART-βCD complexes and physical mixture: (○)ARTβCDsd; (□)ARTβCDslu; (◇)ARTβCDknead and (Δ) physical mixture. Release was into 900 ml of a mixture water: ethanol 90:10 (v/v) for a period of 2 hours at the temperature of 37 °C. Each value is the mean ± standard deviation (n=3).

3.4.1.10. Artemisinin agglomerates manufacturing

The agglomerates between the ART-βCD complex obtained through three different methods (kneading, slurry and spray-drying) and SD excipient microparticles were produced.

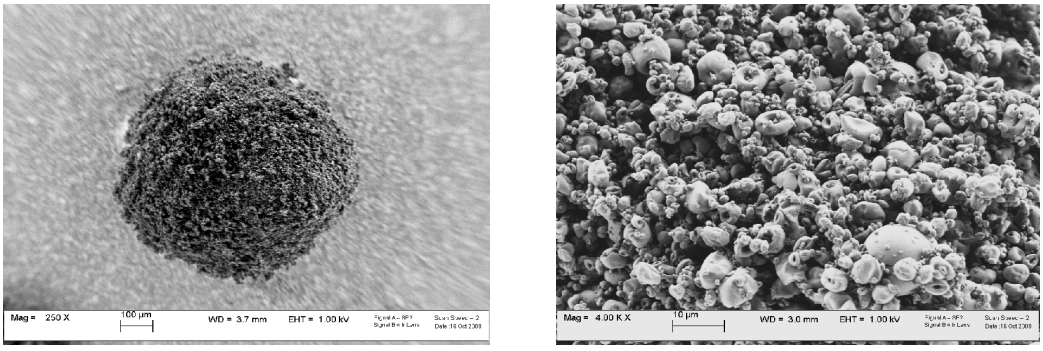
Different ratios of the two particle populations were agglomerated, i.e. (1:1) and (1:2) of ART-βCD complex and SD excipient microparticles, respectively. After initial mixing, the powder mixture underwent sieve vibration as described in the experimental section. In all cases, it was possible to obtain agglomerates with high yields from all the ART-βCD complexes (Table 3.IV).

Table 3.IV. Production yields (%) for agglomerates between the ART-βCD complex and the SD excipient microparticles.

Ratio ART-βCD complex/ sd excip.microp.	Agglomerates (ARTβCDslu)	Agglomerates (ARTβCDknead)	Agglomerates (ARTβCDsd)
1:2	78.7%	67.3%	87.3%
1:1	-	-	92.7%

However, the experimental drug loading of artemisinin determined for the agglomerates was about 120% with respect to the theoretical value. This was probably due to the interference of the excipient microparticles with the artemisinin during the pre-treatment of the samples for the HPLC analysis.

Then, agglomeration by sieve vibration of the ARTβCDsd complex was carried out in absence of excipient microparticles. The yield (%) of the process was very high, 98.1 ± 0.9 (n=3). SEM images of the agglomerates and its surface are shown in the Figure 3.15. The shape of the agglomerate was round (Figure 3.15a) and the ARTβCDsd complex could be easily detected on the surface (Figure 3.15b).



(a).... **(b)**

Figure 3.15. SEM pictures of agglomerates of ART-βCDsd complex ((a) at 250x and (b) at 4000x).

Even if the agglomerates appeared less resistant because of the absence of the excipient microparticles, i.e. of lecithin, in order to reduce the quantity of excipients to administer to the patients the agglomerates were made using only ART-βCDsd. This aspect could be important since for the malaria combination therapy artemisinin agglomerates should have to be administered together with an other drug formulation made of clindamycin

agglomerates. These two drugs are not very potent, so, without considering the excipients, the bulk volume of the final powder to be administered is already quite high for an oral formulation. It also true that in this case the final product would be different from a big tablet, due to the peculiar nature of the agglomerates. In fact, they are soft clusters of microparticles linked by weak bond and when they enter in contact with a liquid they promptly release the microparticles, then reducing the problem related to the high bulk volume.

3.4.2. CLINDAMYCIN

3.4.2.1. Validation of HPLC clindamycin method

Clindamycin was quantified by HPLC (method described in the experimental section). For the method developed at USM, linearity was assessed. As shown in Figure 3.16, the linearity was demonstrated in the concentration range between 0.0001 and 0.062 mg/ml, as proven by the maximum correlation coefficient obtained (R^2=1).

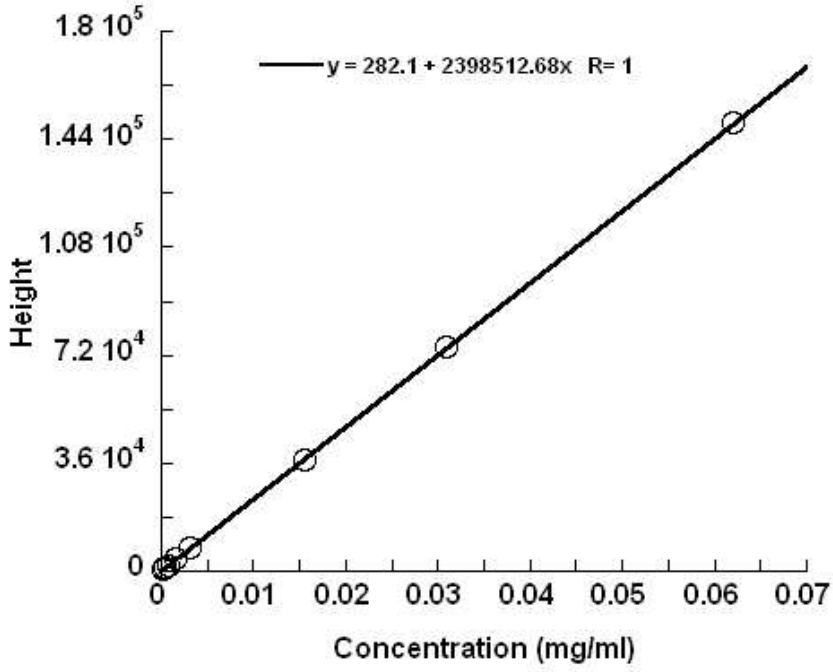


Figure 3.16. Standard curve of clindamycin dissolved in water.

3.4.2.2. Production of clindamycin spray-dried microparticles

Different batches of clindamycin microparticles containing mannitol and lecithin as excipients were manufactured by a spray drying technique. In the first batch the amount of clindamycin was about 20% in weight of the total powder mass. Then, considering the high dosage at which the drug is administered, a second batch with higher drug loading (33%) was manufactured (Table 3.V). The parameters of the processes are reported in the Table 3.VI.

Production yields at the end of the spray-drying process were quite low: 27.7% for the first batch (C20M72L8.SD) and 21.9% for the second one (C33M64L11.SD). Some batches of SD excipient microparticles composed of mannitol:lecithin at a ratio 90:10 were also manufactured. For these batches, production yields were quite low, around 20-21%. A possible cause was that some particles remained stuck inside the evaporation chamber of the spray dryer, probably because the temperature of drying air was not enough high for optimal evaporation.

Table 3.V. Composition (%) of the clindamycin and SD excipient microparticles.

CODE	C20M72L8.SD	C33M64L11.SD	M90L10.SD
Clindamycin	20	33	-
Mannitol	72	60	90
Lecithin	8	11	10

Table 3.VI. Spray drying process parameters for clindamycin microparticles.

Code / Parameter	C20M72L8.SD	C33M64L11.SD	M90L10.SD
T inlet (°C)	98	98	98
T outlet (°C)	59	59	58
Aspiration (%)	50	50	50
Pump (ml/min)	6	7	7

3.4.2.3. Production of clindamycin agglomerates

The SD microparticles of clindamycin, mannitol and lecithin (code C20M72L8.SD and C33M64L11.SD) were used to produce agglomerates by sieve vibration. However, the powders did not agglomerate, due to possible interactions between the clindamycin and the excipients during the spray-drying process. In the first case no agglomerates were produced, while in the second case the final yield was only 4.7%.

Thus, it was decided to produce agglomerates using two populations of microparticles, such as clindamycin raw material and SD excipient microparticles of mannitol and lecithin (90:10). Different ratios between clindamycin and excipient microparticles were tested. At first, two parts of SD excipient microparticles and one part of clindamycin

raw material were mixed and then agglomerated. The yield (%) obtained was very high (Table 3.VII). Therefore, in order to not decrease the amount of clindamycin loaded in the final formulation, the 1:1 ratio between clindamycin and SD excipient microparticles was chosen. The two populations were mixed and agglomerated. Also in this case the yield was high (75.5%), so the latter formulation was selected for further studies. A bigger batch of agglomerates of clindamycin and excipient microparticles mannitol:lecithin (90:10) was produced. In this case, the yield after mixing of the two powders was 96% and the one after vibration 82.1% with a drug loading of 47.8 ± 0.9%.

Table 3.VII. Production yield (%) after powder mixing and agglomeration processes and clindamycin drug loading (%).

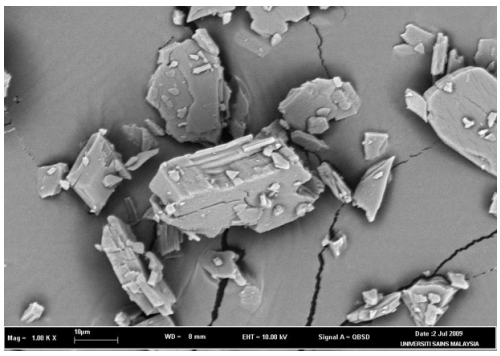
Agglomerate code	Yield after mixing (%)	Yield after vibration (%)	Theoretical Drug loading (%)	Drug loading (%)
C20M72L8.SD	-	-	20	
C33M64L11.SD	-	4.7	33	
Clind.1.M90L10.2	91.4	88.0	33	32.8±0.2
Clind.1.M90L10.1 (batch#1)	95.0	75.5	50	50.3±0.1
Clind.1.M90L10.1 (batch#2)	96.0	82.1	50	47.8±0.1

3.4.2.4. Dissolution Test

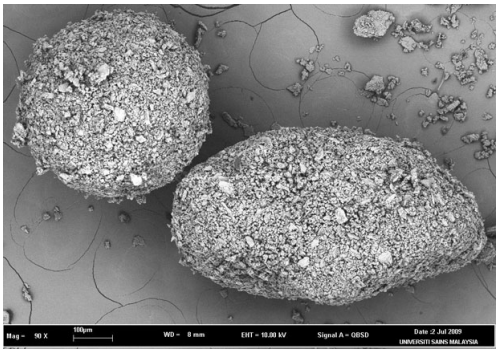
The dissolution test of the agglomerates composed of clindamycin and SD excipient microparticles was conducted in SGF for one hour. The drug was immediately released in less then 5 minutes. In fact, clindamycin is very soluble in water and the presence of the excipient microparticles did not affect drug release.

3.4.2.5. SEM analysis

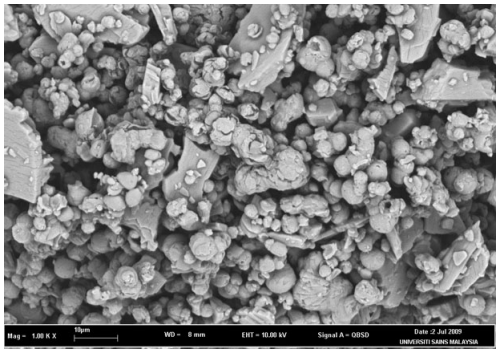
The SEM images of clindamycin raw material and agglomerates of clindamycin and SD excipient microparticles are shown in Figure 3.17. The clindamycin particles presented irregular and flake shape (Figure 3.17a). On the other hand, the agglomerates showed round shape with a rough surface (Figure 3.17b). Looking in detail at this surface (Figure 3.17c) small excipient microparticles were evident surrounding larger clindamycin particles, without visible bridges between them. The agglomerates obtained by vibration showed this particular arrangement of the small and larger particles. The same phenomenon was observed in a work published by Raffin [65] in which pantoprazole gastroresistant microparticles were agglomerated with the same type of excipient microparticles.



(a)



(b)



(c)

Figure 3.17. SEM pictures of clindamycin raw material ((a) magnification 1000x), (b) agglomerates of clindamycin and SD excipient microparticles 1:1 (magnification 50x) and (c) detail of the surface of agglomerates (magnification 1000x)).

3.4.2.6. DSC Studies

The thermogram of clindamycin raw material is shown in Figure 3.18. It presents a broad endothermic peak in the temperature range of 70-100°C, which could be ascribed to dehydration or evaporation of water molecules. The drug also showed a small melting endothermic peak at the temperature of 208.36 ± 0.02°C followed by decomposition.

The thermograms of mannitol, lecithin and mannitol:lecithin (90:10) SD microparticles are shown in Figure 3.19. Lecithin did not show any endothermic peak (Figure 3.19a), whereas mannitol exhibited a sharp melting endothermic peak at a temperature of 168.70 ± 0.03°C (Figure 3.19b). Similarly, the excipient microparticles showed a sharp melting endothermic peak at a temperature of 168.25 ± 0.23°C (Figure 3.19c), corresponding to mannitol fusion.

In Figure 3.20 the thermograms of agglomerates composed of clindamycin and SD excipient microparticles are compared with those of clindamycin raw material and excipient microparticles alone. In the case of the agglomerates where the ratio between clindamycin and SD excipient microparticles was 1:1, it was possible to observe only the endothermic peak of mannitol at the temperature of 166.29 ± 0.24°C. Clindamycin peak was not present, so in order to understand whether the problem was associated to the scan rate, the samples were subjected to the same temperature scan, but at different scan rates (2 °C/min and 20 °C/min). In both cases, clindamycin peak was never detected (data not shown). In order to verify whether the absence of the clindamycin peak was due to an interaction of the drug with the excipients, DSC analysis of the physical mixtures of clindamycin-lecithin (Figure 3.21) and of clindamycin-mannitol (Figure 3.22), in the same ratio used for the agglomerates, were carried out. In the DSC curve of lecithin-clindamycin physical mixture, the clindamycin peak was observed at the temperature of 207°C, followed by an exothermic peak, due to drug degradation. In the case of the clindamycin-mannitol physical mixture, a small peak concerning the melting point of clindamycin was noticed at 195°C followed by the degradation process at about 207°C (Figure 3.22). Even if the amounts of the two powders in the sample were similar (in fact the ratio between mannitol and clindamycin was 46:50 as in the agglomerate formulation) it is possible that the melted mannitol shifted the melting

point of the clindamycin. In order to confirm this hypothesis, it was necessary to use another solid-state analysis such as PXRD for a correct interpretation of the results obtained by DSC.

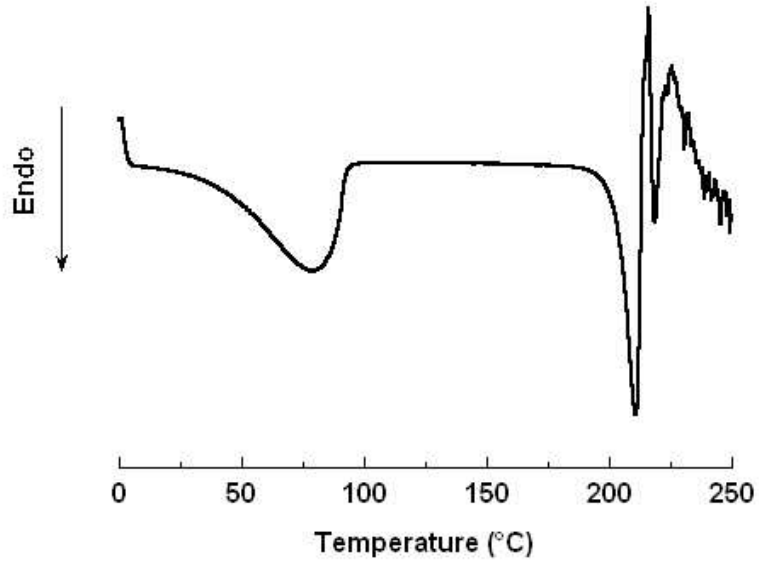


Figure 3.18. DSC thermogram of clindamycin raw material.

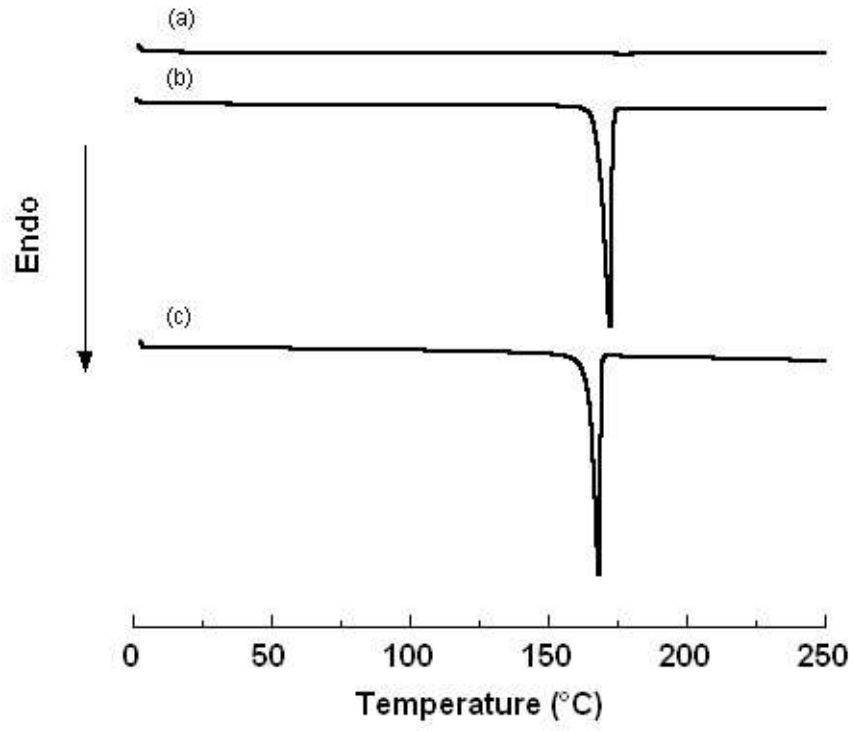


Figure 3.19. DSC thermograms of (a) lecithin, (b) mannitol and (c) mannitol: lecithin (90:10) SD microparticles.

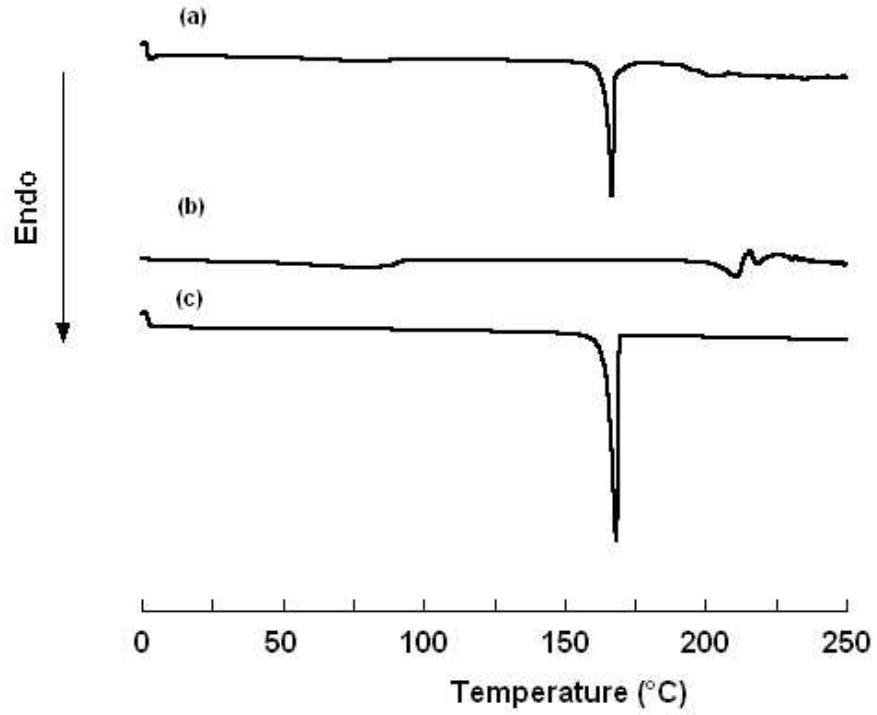


Figure 3.20. DSC thermograms of (a) agglomerates of clindamycin and SD excipient microparticles, (b) clindamycin raw material and (c) SD excipient microparticles.

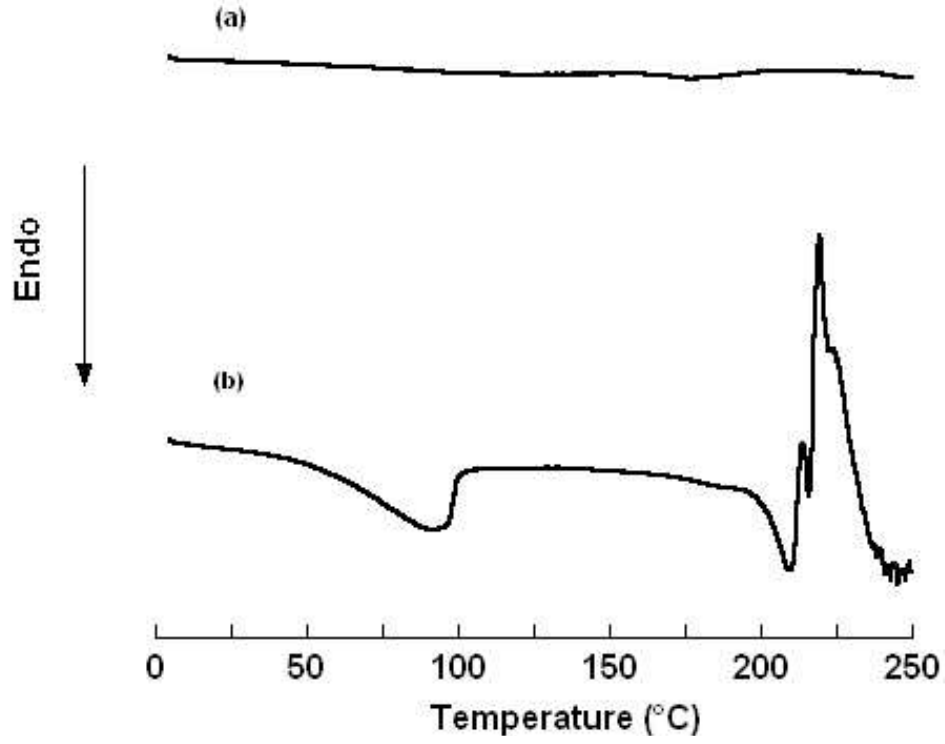


Figure 3.21. DSC thermograms of (a) lecithin and (b) physical mixture of clindamycin and lecithin.

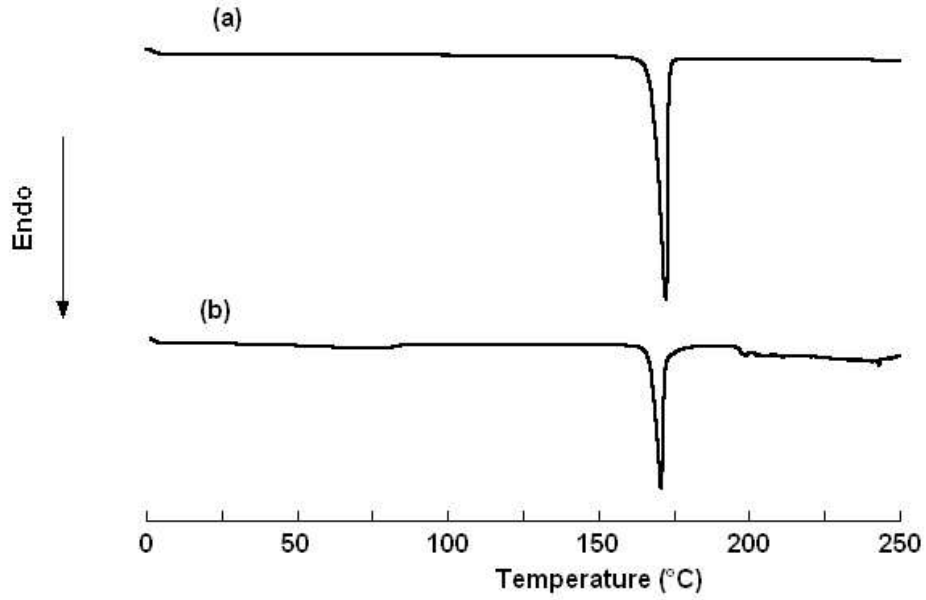


Figure 3.22. DSC thermograms of (a) mannitol and (b) physical mixture of clindamycin and mannitol.

3.4.2.7. PXRD Analysis

The X-ray diffraction patterns of clindamycin, SD excipient microparticles and agglomerates made of clindamycin raw material and excipient microparticles are shown in Figure 3.23.

The X-ray patterns of clindamycin displayed the presence of numerous distinct peaks at 2θ angle of 5.70, 11.55, 15.20, 16.90, 19.60, 22.40 and 40.00, suggesting that the drug was in the crystalline form. The agglomerates showed a similar diffraction pattern, but lower peak intensities, suggesting the clindamycin keeps its crystalline form. No new peaks were observed, suggesting the absence of interaction between the drug and the excipient microparticles confirmed the DSC results.

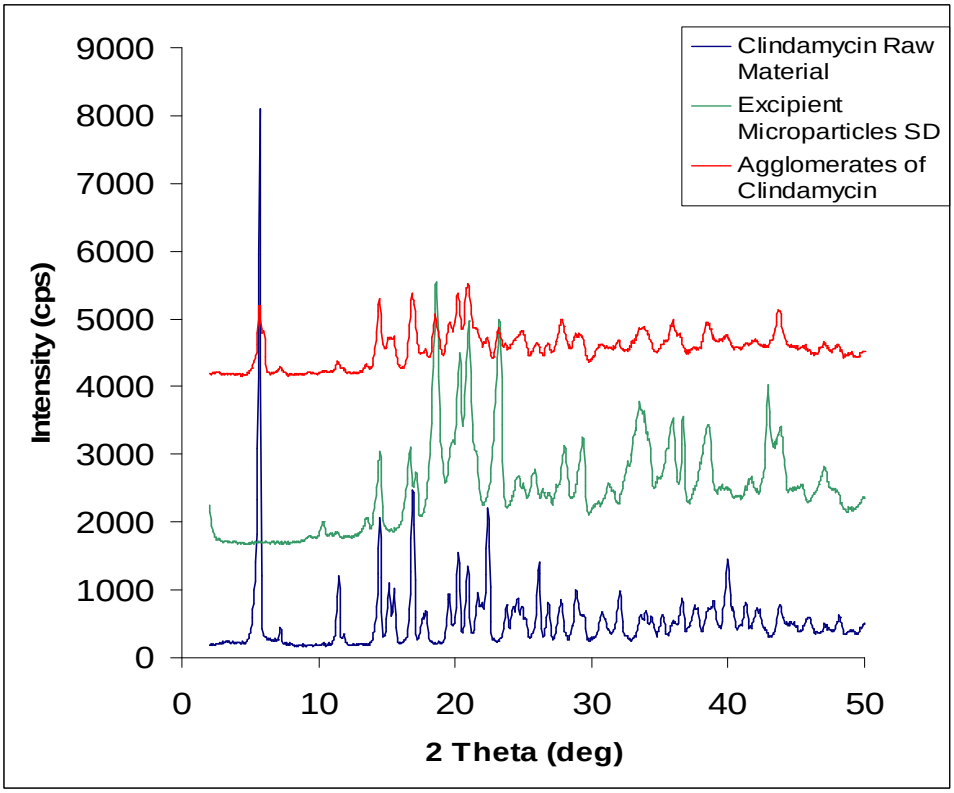


Figure 3.23. X-ray diffraction profiles: (blue) clindamycin, (green) sd mannitol-lecithin (90:10) microparticles, (red) agglomerates of clindamycin and SD excipient microparticles.

3.5. CONCLUSIONS

The ART-β-CD complex could be prepared by different methods: slurry, kneading and spray dried technique. From the characterization data collected, it was noticed that in the case of the slurry and kneading methods, the complexation degree was not satisfactory. In fact, the complexation degree was only 11% and 35% in the case of ARTβCDslu and ARTβCDknead, respectively. On the other hand, by spray-drying a solution of drug and β-CD a complexation degree of 70% was obtained. Agglomerates from mixture of complexes and excipient microparticles were prepared. Since an inteference of mannitol-lecithin microparticles with the artemisinin during the HPLC analyses was found out, it was decided to prepare soft agglomerates of only ARTβCDsd. These agglomerates were less resistance due to the absence of the binder, as lecithin, but there was the advantage that the drug loading was not reduced by the presence of high amount of excipients.

Moreover, microparticles containing clindamycin were manufactured by spray-drying and then characterized. These microparticles were not able to agglomerate per se, so the agglomerates using clindamycin raw material and mannitol-lecithin 90:10 SD excipient microparticles were manufactured. The drug was immediately released in simulated gastric fluid, so the excipient microparticles did not affect the release. The DSC analyses and the X-ray studies showed no interactions between the drug and the excipients.

In view of administering together the two agglomerates, in vivo (in animals) study of ARTβCDsd agglomerates and clindamycin and excipient microparticles agglomerates will be performed.

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CHAPTER 4

SODIUM LEVOTHYROXINE AGGLOMERATES AS BUCCAL DELIVERY SYSTEM

4.1. INTRODUCTION

4.1.1. Normal Thyroid Gland Function

Thyroxine (3,5,3',5'-tetraiodothyronine, abbreviated as T_4) and 3,5,3'-triiodothyronine (T_3) are two fundamentally thyroid hormones very important for a normal growth and development secreted by the follicular cells of the thyroid gland. They are synthesized via the iodination and covalent bonding of the phenyl portions of tyrosine residues found in an initial peptide, thyroglobulin, which is secreted into thyroid granules. These iodinated diphenyl compounds are cleaved from their peptide backbone upon being stimulated by thyroid stimulating hormone.

Both T_4 and T_3 are transported in blood, in particular 90-95% of the secreted T_4 is protein bound, principally to thyroxine-binding globulin (TBG), and, to a lesser extent, to thyroxine- binding prealbumin (TBPA) and serum albumin (TBA). T_4 is involved in controlling the rate of metabolic processes in the body and influencing physical development [1].

T_4 is considered a prohormone and a reservoir for the active thyroid hormone T_3 which is about four times more potent. T_4 is converted in the tissues by deiodinases, including thyroid hormone iodine peroxidase (TPO), to T_3.

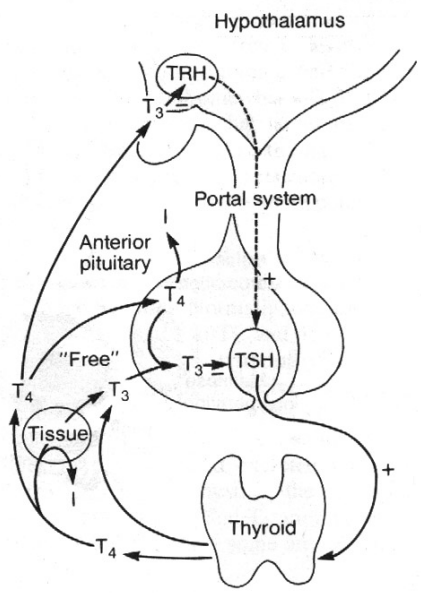


Figure 4.1. Schematic representation of the thyroid hypothalamic- pituitary- thyroid axis [2].

The thyroid hormones synthesis and secretion were regulated by the hypothalamic-pituitary-thyorid axis (Figure 4.1). Briefly, myriad neural inputs influence the hypothalamic secretion of the thyrotropin-realising hormone (TRH). TRH stimulates release of thyrotropin (TSH, thyroid-stimulating hormone) from the anterior pituitary. The TSH stimulates the synthesis and release of the thyroid hormones T_3 and T_4, which both feed back to inhibit the synthesis and release of TRH and TSH. Moreover, somatostatin, dopamine and high concentrations of glucocorticoid can inhibit the TRH action [1].

Thyroid hormones regulate multiple metabolic processes and play an essential role in the normal growth, development, normal maturation of the central nervous system and bone. The metabolic action of thyroid hormones include augmentation of cellular respiration and thermogenesis, as well as metabolism of proteins, carbohydrates and lipids. The protein anabolic effects of thyroid hormones are essential to normal development.

4.1.2. Dysfunction and effects of thyroid hormone on target tissue

Normal thyroid function requires obviously an adequate intake of iodine, if there is a deficiency of dietary iodine, the thyroid will not be able to make thyroid hormone. The lack of thyroid hormone will lead to decreased negative feedback on the pituitary, leading to increased production of TSH which causes the thyroid to enlarge endemic colloid goiter. This has the effect of increasing the thyroid's ability to trap more iodide, compensating for the iodine deficiency and allowing it to produce adequate amounts of thyroid hormone. An other dysfunction is hypothyroidism that generally derivates by two causes. The first is a result of previous (or currently ongoing) inflammation of the thyroid gland, which leaves a large percentage of the cells of the thyroid damaged (or dead) and incapable of producing sufficient hormone. The most common cause of thyroid gland failure is called autoimmune thyroiditis (also called Hashimoto's thyroiditis), a form of thyroid inflammation caused by the patient's own immune system.

The second major cause is the broad category of "medical treatments." The treatment of many thyroid conditions warrants surgical removal of a portion or all of the thyroid gland. If the total mass of thyroid producing cells left within the body are not enough to meet the needs of the body, the patient will develop hypothyroidism.

As mention before, the thyroid hormones are critical for normal bone growth and development. In children, hypothyroidism can cause short stature and delayed closure of the epiphyses. Biochemical studies have shown that T_4 can effect the expression of various bone markers in serum, reflecting changes in bone formation [3].

Thyroid hormone powers systemic vascular resistance, increases blood volume, and has inotropic and chronotropic effects on cardiac function [4]. The combination of these effects on both the circulation and the heart itself results in increased cardiac output. Hyperthyroid patients have a high output circulation state, whereas hypothyroid patients have low cardiac output, decreased stroke volume and increased systemic vascular resistance [4].

Thyroid hormones play important roles in the development and function of brown and white adipose tissue [5]. Thyroid hormones has multiple effects on liver function including stimulation of enzymes regulating lipogenesis and lipolysis as well as oxidative processes [6]. It has been appreciated for many years that hypothyroidism is associated with hypercholesterolemia with elevated serum intermediate and low-density lipoprotein (LDL) cholesterol concentrations [5]. The major mechanism for these effects may be lowered cholesterol clearance resulting from decreased LDL receptors.

Neonatal hypothyroidism due to genetic causes and iodine deficiency in humans can cause mental retardation and neurological defects. Studies in hypothyroid neonatal rats have shown that absence of TH causes diminished axonal growth and dendritic arborization in the cerebral cortex, visual and auditory cortex, hippocampus and cerebellum [7]. In the cerebellum, absence of thyroid hormones delays proliferation and migration of granule cells from the external to the internal granular layer.

Thyroid hormones have also influence on the sexual apparatus. Normal thyroid hormone levels are important for maturation of the testes in prenatal, early postnatal and prepubertal boys [6]. The T_4 hormonal dysfunctions can affect also the female reproduction including estrogen and androgen metabolism, sexual maturation, menstrual function, ovulation, fertility [6].

4.1.3. Hypothyroidism treatment

Sodium levothyroxine (Figure 4.2), a stereoisomer of physiological T_4, is the most commonly used synthetic thyroxine form. Levothyroxine at doses individualized according to patient response, is effective as replacement of supplemental therapy in hypothyroidism of any etiology, except transient hypothyroidism during the recovery phase of subacute thyroiditis.

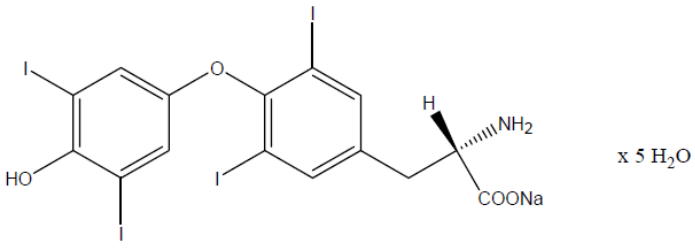


Figure 4.2. Chemical structure of penthydrate levothyroxine.

Dosages vary according to the age groups and the individual condition of the patient, body weight and compliance to the medication and diet. The common daily dosage of T_4 is 2.25μg/Kg for an adult, whereas in the case of child the dosage is reduced until 1/6. However, monitoring of the patients condition and adjustment of the dosage is periodical and necessary.

Levothyroxine is taken on an empty stomach approximately half an hour to an hour before meals to maximize its absorption, often with water to ease the swallowing and help the tablet dissolution for the drug absorption. There are also foods and other substances that can interfere with absorption of thyroxine replacement.

Thyroid hormones are usually not dangerous for pregnant women or nursing mothers, but they should be given under doctor's supervision. One exception is that thyroid hormones may aggravate heart conditions, especially in older patients; therefore, doctors may start these patients on a lower dose and work up to avoid risk of heart attack.

4.1.4. Pharmacokinetics of levothyroxine

After oral administration of T_4, the absorption from the gastrointestinal (GI) tract ranges from the 40% to 80%. The majority of T_4 dose is absorbed from the jejunum and the upper ileum. The T_4 absorption is increased by fasting, and decreased in malabsorption syndromes and by certain foods such as soybean infant formula. Dietary fiber decreases bioavailability of T_4. Absorption may also decrease with age. In addition, many drugs and foods affect T_4 absorption. As mentioned before, circulating thyroid hormones are bound greater than 99% to plasma proteins, including TBG, TBPA and TBA. The higher affinity of both TBG and TBPA for T_4 partially explains the higher serum levels, slower metabolic clearance, and longer half-life of T_4 compared to T_3. Protein-bound thyroid hormones exit in reverse equilibrium with small amount of free hormone. Only unbound hormone is metabolically active. Many drugs and physiologic conditions affect the binding of thyroid hormones to serum protein. Moreover, the thyroid hormones do not readily cross the placental barrier. T_4 is slowly eliminated. The major pathway of thyroid hormone metabolism is through sequential deionination. Approximately eight-percent of circulating T_3 is derived from peripheral T_4 by monodeionidation. The liver is the major site of degradation for both T_4 and T_3, but they are also metabolized via conjugation with glucuronides and sulfates and excreted directly into the bile and gut where they undergo enterohepatic recirculation. The major elimination of thyroid hormones is by kidneys. A portion of the conjugated hormone reaches the colon unchanged and is eliminated in the feces. Approximately 20% of T_4 is eliminated in the faeces [8].

4.1.5. Oral administration of levothyroxine

Levothyroxine stability was a crucial point of a formulation. The Food and Drug Administration (FDA) announced that the all drug products orally administered containing levothyroxine sodium were considered as new products by Thursday August 14, 1997. According to the FDA, “no currently marketed orally administered levothyroxine sodium product has been shown to demonstrate consistent potency and stability, and thus, no currently marketed orally administered levothyroxine sodium

product is generally recognized as safe and effective" [9]. Moreover, T_4 is sensitive to temperature, moisture, pH and oxidation. Won studied the kinetics of degradation of T_4 sodium in solution and in solid state. He concluded that in solution T_4 sodium followed first order kinetics of degradation. It was found that as the pH of the solution was increased, the degradation reduced [10]. Due to the stability and kinetic problems, the challenge could be the development of a levothyroxine formulation suitable for an administration route alternative to the oral one.

4.1.6. Buccal administration

Amongst the various routes of drug delivery, oral route is perhaps the most preferred to the patient and the clinician alike. However, peroral administration of drugs has disadvantages such as hepatic first pass metabolism and enzymatic degradation within the gastrointestinal (GI) tract, that prohibit oral administration of certain classes of drugs, especially peptides and proteins. Consequently, other absorptive mucosa are considered as potential sites for drug administration as the oral transmucosal route of delivery [11]. Within the oral mucosal cavity, delivery of drugs is classified into three categories: (i) sublingual delivery, which is systemic delivery of drugs through the mucosal membranes lining the floor of the mouth, (ii) buccal delivery, which is drug administration through the mucosal membranes lining the cheeks (buccal mucosa), and (iii) local delivery, which is drug delivery into the oral cavity [11]. Oral transmucosal absorption is generally rapid because of the rich vascular supply to the mucosa and the lack of a stratum corneum epidermidis. This minimal barrier to drug transport results in a rapid rise in blood concentrations. The oral transmucosal route has been used for many years to provide rapid blood nitrate levels for the treatment of angina pectoris. The drug appears in blood within 1 minute, and peak blood levels of most medications are achieved generally within 10 to 15 minutes, which is substantially faster than when the same drugs are administered by the orogastric route [12]. Moreover, oral transmucosal administration has the advantage of avoiding the enterohepatic circulation and immediate destruction by gastric acid or partial first-pass effects of hepatic metabolism.

4.2. AIM OF THIS SECTION

For the treatment of hypothyroidism sodium levothyroxine is the drug of choice. It is usually administered in the form of oral tablets with strengths ranging between 25 and 300 mcg. However, a lot of commercial tablets of T_4 presented erratic stability profiles, thus a new solid dosage form of T_4 agglomerates was studied for buccal administration. First, microparticles containing sodium levothyroxine were manufactured by a spray-drying technology. Then, the best formulation of T_4 microparticles was agglomerated by vibration on sieves to obtain soft clusters of microparticles called “chimerical agglomerates”. These globules have the peculiar characteristic to be easily dispersible in the mouth in the presence of a minimum volume of saliva. T_4 microparticles and agglomerates were characterized with respect to drug loading, flowability properties, morphology, diffusion across a model membrane and stability. In fact, we expect that the T_4 might be absorbed through the buccal mucosa and, in case the product (i.e. the drug) is then swallowed, also across the intestinal mucosa.

4.3. MATERIALS AND METHODS

4.3.1. Materials

Levothyroxine sodium pentahydrate was supplied by Sigma-Aldrich (Steinheim, Germany). The batches used were #073K1491 and #075K1854. Liothyronine was also supplied by Sigma-Aldrich (Steinheim, Germany). The batches used were #034K1442 and #085K10272. Mannitol (Eur. Ph.) was kindly donated by Lisapharma (Como, Italy), whereas lecithin (Lipoid S45) was supplied by Lipoid AG (Ludwigshafen, Germany). All other chemicals were of analytical grade.

4.3.2. Methods

4.3.2.1. Validation of T_4 HPLC assay

The samples of T_4 and its first metabolite T_3 were determined by HPLC assay. All samples were analyzed using an Agilent 1100 Series HPLC system equipped with an autosampler and a UV detector set at 225 nm. The column was Luna CN 150 x 4.6 mm, (particle size 5μm) and the mobile phase methanol/water 50:50 with 0.l% (v/v) phosphoric acid. The flow rate was set at 0.8 ml/min. The injection volume was 40 μl. Linearity, limit of quantification (LOQ) and repeatability of the method were performed.

4.3.2.2. Solubility studies

An excess amount of T_4 (50 mg) was added to 5 ml of water and phosphate buffer saline pH 7.4 (PBS), respectively. The samples were left to equilibrate at room temperature protected from light and under continuous stirring for 6 hours. After centrifugation, the samples were diluted and analyzed by HPLC.

4.3.2.3. Manufacturing of spray dried (SD) microparticles

T_4-loaded microparticles were manufactured by spray drying. The drug and the excipient were sprayed together (i.e., from the same solution). The excipients selected were mannitol and lecithin at the ratio of 92:8, respectively. The theoretical amount of T_4 in the microparticles was fixed at 2 μm per mg of sd microparticles. First, mannitol was dissolved in water, while lecithin and T_4 were dissolved in methanol due to the low solubility of T_4 in water. Before spraying, the methanolic phase was added to the aqueous solution dropwise (formulation #A). In the formulation #B of T_4 microparticles, the pH of the solution to be sprayed was increased until pH 11 by addition of NaOH 1M, since it was suggested by Won that the T_4 is more stable in alkaline solution [1].

For all batches the total solid concentration in the solution to be sprayed was 2% (w/v). The solvent system was a 8% (w/w) methanolic solution. The yield (%) of the spray-dried process is reported as a percentage in weight of the product recovered over the total components initially weighed.

4.3.2.4. Soft agglomerate preparation

For the preparation of agglomerates starting from T_4 microparticles (formulation #A), the powder was put on top of two stacked sieves with nominal apertures of 850 and 106 μm, respectively (10 cm diameter sieves, Endecotts Ltd, London, UK), which were vibrated for 5 min on a laboratory sieve shaker (amplitude 3-4; Analysette 3 Fritz model, Fritsch GMBH, Idar-Oberstein, Germany). Agglomerates between 106 and 850 μm were collected. The process was repeated five times recycling the non-agglomerated powder and the oversized agglomerates. Agglomerates were characterized with respect to drug loading, morphology and drug dissolution.

4.3.2.5. Characterization of primary microparticles and agglomerates

4.3.2.5.1. Stability of the microparticles

Two batches of T_4 microparticles formulation #A were stored in closed vials at room temperature (24-25 °C); another batch of formulation #A and on batch of formulation #B were stored at 40 °C for a total of six months. Moreover, a physical mixture of T_4 raw material and SD excipient microparticles prepared by mixing the two powder populations with a spatula, was stored in vials at 40 °C for six months and assessed for stability as well. This served as a "control" because T_4 was not processed by spray drying, but simply stored in the presence of the selected excipients. In this way it was wanted to evaluate any possible effect of the spray drying process on T_4 loading. Every month the drug content of each batch was measuring in triplicate.

4.3.2.5.2. Morphology and particle size studies

Particle size analysis was performed on T_4 spray-dried microparticles (formulation #A) by laser light scattering on a Malvern Mastersizer (Malvern Instruments, Mastersizer, UK). A small amount of microparticles was suspended in ethyl acetate, in which microparticles are insoluble. Then the sample was sonicated to eliminate the aggregates and some drops diluted inside the analysis chamber filled with ethyl acetate to be analyzed. The results are the average of three analyses. The values ($D_{v,\ 0.5}$) were expressed for all formulations as mean size range. Morphology and surface characteristics of the microparticles of both formulations #A and #B and of the agglomerates obtained from microparticles formulation #A were coated with gold at room temperature, then the surface appearance of the microparticles was studied by scanning electron microscopy (SEM, a 360 Stereoscan Cambridge Instruments Ltd., Cambridge, UK) using accelerating voltage of 20 kV.

4.3.2.5.3. Drug loading assay

Accurately weighed samples of microparticles or agglomerates (30 mg) were dissolved in 5 mL of water:methanol 50:50 (v/v). After sonicating for 10 minutes, the samples were diluted 1:10 with the same solvent and then HPLC analyzed. For each batch, three analyses were performed.

4.3.2.5.4. In vitro dissolution

To evaluate the dissolution behaviour of the agglomerates, 100 mg of the sample were introduced into a becker containing 100 ml of distillated water at 37 °C under stirring. 0.5 ml samples were withdrawn at fixed time points for 30 minutes and immediately analyzed by HPLC.

4.3.2.5.5. Density measurements

A certain amount of T_4 agglomerates was weighed and poured into a dry 10 ml graduated cylinder. The bulk volume Vb and the tapped volume Vt after 1250 taps on a tap density apparatus (Erweka SVM 12) were used to calculate (in g ml-1) the bulk density (*ρbulk*) and the tapped density (*ρtapped)*. Measurements were performed two times. Then, the Carr Index (or Compressibility Index, CI%) values were calculated to express the flowability of the microparticles, as follows:

$$CI = 100\ (\rho tapped - \rho bulk)/\ \rho tapped \qquad (1)$$

where *ρtapped* is the tapped bulk density of the powder and *ρbulk* is the freely settled bulk density of the powder.

4.3.2.5.6. In vitro diffusion experiments with Franz cells

The T_4 agglomerates were manufactured envisaging a systemic treatment with thyroid hormone via buccal administration. Hence, preliminary diffusion experiments were carried out to understand the capability of the drug molecule to diffuse across a model membrane of regenerated cellulose cut from a dialysis tubing membrane as barrier (MW cut-off 12,000–14,000 Da, tubing width 32–34 mm, Dexstar Visking, Medicell International Ltd, London, UK), eliminating the variables on drug flux related to the use of a biological membrane. Experiments were performed using vertical Franz type diffusion cells (Vetrotecnica, S.r.l., Padova I) with 0.58 cm^2 of diffusion area. The receptor compartment (3.9-4.2 ml average volume) was filled with water or phosphate buffered saline (PBS). In order to assess the proper cell assembly, the donor compartment was filled with the same liquid. After filling the receptor compartments, the assembled system was allowed to equilibrate at 37 °C for half an hour. Then, after removing the saline from the donor, either 0.4 ml of T_4 saturated solution or a fixed amount of microparticles was introduced into the donor compartment, which was closed with a screw-cap to prevent evaporation. The receptor solution was magnetically stirred to avoid any boundary layer effect. All experiments were carried out over a 2-h period of time. At predetermined time-points, samples were withdrawn from the receptor compartment and immediately analyzed for T_4 content. At the end of the experiment, the donor solution was quantitatively collected and assayed in order to measure the residual T_4 amount and calculate the mass balance. All experiments were replicated at least three times; results are expressed as the mean ± standard deviation.

4.4. RESULTS AND DISCUSSION

4.4.1.Validation of HPLC assay

The T_4 and T_3 were quantified by HPLC. A calibration curve was constructed using three concentrations of T_4 and T_3 dissolved in water and methanol (50:50) as reported in the Figure 4.3. Each sample was injected three times. The correlation coefficient (R^2) for the calibration curve was 1 in both cases in the range of 0.4-60 μg/ml, indicating excellent linearity.

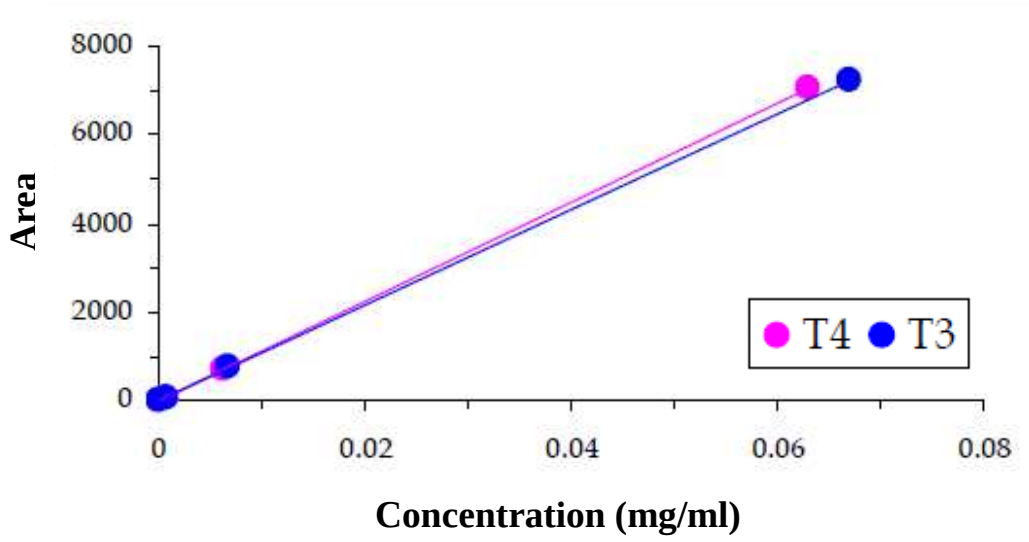


Figure 4.3. Area versus concentration of T4 and T3 in a 50% methanolic solution.

The repeatability of the method was also evaluated: the same sample was injected 6 times consecutively. The resulting areas and the relative standard deviation (RDS%) are reported in the Table 4.I. The obtained RSD of 0.16%, was widely under the limit reported in the European Pharmacopoeia 6^{th} Ed., that is fixed at 0.85%. Finally, the limit of quantification (LOQ) was evaluated and the value was 0.063 μg/ml.

Table 4.I. Data of repeatability.

INJECTION	AREA
1st	1565.8
2nd	1565.3
3rd	1563.3
4th	1561.7
5th	1562.9
6th	1568.8
Average	**1564.6**
Standard Deviation	**2.55**
RDS(%)	**0.16**

4.4.2. Levothyroxine solubility

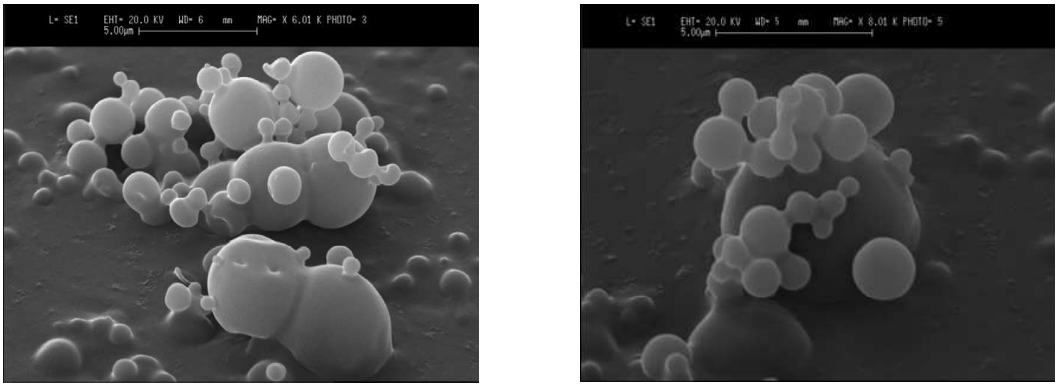
Levothyroxine is classified a very slightly water-soluble compound [13], despite the fact of being used as sodium salt. However, the experimental value of T_4 solubility in water at room temperature resulted high 2.1 ± 0.3 mg/ml. However, the solubility of T_4 in the presence of salts was problematic. In fact, the solubility of T_4 sodium salt in PSB pH 7.4 dramatically decreased (0.05 ± 0.04 mg/ml) in comparison with the behaviour in water. A similar value was reported also in work by Padula at al. [2]. This reduction of solubility is probably due to a salting out effect [14].

4.4.3. Manufacturing of T_4 spray dried microparticles

Sodium levothyroxine microparticles were manufactured by a spray drying technique, as it is a fast and cheap process. The amount of hormone to be administered to the patience has to be carefully controlled and accurate, with a daily dose per os ranging between 20 and 300 μg. For this reason, it was impossible to produce microparticles only composed by T_4, because the administration of these extremely low doses would never be accurate nor reproducible. It was necessary to add excipients to increase the mass bulk of the formulations. Hence, the T_4 loading in the microparticle system was fixed at 2 μg per mg of microparticles. Mannitol was selected as diluent, because it is an

inert and safe material that dissolves very fast in water releasing immediately the drug. Moreover, it was demonstrated that it was a stable excipient for an oral dosage formulation [3]. It also has favourable organoleptic characteristics, making it particularly suitable for an orodispersible product. Since the powder of microparticles had to be agglomerated, it was necessary to add a certain amount of a binding agent: in this case soybean lecithin was selected. The ratio between mannitol and lecithin was fixed at 92:8, respectively. Several microparticle batches containing T_4 (formulation #A) were manufactured at the Inlet Temperature of 100 °C. The process yield (%) was 56 ± 5%. This value was particularly satisfying knowing the generally low production yields encountered with laboratory scale spray-dryers as reported in the work by Giudenchi [4]. The microparticles of the formulation #B were manufactured increasing the pH of the spray-drying solution at 11 by adding a 1 M NaOH solution dropwise. To check whether the pH of the obtained microparticles may remained alkaline, 50 mg of microparticles were dispersed in 0.5 ml of water and the pH measured. The pH value was still alkaline, but further dilution of the solution of ten times decreased the pH to a value of 7. The actual drug loading in the microparticles was 2.03 ± 0.03 μg T_4 per mg of microparticles. Since T_4 is more soluble in the presence of an organic solvent and given that the drying phase resulted to be suboptimal during the spray-drying process (the chamber wall was all covered with the microparticles), another batch of T_4 microparticles was produced spraying a liquid system in which the solvent was a 50:50 methanol/water solution at the same Inlet Temperature (100 °C) as for the other batches (formulation #C). The aim was to facilitate the evaporation of the solvent. The yield obtained was 48.5% and the drug loading of this batch was 1.99 ± 0.01 μg per mg of microparticles.

4.4.4. SEM images



(a) (b)

Figure 4.4. SEM images of T_4 spray-dried microparticles: (a) formulation #A; (b) formulation #B.

T_4 spray-dried microparticles formulation #A are shown in Figure 4.4a. The microparticles had a typical round shape of spray-dried products and evidenced a tendency to form clumps almost looking fused together in some points. The microparticles had different sizes: the bigger ones had a diameter in the range of 3-4 μm, whereas the diameter of smaller ones was lower then 1 micron. As a typical micronized powder, the overall small particle size causes flowability problems, which as a consequence may lead to erratic dose metering during product use. Thus, agglomeration of the spray dried microparticles could resolve this drawback, increasing the handling properties and flowability characteristics of the primary microparticles.

The T_4 SD microparticles of formulation #B, which were manufactured changing the pH of the solution to spray, are shown in Figure 4.4b. No morphological differences were observed with respect to formulation #A. Similarly, the T_4 microparticles manufactured by spraying a 50% (v/v) of a methanolic solution (formulation #C) presented no morphological differences from the T_4 microparticles of formulation #A (image no shown).

4.4.5. Particle size analysis

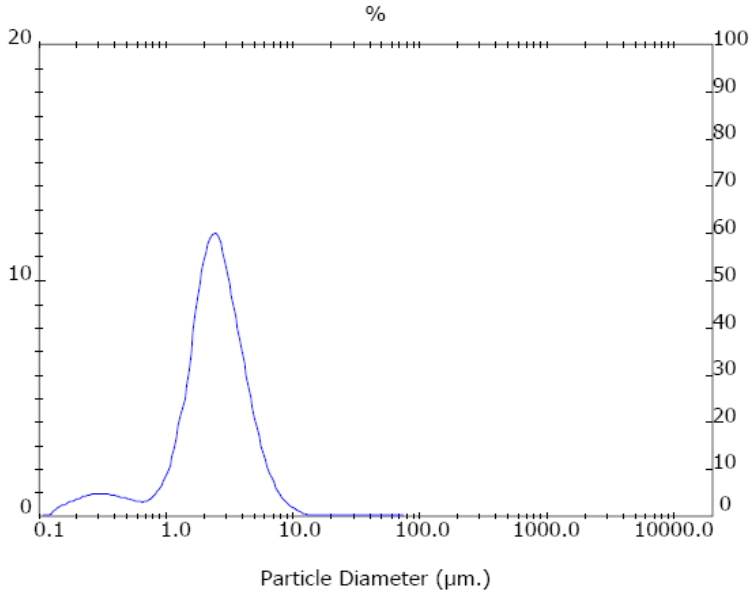


Figure 4.5. Size distribution of T_4 microparticles (formulation #A).

From Figure 4.5 it possible to observe that most of the T_4 microparticles had a diameter in the range of 1-10 µm. The $D_{v,\,0.5}$ for the three different batches had a value of 2.5 ± 0.1µm confirming that, in general, the size of the spray-dried microparticles was homogeneous, even if there was small subpopulation of microparticles with a diameter lower than one micron as shown in the SEM images.

4.4.6. Flowability properties

The measurement of the bulk densities was useful to determine the flowability properties of the T_4 spray-dried microparticles. From these density values the Carr index or compressibility index was calculated according to equation (3). Data are reported in Table 4.II.

Table 4.II. Tapped and bulk densities and Carr index of microparticles.

Tapped density	**0.25 g/ml**
Bulk Density	**0.34 g/ml**
Carr Index	**28**

A Carr index greater than 25 is considered to be an indication of poor flowability, whereas below 15, it shows good flowability. The T_4 microparticles had a value of 28, confirming the tendency of packing and handling problems during their manipulation.

4.4.7. Stability of the T_4 SD microparticles

Drug product stability is considered one of the most important requirements for product quality. Only stable preparations would promise precise delivery of the drug to the patients. Expiration date of any drug product is defined at the end of scientific studies at normal and/or stressed (accelerated) conditions on a certain number of batches. Sodium levothyroxine products for oral use have drawn the attention of the FDA Advisory Committee after some products on the market were found with a drug content outside the specification limits of 90–110% of labelled dose. In particular, the actual drug content resulted to be below the limit of 90% of labelled doses before the expiration date. Obviously, this raised an alert concerning these products for the clinical consequences of using underdosed drugs. Consequently, there were numerous recalls of levothyroxine products from the market. The low drug content was motivated based on the poor stability of this drug molecule in the solid state (FDA). Furthermore, erratic potency and stability issues raised further concerns in the physicians regarding the possibility to substitute levothyroxine brand products with marketed generic ones.

Based on this, stability tests at room temperature and at 40 °C for a period of six months were carried out to evaluate the possible degradation of the drug in the microparticles. Each month T_4 loading in the microparticles was evaluated. The formulation considered were formulation #A), formulation #B (microparticles produced by spraying an alkaline solution), formulation #C (microparticles obtained spraying a methanolic solution) and the physical mixture of drug plus excipients (see Table 4.III). In the case of formulation #A, two batches underwent the study at room temperature, whereas only one batch was stored at 40 °C. The results show that at room temperature for both batches, the drug loading, expressed as μg of T_4 per mg of microparticles did not significantly vary during the six months checked. The drug content remained between the range of ±10% of the initial loading. It is possible to affirm that the microparticles of formulation #A were

stable at room temperature for a period of six months. Moreover, the batch of formulation #A stored at 40 °C showed a stable trend even if the range of variation was wider, as the T_4 loading at month 3 and 5 was less then 90%, but higher than 85% of initial drug loading. In the case of formulation #B, T_4 loaded in the microparticles manufactured by spraying in alkaline conditions degraded during the six months of storage at a temperature of 40°C. The high pH in combination with the higher temperature of storage seemed to have a negative effect on the stability of the microparticles, even if a previously study in which the same type of microparticles had been stored at room temperature for six weeks showed that T_4 loading remained within the range of ±10% with respect to the drug loading at time zero (data no shown).

The stability profile in accelerated conditions of the microparticles of formulation #C (manufactured by spraying a 50:50 water/methanol solution) was similar to that of the batch of formulation #A kept at 40 °C, even if the value at month 6 was a slightly lower than minimum acceptable value. This result was predictable, since the operative conditions and the composition were the same. In the case of the physical mixture, the stability study showed a variability profile over the six months. Moreover, the standard deviations of the 1st, 3rd and 6th months were high. Given that the amount of drug compared to that of excipient microparticles was very low in the mixture, it is likely that T_4 was not uniformly distributed in the mixture. It possible to state that in the SD microparticles containing T_4 the drug loading was stable than in the physical mixture. Moreover, the alkaline pH accelerated the process of degradation of T_4 at 40 °C.

Table 4.III. Stability tests of the different formulations.

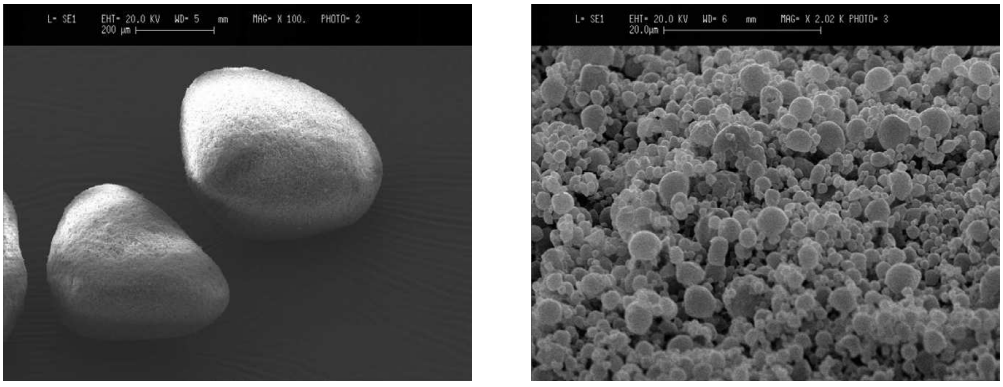
	0 month	*1st month*	*2nd month*	*3rd month*	*4th month*	*5 th month*	*6th month*
Formulation #A (I batch-25°C)	2.00±0.02 (100.0%)	1.99±0.01 (99.1%)	2.03±0.07 (101.1%)	1.97±0.02 (98.2%)	2.08±0.08 (103.3%)	1.94±0.03* (96.6%) **165 days*	2.02±0.09 (100.4%)
Formulation #A (II batch-25°C)	1.88±0.04 (100%)	1.88±0.023 (99.6%)	1.85±0.02 (97.9%)	1.84±0.03 (97.8%)	1.91±0.11 (101.3%)	1.84±0.04 (97.7%)	1.85±0.01 (98.2%)
Formulation #A (40°C)	1.94±0.13 (100.0%)	1.82±0.01 (94.0%)	1.83±0.03 (94.1%)	1.74±0.017 (89.6%)	1.83±0.08 (94.3%)	1.72±0.012 (88.4%)	1.70±0.02 (100.4%)
Formulation #B (40°C)	2.03±0.082 (100.0%)	1.52±0.05 (74.9%)	1.31±0.18 (64.6%)	0.98±0.20 (48.4%)	0.97±0.05 (48.0%)	0.94±0.023 (46.3%)	0.89±0.03 (43.7%)
Formulation #C (40°C)	1.99±0.012 (100.0%)	1.91±0.03 (96.1%)	1.83±0.05 (92.0%)	1.79±0.017 (90.0%)	1.68±0.01 (84.5%)	1.76±0.04 (88.6%)	1.65±0.01 (83.1%)
Physical mixture (40°C)	2.23±0.19 (100.0%)	2.31±0.01 (103.1%)	2.28±0.05 (101.7%)	1.75±0.23 (77.9%)	2.15±0.11 (96.1%)	1.82±0.03 (81.2%)	1.86±0.12 (83.2%)

4.4.8. Agglomerate manufacturing and characterization

The microparticles of formulation #A could be used as they are for buccal administration, but due to their scarce flow properties they are not very easy to be dosed and poured into the mouth. For this reason, their transformation into soft spherical agglomerates, through the improvement of the flowability and the compressibility index, could improve the metering. The agglomerates are globules obtained from the agglomeration of the microparticles, identified as “primary” because they represent the original building blocks for the formation of the agglomerates. These primary microparticles need to be formulated with a composition suitable for determining particle aggregation in a controlled manner (Figure 4.6). The agglomerates were manufactured by vibration on sieves. The yield of the process was quite high (2%). The agglomerates were defined “chimerical” because their big dimensions (bigger than those of the original microparticles) were not permanent, but change quickly after administration.

Figure 4.6. Chimerical agglomerates manufactured on the sieve with nominal apertures of 106µm.

The drug loading of the original primary microparticles was not affected by the agglomeration process. In fact, it had the same value of T_4 loading measured on the microparticles before agglomeration (1.6 µg T_4 /mg agglomerates). It is possible to conclude that the agglomeration step did not affect the drug loading since it is a mild process. The SEM image of agglomerates is shown in Figure 4.7a. The agglomerates presented a round, but not perfectly spherical shape and an average size of 200-300 µm. A detail of the surface is shown in Figure 4.7b: it possible to recognize the primary microparticles that interact with each other through weak bonds.



(a) **(b)**

Figure 4.7. SEM images of (a) chimerical agglomerates containing T4 (100x) and (b) detail of the agglomerates surface (2000x).

The flowability properties of the agglomerates were calculated and reported in Table 4.IV. The agglomeration process had a positive effect on the product flowability. In fact, the agglomerates had a Carr Index value lower than the microparticles and flowed very easily on every type of surface.

Table 4.IV. Values of tapped and bulk densities and Carr index of agglomerates containing T_4.

Tapped density	**0.42 g/ml**
Bulk Density	**0.45 g/ml**
Carr Index	**10**

Moreover, a dissolution test was performed to evaluate the dissolution rate of T_4 from the agglomerates: this rate should be very high and almost instantaneous also in the presence of a very small liquid volume as it is that of the saliva on the buccal mucosa. The experiments demonstrated that drug release was immediate, assuring a good availability of the drug at absorption site.

4.4.9. In vitro diffusion experiments with T_4 microparticles

In vitro diffusion studies were conducted to evaluate the release kinetic of T_4 from the formulation #A microparticles compared to a T_4 saturated aqueous solution. Vertical Franz cells were used for these experiments assembled with a dialysis cellulose membrane as model barrier. Since the solubility of T_4 in PBS was very low (0.05 mg/ml), preliminary diffusion studies were performed using water (where the solubility is higher) as the medium to be introduced in the receptor compartment. Figure 4.8 shows that the T_4 (%) permeated during three hours was higher in the case of the saturated solution compared to the formulation #A microparticles. This result was predictable since the amount loaded and the concentration of T_4 in the donor compartment at time zero were different in the two cases. In fact, in the case of the saturated solution, 0.4 ml of solution were introduced in the donor with a measured concentration of 2.08 mg/ml of T_4 (HPLC assay), which means that an amount of 840 μg of T_4 was actually present in the donor. Moreover, the saturated solution was filtered prior to use, in order not to have undissolved drug in the donor at time zero. On the other hand, in the case of the solid formulation 10 mg of formulation #A microparticles were introduced in the donor together with 0.3 ml of water (for powder dissolution). This means that 20 μg of T_4 were present in the donor, assuming an attended concentration of 0.07 mg/ml in case of complete drug dissolution in the available liquid. This difference in donor concentration could explain the result.

The diffusion profile of T_4 raw material was also studied to understand whether the difference in permeation between the microparticles and the raw material might be due to the competition for the solvent (water) between mannitol and levothyroxine. In other words, we hypothesized that the lower T_4 diffusion observed with the microparticles was due to incomplete drug dissolution in the donor due to insufficient solvent and to the presence of mannitol, competing with T_4 for the dissolution in the aqueous solvent.

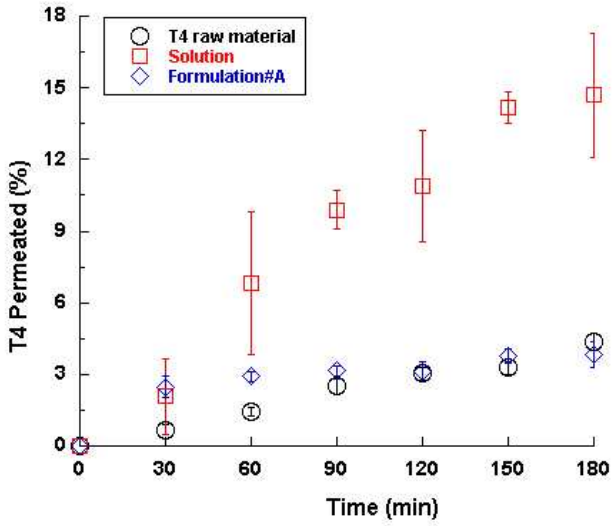


Figure 4.8. Permeation profiles of T4 across a dialysis membrane, from a T4 saturated water solution (□), T4 **raw material (○) and formulation #A microparticles (◇). Average values ± s.d..**

The permeation profiles were similar in the case of the raw material in comparison with the microparticles, suggesting that the excipients did not influence the permeation of the drug. We concluded that the low permeation of T_4 observed for the solid formulation was due to the difficulty of T_4 to dissolve in such a little volume of solvent, even if the experimental solubility of T_4 in water (2.0 mg/ml) resulted to be higher than the concentration expected upon complete dissolution of the T_4 loaded in the donor in the presence of 300 microliters of water.

4.5. CONCLUSIONS OF THIS SECTION

The study concerned the possibility to manufacture T_4 microparticles by spray drying technique to be then agglomerated. The aim of this part of the work was to develop a dosage form for buccal administration of the thyroid hormone to be proposed in alternative to oral tablets. One of the main requirements for this new product should be a suitable stability profile over a shelf-life period equivalent to that of the reference product (T_4 tablets).

It was decided that the theoretical drug loading for such dosage form was 2 μg /mg of powder and the excipient selected were mannitol and lecithin. Different operating conditions were selected trying to obtain T_4 microparticles stable in the period of six months.

The T_4 microparticles (formulation #A) were stable over a period of six months both at room temperature (25-27 °C) and 40 °C. The formulation obtained by spraying an alkaline solution (formulation# B) presented similar morphology in comparison with formulation #A. However, the drug loading of these microparticles was not stable during the stability test at accelerated conditions. The T_4 microparticles of formulation #C were similar to formulation #A in terms of stability.

Hence, it is possible to produce agglomerates starting from primary T_4 spray-dried microparticles without affecting the microparticle drug loading, but definitely improving relevant technological properties of the microparticles such as the flowability. The higher flowability of agglomerates improves the handling properties of the T_4 microparticles, which is an important requirement of the formulation envisaged for use as a buccal drug delivery system.

4.6. REFERENCES

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5. GENERAL CONCLUSIONS

In this PhD thesis the applications of the soft agglomeration technology were studied.
In the case of the mesalazine, it was discovered that the drug can be entrapped inside lipid excipients to obtain a gastroresistant microcapsules. However, these lipid microcapsules tend to float and they can not be mixed in the fluid to be administered. In order to overcome this inconvenient, the lipid microcapsules were agglomerated with the excipient microparticles. The agglomeration process was able to improve the wettability of the lipid microcapsules. Therefore, the mesalazine agglomerates in reality are lipid microcapsules coated with layer of excipient microparticles.

Concerning the possibility to obtain an extemporaneous formulation of artemisinin-combination treatment (ACT) as recommended by WHO, formulations based both on artemisinin – β-cyclodextrin complex agglomerates and clindamycin agglomerates have been obtained. Initially, it was studied and characterized the artemisinin complex obtained by spray drying. Then, agglomerates using the complex were produced. Moreover, agglomerates using the clindamycin raw material and spray-dried excipient microparticles were manufactured. The agglomerates will be mix together in order to obtain a combined agglomerate formulation for the use.

Concerning the possibility to obtain agglomerates of sodium levothyroxine and since some commercial oral solid form products were recalled from FDA due to a not stable profile, it was studied a powder obtained spraying sodium levothyroxine together with excipients such as mannitol and lecithin. The agglomeration process improved the handling properties without changing the primary microparticles. The sodium levothyroxine agglomerates can be buccal administrated because the microparticles can dissolve in a minimum volume of liquid as the saliva. Moreover, if a fraction of the dose is not absorbed in the month, it can be absorbed through the gastrointestinal tract.
The aspects of the powder agglomeration technology, explored in this thesis, demonstrated the versatility of this technology. It can be used successfully for the parallel development of “the classic oral form” as tablets or capsules for a paediatric or elder people administration.