POLITECNICO DI TORINO

Master's Degree in Biomedical Engineering

Master's Degree Thesis

Topical formulation of a novel drug for the treatment of gouty arthritis

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Abstract

The main objective of this work is to provide a topical formulation of a novel thiocolchicoside derivative drug to treat gout inflammation. The drug has been tested in cell-based assays and showed promising effects but it has not yet been formulated for clinical applications, hence there is a great need to find a delivery method that maximizes the therapeutic effect and minimizes detrimental side effects. The latter aspect is of importance due to general toxicity of the compound in question.

Gout is one of the most common and painful inflammatory diseases that can affect human beings. In the last two decades, the incidence of gout among adults (age ≥ 18 years) doubled. According to Elfishawi et al. in the 1989–1992 time period the incidence, in USA, was 66.6/100,000, whereas it was 136.7/100,000 in the 2009–2010 time period [1]. Gout attacks occur after the precipitation of sodium urate crystals in joints and they are often associated with hyperuricemia. The use of colchicine in treating gout attacks is known to be effective in most cases, but it is often the therapeutic of last resort, because of the potentially serious and sometimes unbearable side effects that can occur. Colchicine is commonly used as a chemotherapeutic agent or as an anti-inflammatory agent but it often triggers strong side effects, especially on patients suffering from co-morbidities. In previous studies, the basic structure of colchicine was computationally designed by adding different groups such as alkanes, alkenes, esters, ethers, aromatics in different positions

In this way, many derivatives were generated and each one of them was characterized by different affinities in the bond between the drug and its protein target, which is tubulin. The thiocolchicoside derivative used in this work, the CCI-001, was initially designed for bladder cancer chemotherapy but modeling and in vitro assays results show CCI-001 as a potential candidate to treat gout due to its efficacy and reduced side effects. The CCI-001 compound has been approved for clinical trials for bladder cancer but it can also be repurposed as an effective therapeutic agent for gout. Topical delivery rather than enteral or parental routes was chosen for several reasons that are explained below. A very simple protocol containing reagents and conditions of reactions to formulate an inexpensive and simple gel was prepared and, then, the formulation was implemented in the laboratory. Once the formulation was generated, a very thorough characterization has been carried out on the final product to evaluate pH, viscosity, and stability.

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List of abbreviation

MSU	Monosodium Urate Crystals
SUA	Serum Uric Acid
CVD	Cardiovascular Diseases
CKD	Chronic Kidney Diseases
NSAID	Nonsteroidal anti-inflammatory drugs
ULT	Urate Lowering Therapy
FDA	Food and Drug Administration
CADD	Computer-aided Drug Design
SBDD	Structure-based Drug Design
LBDD	Ligand-based Drug Design
NMR	Nuclear Magnetic Resonance
QSAR	Quantitative Structure-Activity Relationship
SC	Stratum Corneum
AUC	Area Under the Curve
MTC	Minimum Toxic Concentration
MEC	Minimum Effective Concentration
ROA	Route Of Administration
MD	Molecular Dynamics simulation
LAGEPP	Laboratory of Automatic Control, Chemical and Pharmaceutical Engineering
C-980	Carbopol 980
TEA	Triethanolamine
HPLC	High Pressure Liquid Chromatography
dTEA	Diluted Triethanolamine
ROS	Reactive Oxygen Species
DMPK	Drug Metabolism and Pharmacokinetics
ADMET	Absorption, Distribution, Metabolism and Excretion

1 Introduction

1.1 Gout

Gout is the most common form of inflammatory arthritis and it is caused by elevated urate serum levels, known as hyperuricemia. Hyperuricemia can lead to the deposition of monosodium urate crystals (MSU) in most joints, but also in tendons and other tissues. This results in recurrent episodes of acute inflammation, known as gout flares, which are extremely painful and debilitating for patients who experience them [2]. The sites of inflammation are typically localized, most commonly to the joints in the big toe, which makes topical application of medication a logical choice of the method of administration.

1.1.1 Epidemiology of gout

Understanding trends in gout prevalence in the world is very important to facilitate adequate health-care resource planning. Very recent studies have shown that the highest prevalence of gout in the world has been reported in Oceanic countries. In specific ethnic groups, such as Taiwanese Aboriginals and Maori, prevalence exceeds 10%. The prevalence of gout in the USA for adults affected in 2007–2008, is also high with 3–4%. Also in Europe gout affects commonly the population: recent studies in France, Germany, Greece, Italy, Netherlands, Spain and the UK revealed a gout prevalence ranging from 1% to 4% for the period 2003–2014. The prevalence of gout varies among Asian countries, but new data from China and South Korea confirm that the incidence is increasing. Unfortunately, data are scarce concerning the prevalence of gout in Africa [2].

The difference in relative risk in men and women can be also analyzed. For both men and women, the risk increases with the increase of serum uric acid (SUA) in plasma but, at a given concentration of SUA, in men the risk is higher, as we can see in Figure 1 [3].

Other than hyperuricemia other factors, such as genetics, diet, obesity and comorbidities must be considered in the pathogenesis of gout. An association between gout and dietary factors has been recognized for centuries. Some foods and drinks, such as red meat, seafood, sugar-sweetened and alcoholic drinks increase the risk of gout, while low-fat dairy products, vitamin C and coffee are recognized to be potentially protective [2]. As regards the associations between gout, hyperuricemia and comorbidities, many studies confirmed that the link exists and it is complex because some diseases predispose to hyperuricemia. Recent

research has confirmed that traditional cardiovascular risk factors (such as hypertension and hyperlipidemia), CVD and CKD are risk factors for gout, and there is also a well-recognized association of gout with subsequent CVD and renal disease [2].

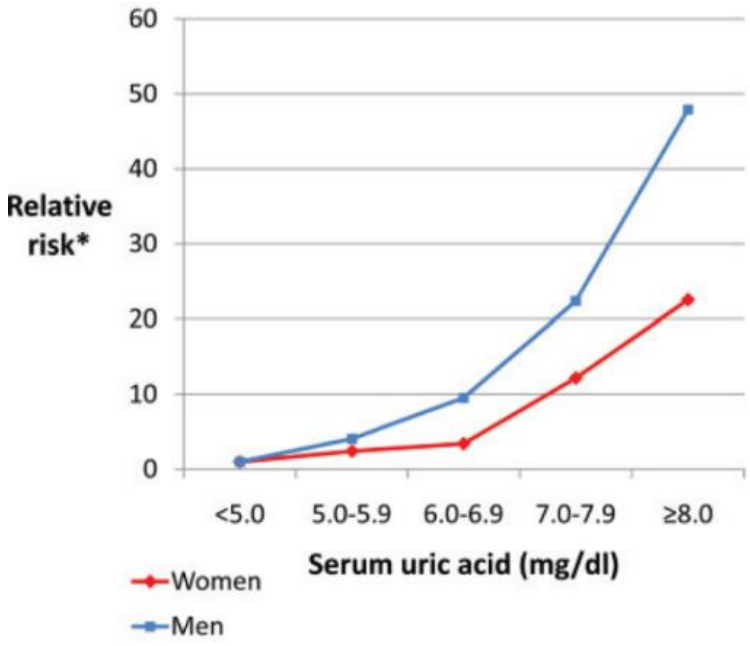

Figure 1: Risk of developing gout according to serum uric acid [3]

1.1.2 Pathophysiology of gout

Patients that suffer from hyperuricemia can develop a condition known as gouty arthritis. Hyperuricemia consists of elevated urate concentrations at sites of deposition and, in gout patients, this happens because of overproduction and underexcretion of urate. At this point, MSU deposition within joints occurs, activating cells of the innate immune system [4]. Therefore, for gouty arthritis to appear three pathophysiological checkpoints are required: hyperuricemia, MSU crystals deposition in situ and the acute inflammatory response, as shown in Figure 2.

Cells of the innate immune system recognize MSU as a foreign substance and they initiate an inflammatory process to get rid of it. The first cells that come into play are macrophages which phagocytose MSU. This event triggers the formation of a scaffold of proteins called inflammasome. The inflammasome is responsible for the production of a biologically active interleukin called IL-1β that gets released from the cell, attracting other inflammatory

mediators, thereby perpetuating inflammation. The secretion of IL-1β and other cytokines increases the recruitments of neutrophils to the site of MSU deposition. Unfortunately, inflammation ultimately leads to the destruction of the joint tissues. Interestingly, MSU crystals alone may not be sufficient to trigger the activation of IL-1β, instead seem to require co-stimulation with free fatty acids or lipopolysaccharide to release IL-1β. Given that, the consumption of alcohol or a large meal can lead to an increase in free fatty acid concentrations, it stands to reason that the involvement of free fatty acids in triggering the release of IL-1β may be an important factor in the development of gouty arthritis flares [5] [6].

Figure 2: Gout pathophysiological checkpoints [7]

1.1.3 Gout treatments

Gout treatment aims to both lower urate serum levels, through the so-called Urate Lowering Therapy (ULT), and decrease inflammation and pain during flares. ULTs include allopurinol and febuxostat that interfere with purine synthesis, whereas acute gout attacks can be controlled by nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids and colchicine. NSAIDs are used to relieve some symptoms, such as inflammation, swelling, stiffness, and joint pain, but carry the risks for stomach pain, bleeding and ulcers [8], [9]. Side effects of corticosteroids include mood changes, increased blood sugar levels and elevated blood pressure [8]. Colchicine inhibits the polymerization of tubulin by binding to tubulin dimers and making them assembly incompetent thus preventing the activation of the inflammasome. Efficacy of colchicine for the treatment of gout flares has been demonstrated by randomized controlled trials, but oral administration is associated with side effects such

as vomiting and diarrhoea that may precede rare adverse effects including muscle damage, neuropathy, multiple organ failure and bone marrow suppression.[8], [10].

1.2 Colchicine

Colchicine, an alkaloid extracted from the plant Colchicum autumnale, is the fastest-acting drug among the currently available for the control of an acute attack of gout. Colchicine summary formula is $C_{22}H_{25}NO_6$, its molecular weight is 399,437 g/mol and its main chemical structure is characterized by three benzene rings, as shown in Figure 3 [11]. The A-ring is a trimethoxyphenyl ring, B-ring is a saturated seven-membered ring containing an acetamido group and C-ring is a tropolone ring. A and C rings are held together in a very rigid configuration by the B-ring [12]. Colchicine reduces inflammation by a combination of some effects: it inhibits the migration of neutrophils and leucocytes, induced by monosodium urate accumulation and it helps the suppression of superoxide production by neutrophils [13]. Microtubules have been reported to provide the platform for mediating the assembly and activation of gout inflammasome and the colchicine's effectiveness can be explained by its inhibitory effect on microtubule polymerization, once it binds with free tubulin dimers [6]. Colchicine's high-affinity binding to tubulin can be elucidated by the presence of structural features of this molecule and it is due mostly due to A and C rings. Even though the bond is made possible by A and C rings, the presence of the side chain at the C-7 position of the B-ring influences several aspects of the interaction, such as activation energy, binding kinetics, and association/dissociation rates [6], [14],[15]. Colchicine non-covalent bond to tubulin follows stoichiometric proportion (one mol per one mol), its binding process is via a slow, two-step and strong bimolecular reaction, that induces a conformational change in the tubulin protein molecules [12]. Factors such as tubulin`s concentration, pH and temperature may affect the binding activity [12],[16].

Despite being the fastest and most effective drug against acute gout attacks, colchicine oral or parenteral administration displays a high risk/benefit ratio and a narrow therapeutic window due to severe adverse effects. The side effects are mostly due to the fact that colchicine binds preferably with β-tubulin IV, which is one of the most abundant tubulin isotypes in non-pathologic tissues [17], [18]. Upon oral administration, colchicine causes nausea, vomiting, diarrhea and stomach upset. Moreover, colchicine is always avoided for chronic kidney's disease (CKD) gout patients because 10-20% of bioactive colchicine is

eliminated by the kidney [18], [19]. Lowering the dose is not an option because of the very narrow therapeutic window. For these reasons, CKD patients, among all the others, would greatly benefit from a new medication, characterized by less toxicity and more anti-inflammatory effect.

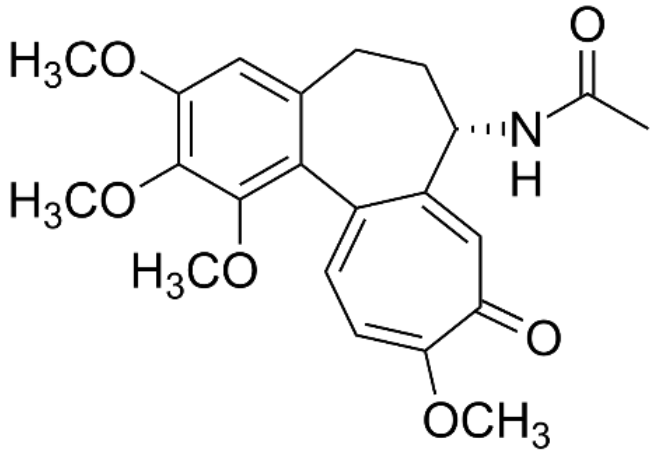

Figure 3: Basic structure of colchicine [20]

1.2.1 Colchicine effect on tubulin

Tubulin isotypes are homologous proteins consisting of a sequence of approximately 450 amino acids and, in particular, they are dimer of two 55-kDa polypeptides known as α-tubulin and β-tubulin. The α-tubulin and β-tubulin are approximately 40% identical at the sequence level [21], but differences in amino acid sequences can be found among all the tubulin isotypes, which are expressed by different genes, and as a function of these differences, various α and β isotypes of tubulin can be distinguished [22]. α and β tubulin heterodimers polymerize into microtubules, which are composed of 13 protofilaments assembled around a hollow core. Microtubules are major components of the cytoskeleton of all eukaryotic cells, and they are rigid hollow cylinders approximately 25 nm in outer diameter, as shown in Figure 4. They are involved in mitosis, cell motility, intracellular transport, and maintenance of cell shape [22]–[24].

A fundamental step in the drug developing process is the understanding of how colchicine inhibits tubulin polymerization. Microtubule-binding compounds are classified as stabilizing or destabilizing agents based on whether they promote tubulin polymerization or depolymerization. Colchicine and combretastatin, for example, cause microtubule

destabilization by preventing free tubulin dimers from being incorporated into the microtubule structure [25].

The α and β tubulin heterodimers polymerize into microtubules and colchicine acts on tubulin by binding the unpolymerized soluble tubulin and forming a tubulin-colchicine complex, which is poorly reversible. This complex binds to the ends of microtubules and brings a conformational change that prevents the elongation of the microtubule polymer [13]. At low concentrations, colchicine arrests microtubule growth and, at higher concentrations, it promotes microtubule depolymerization.

In gout treatment, colchicine was found to significantly affect the deformability and motility of human neutrophils in confined spaces, emphasizing the role of the cytoskeleton as a pharmacologic target during any inflammatory process in which activated neutrophils are involved [13], [26], [27].

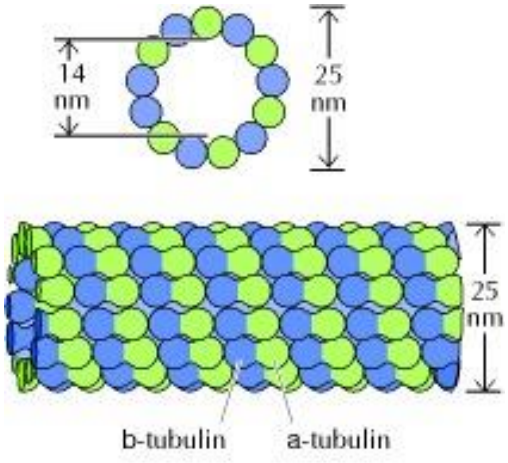

Figure 4: Structure of a microtubule [24]

Figure 5: Colchicine's effect on microtubules [26]

Toxicity has been the main reason why this drug did not pass all the tests for its approval by the FDA as a first line of gout treatment. But, since the efficacy of the drug is known and widely recognized, it would be unfortunate not to use it in this kind of pathology. This idea leads to the realization, through rational drug design, of a new derivative drug, which combines the efficacy of colchicine with more controlled toxicity [18]. This novel drug will be topically applied and delivered through a cream or a gel to the site of inflammation.

1.3 Drug discovery and development

Drug discovery and development are the processes used to find a new compound therapeutically useful in treating a disease. Drug discovery consists in the identification of a new molecule with desired effect on a specific target, using different paths. Researchers commonly discover a new drug by screening chemical libraries, by discovering novel insights into a molecular disease process, or by using existing treatments on other diseases, which is referred to as repurposing [28]. Once the molecule has been identified, the development begins. In the initial stage, experimental assays are carried out on cells (in vitro experiments) and then on animals (in vivo experiments). In vivo assays are intended to gather pharmacokinetic information (how the novel compound is absorbed, distributed, metabolized and excreted), but also information about its potential benefits, its mechanisms of action, its best dosage and its side effects (toxicity) [28]. At this stage, several compounds may be potential candidates for development as a medical treatment, however, after early testing on cells and animals, only a few compounds are eligible to pass onto the next testing phase. The development stage starts from microorganism and animal studies and continues through clinical trials to possibly end with a single drug candidate receiving regulatory approval. After passing the preclinical trials on animals, the new compound is tested on humans, and this stage is challenging in terms of a high failure rate. Indeed, the success percentage for new drug approvals remains low due to safety and efficacy failures when it comes to humans because pathologies and metabolism of drugs work differently in animals than in humans. Also, preclinical studies are carried on young, healthy animals, in contrast with human patients [29], [30].

To follow the proper steps shown in Figure 6, huge investments of money and time are requested from the pharmaceutical companies with no guarantees that the novel drug will be put in the market. The cost of developing a single new drug, including commercialization,

varies from US $ 800 million to US $ 1.7 billion [31]. Once started, the process can be stopped at any investigative stage, however, the money invested by the company cannot be recovered [31]. In the last decade, various approaches have been developed to shorten the research and development cycle, reduce expenses and risk of failure in drug discovery. Considerable interest has grown in the field of mathematical and computational modeling which allows to predict virtually all the possible interactions between drug molecules and proteins.

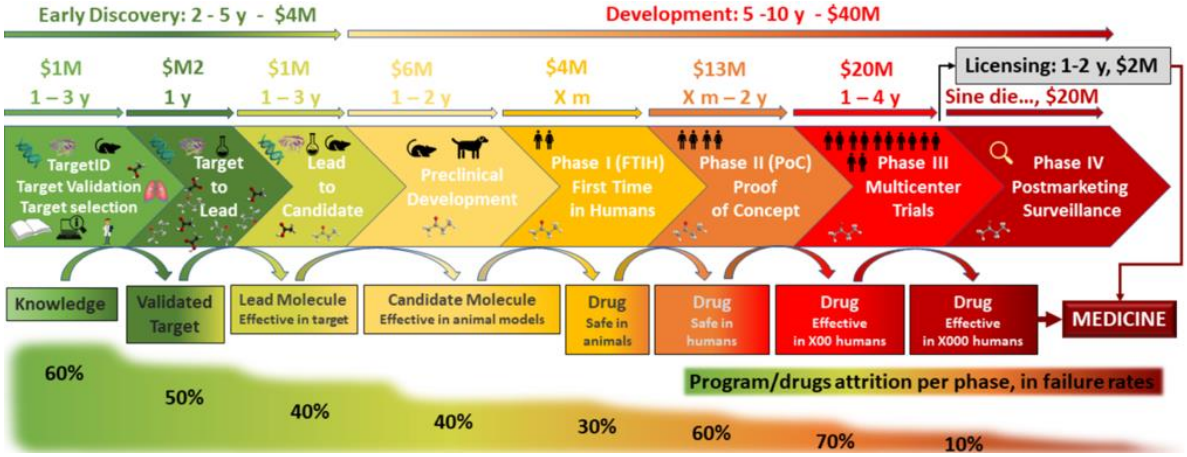

Figure 6: Drug development process [32]

1.3.1 Mathematical modeling in drug design

Mathematical modeling is a fundamental tool for solving problems arising from the study of complex systems, whether originating from physical, chemical, or biological sciences or from economic and social systems. A mathematical model is basically a set of equations that define the evolution of a state variable over an independent variable [33]. Mathematical models have several advantages since mathematics is a very precise language with well-defined rules for manipulations handled by computers performing numerical calculations. The majority of interacting systems in the real world are far too complicated to be modeled in their entirety. Thus, the first step to build a good model is identifying the most important real system components, excluding all the other elements. The outputs' correctness depends upon the state of system knowledge and how the model is built [34]. When the equation's number and the variable state dimension equals, then the model is defined as consistent.

To build a robust model, the discretization of the system is necessary for an approximation of the physical reality, but it leads to a difference between what the model predicts and what actually happens. This difference must be negligible. Uncertainty is related to several factors such as the infinite number of physical variables, the mathematical problem and the measurements affected by undefined errors [33], [34].

Generally, mathematical methods can be classified according to the type of outcome and the dimensional level used to describe the system, as shown in Figure 7.

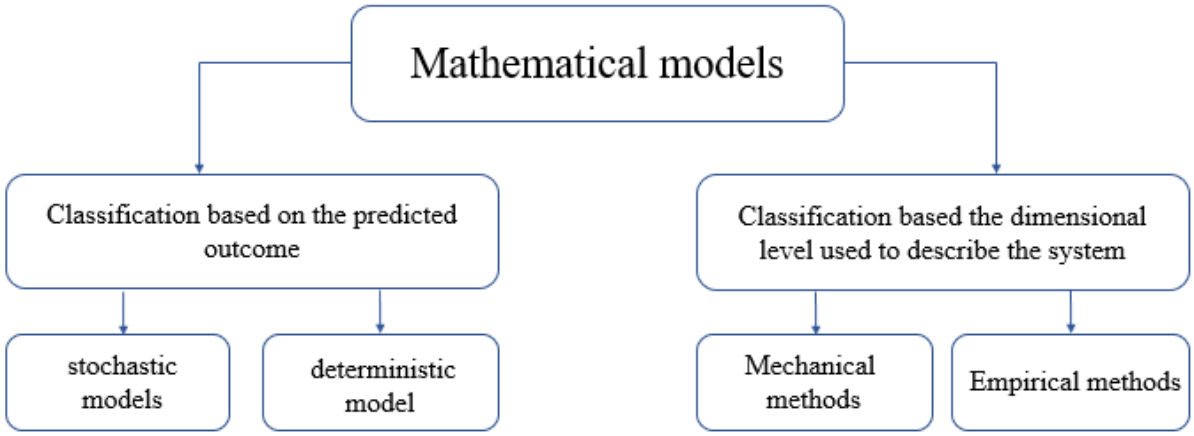

Figure 7: Classification of mathematical models

Stochastic models consider all the random variables of the problem, so the outcome is always different, while in the deterministic models all the random variables are ignored. In the mechanical methods all the informations about how a process evolves and changes are taken into account, in the empirical methods, instead, all these informations are neglected. Only the conditions linked to every change are considered [33], [34]. In drug design, the commonly used approaches can be categorized into structure-based drug design (SBDD), ligand-based drug design (LBDD) and sequence-based approaches. SBDD methods are based on the knowledge of the target macromolecule structure. This knowledge is acquired from crystal structures, NMR data and homology models. When three-dimensional (3D) structures of potential targets are not available, LBDD tools can provide crucial insights into the nature of the interactions between drug targets and ligands. These tools include quantitative structure-activity relationship (QSAR), pharmacophore modeling, molecular field analysis and 2D or 3D similarity assessment. In recent years, when either target structures and ligand information were not available, sequence-based approaches have been

adopted. They use bioinformatics methods to analyze and compare multiple sequences. Currently, all single methods are unable to fulfill the practical needs of drug discovery and development. Therefore, combinational and hierarchical strategies that employ multiple computational approaches have been frequently and successfully used. To access the wide chemical diversity and to follow the rules dictating a pharmacokinetic or metabolic profile (absorption, distribution, metabolism and excretion), computer-aided techniques are essential [35].

1.4 Novel Colchicine derivative

Prof. Jack Tuszynski and his team (Division of Experimental Oncology, Cross Cancer Institute and the University of Alberta, Edmonton, Canada) designed the so-called CCI-001, a novel colchicine derivative, with reduced general toxicity and increased selectivity and specificity. The summary formula of CCI-001 is $C_{23}H_{27}NO_6S$, it is characterized by a molecular weight of 445,5 g/mol and the main structure is shown in Figure 8. CCI-001's LogP is 2.58 and its solubility in water at pH= 7.4 is of 0,007 mg/mL [18]. In the rational design of Colchicine derivatives the main steps that were followed, are shown in Figure 9. The thiocolchicoside derivative used in this work, the CCI-001, was initially designed for bladder cancer chemotherapy but modeling and in vitro assays results show CCI-001 as a potential candidate to treat gout due to its efficacy and reduced side effects [36].
The CCI-001 compound has been approved for clinical trials for bladder cancer but it can also be repurposed as an effective therapeutic agent for gout, provided a proper formulation as a topical medication.

It is known that colchicine preferentially binds β-tubulin IV isotype (and also to a lower degree other tubulin isotypes) and this causes toxicity. But, if the basic structure of colchicine (Figure 3) is modified to bind preferentially another β-tubulin isotype, theoretically, colchicine will attack mainly the cells which overexpressed that β-tubulin isotype. So far, there is no evidence of cells with identical expression of β -tubulin isotypes and this can be used for our purpose [18]. Neutrophils accumulate in specific sites causing gout inflammations, so they're the target cells for the purpose of anti-gout therapy development. The identification of the isotype expressed by neutrophils makes it possible to modify the colchicine in a way that the affinity with neutrophils is enhanced.

The innovative idea is, thus, to target the β-tubulin isotype cluster of neutrophils, which is isotype VI, to generate a less toxic and more potent drug. This will be extremely useful because the novel drug will be, not only less toxic but also effective in lower doses [18]. In a more effective way, the novel colchicine derivative will bind specifically β-tubulin VI, influencing cell activities known to be central in inflammatory pathways to the pathogenesis of gout. It has been demonstrated through in vitro and in vivo experiments that CCI-001 is able to downregulate MSU-induced neutrophil responses [6], [18]. This property has been assessed observing that CCI-001 can inhibit the production of several molecules known to increase the inflammation response, such as IL-8, cytoplasmatic calcium and reactive oxygen species (ROS) [37].

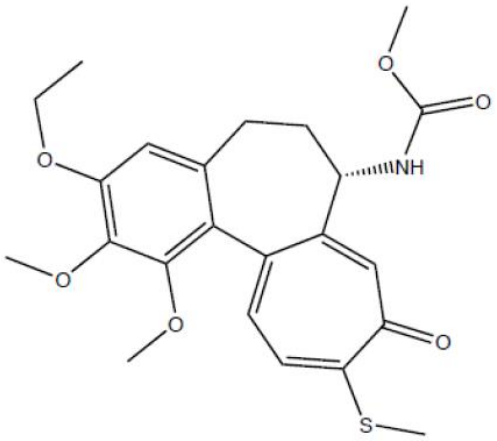

Figure 8: Chemical Structure of CCI-001 [18]

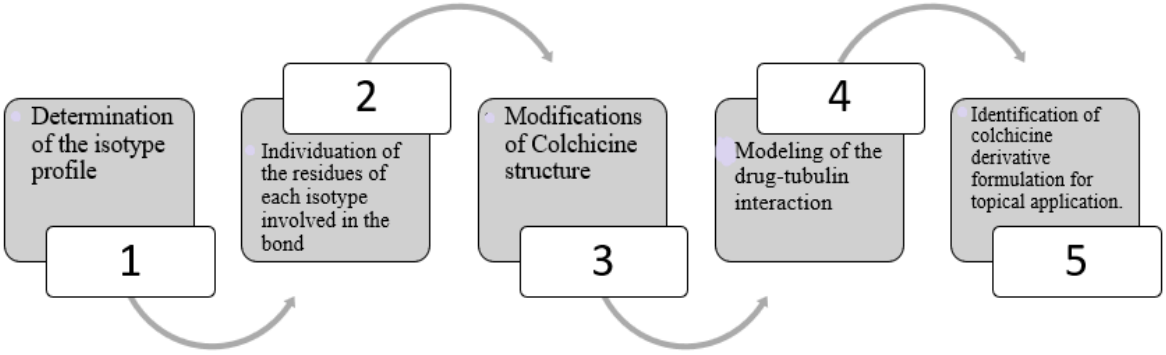

Figure 9: Rational Design of Colchicine derivative

1.5 Development and optimization of a Topical therapeutic product. Principle and Criteria

Topical and transdermal medications have been widely used in therapy in the last decades. Nevertheless, there is significant confusion between the two terms, which are wrongly used interchangeably. While both topical and transdermal products are applied to the skin, they deliver active agents to different sites. Topical medications are designed specifically with the intent to release the drug in the same site of the application. Transdermal medications, instead, are designed to use the skin as a vector. The main idea at the base of transdermal medications is to use a specific technology that makes it possible for the drug to penetrate the skin barrier and to reach systemic circulation [38].

Skin is the most extensive organ of the body comprising about 10% of the entire body mass. It is composed of three distinctive layers: the epidermis, the dermis and the hypodermis (Figure 10)**.**

The epidermis is the outermost layer; its cells are renewed on daily basis and it has four or five different layers, depending on the location in the body. Epidermis with four layers is referred to as “thin skin”, while the one with five layers is known as “thick skin”.The dermis is the thickest layer of skin and contains nerves, blood vessels, sweat glands, oil glands and hair follicles. The hypodermis is the deepest layer of the skin. This layer is made up mostly of fatty tissue, which helps to isolate the body and as energy storage [39], [40]. Therefore, the main differences between topical and transdermal deliveries are that topical medications must guarantee that the concentration of the drug is maximized in one of the three layers of the skin, while it must be minimal in the systemic circulation. Conversely, in transdermal delivery, the concentration of the drug must be maximized in the systemic circulation and minimized in the different layers of the skin [41].

Every layer of the skin is characterized by specific structures and functions [38] and for this reason, different routes of penetration are needed. In particular, three of them have been investigated in literature: the transcellular route through the stratum corneum (SC), the intercellular route, and the anexial route through hair follicles, sweat glands, and sebaceous glands [42]. SC is the layer that affects drug absorption the most [43]. It is the outer layer of the epidermis and it is made of 15 to 20 layers of corneocytes. Its structure consists of a dense aggregate of adherent cells, packed with lipid [44]. In its dry state has a thickness of 10 to 15 μm, while, when hydrated, it swells, and its thickness may reach up to 40 μm,

increasing its permeability. The role of SC in limiting drug absorption can be explained by its high density. According to [44] stratum corneum seems to be between three and five orders of magnitude less permeable than the dermis. While, regarding permeability, there is no significant difference between epidermis and stratum corneum alone. According to its structure, SC allows penetration of polar molecules through the water accumulated in the layer, while non-polar molecules may be able to dissolve through the lipid membrane. In both cases, the diffusion through SC is a difficult, slow and completely passive process [42], [45], [46].

Follicles and glands, instead, are present as open pores underneath the skin surface and this allows the penetration both of polar and non-polar molecules [42], [47], [48].

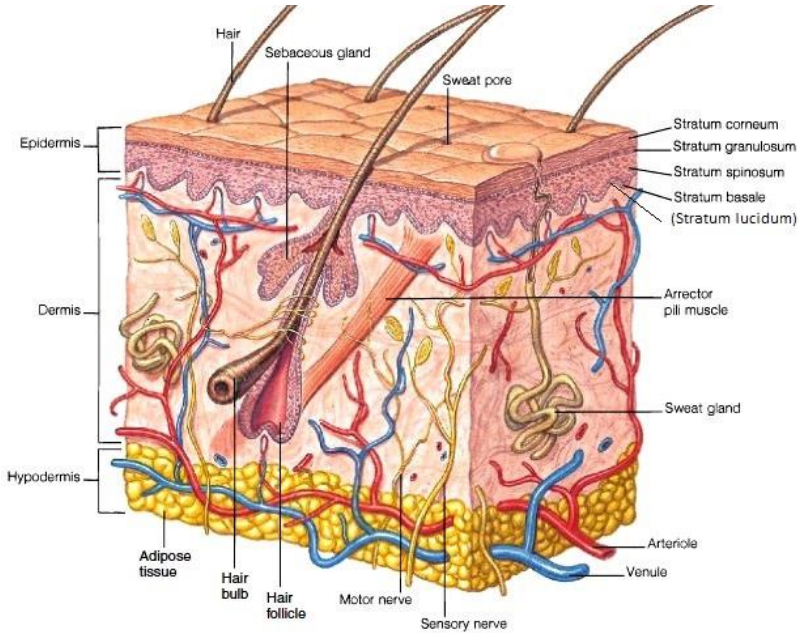

Figure 10: A cross-sectional view of the skin structure [39]

Since the skin and, in particular, the SC works as an active barrier, to have correct penetration and release of the drug an optimal topical formulation is required. Drug transport is a process comprising different steps: *a)* drug dissolution and release from the formulation; *b)* drug partitioning into the stratum corneum; *c)* drug diffusion across the stratum corneum; *d)* drug partitioning from the stratum corneum into epidermis; *e)* diffusion across the viable epidermis layers into the dermis [43].

In this perspective, several aspects regarding both the formulation and the drug must be taken into account. Referring to formulations, properties such as pH, viscosity, organoleptic

properties and stability can affect the behavior of the final product. For example, pH in formulations is extremely important because many drugs are weak acids or bases. Setting the pH in topical formulations of these compounds influences the degree of ionization. According to the pH partition hypothesis, it is generally accepted that the non-ionized species of an acidic or a basic molecule is more permeable across biological barriers than the ionized [49]. The human skin pH values range between 4.1 and 5.8, depending on the location in the body, so the application of formulation with high or very low pH values can harm the skin. Hence a moderate pH value could be more appropriate for topical delivery. Since skin has a pI~4, a proper pH value for topical delivery system must be around 5 [50]. Lower pH values are also acceptable, but they may increase the risk of irritation reaction [50].

Also, viscosity must be carefully evaluated, because differences in viscosity can affect the permeation of the drug through the skin. According to Bolla et al. and to Kadhum et al. [51], [52], lower viscosity can enhance the permeation of the drug. That can be explained considering that with lower viscosity of the vehicle the drug is more easily released. Properties such as concentration and physical state of the drug in the vehicle are equally important. The concentration of the drug in formulations has a key role because the flux increases with the increasing concentration of the dissolved drug. At a higher concentration above the solubility, the excess drug works as a storage and this helps in maintaining constant flux for a prolonged period, according to Fick's law [43]. The physical state of the drug in the formulation can affect the permeation, as well: enhanced permeation is attributed to the solubilized drug. Thus, the solubilized systems have advantages such as increased efficacy at lower concentrations and low drug irritation potential [43]. Major attention must be also focused on the effects of the excipients. Drug interaction with wrong excipients can alter its ability to permeate through the skin, its ability to be not metabolized in the skin, its ability to stay dissolved at the right concentration and its capability to achieve desired release rates. Moreover, the right excipient selection can minimize surface adsorption, have less immunogenicity, and avoid degradation. A small change in the formulation can make a large difference in the efficacy of topical treatments. The formulation ensures that the drug substance is delivered to the right target site and that it maintains dosage integrity, drug transport, and active duration [41], [53].

To treat gout the best choice would be using a topical formulation of the drug, because the intent is to ease the pain up. To do so the action must be localized within the skin because

the insufferable pain gout patients suffer from is caused by extravasation of all components from the inflammation area. In this way, the drug won't be curing the disease, but it will treat symptoms. The topical administration of CCI-001 rather than oral is preferable, in this scenario, for several reasons: first of all, by using a topical delivery, the activity within the local site of inflammation is enhanced, keeping the systemic side effects completely under control and guaranteeing that the drug is delivered selectively to a specific site. Secondly, topical delivery makes it possible to avoid the first-pass metabolism, providing effectiveness in low doses and by continuous drug input. Last, but not least, topical delivery is preferred by the patient because it is easy to apply and it is suitable for self-medication [54].

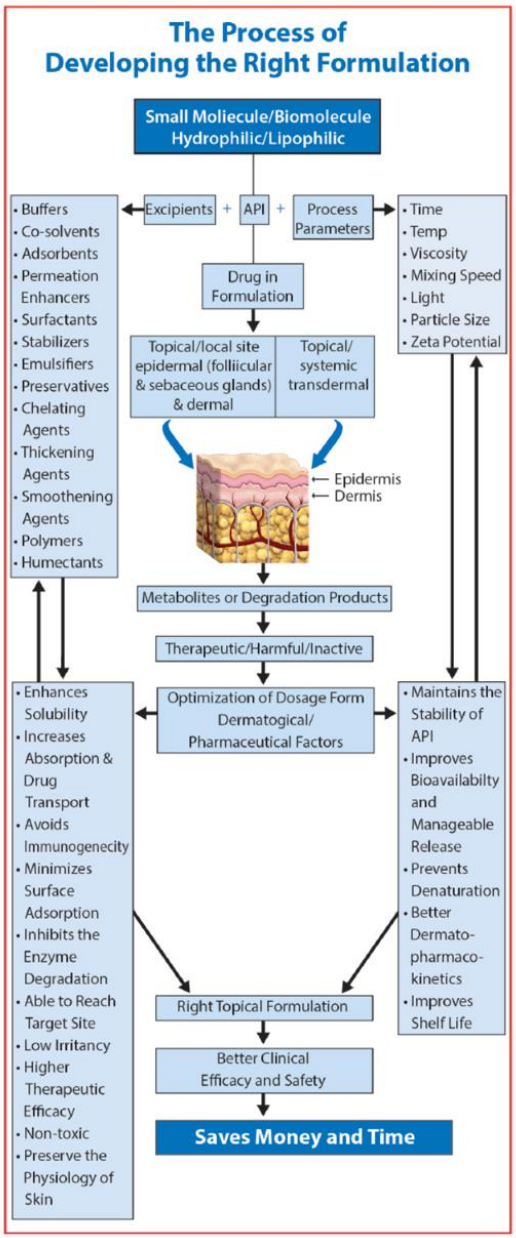

Figure 11:Development of the right formulation [41].

1.6 Bioavailability of a drug

When studying a new drug formulation, it is of paramount importance to know what the minimally effective and maximally tolerated toxic values of a compound are for the human body. One parameter that can give us some insight is: the bioavailability; defined as the fraction of a drug that reaches systemic circulation unaltered. This definition can be applied when systemic circulation is the vehicle through which the drug can reach the target. When the drug is administrated topically, bioavailability is more rigorously defined as the "temporal pattern of free drug concentration at the target site" [55]. With this approach, though, bioavailability remains largely theoretical due to the difficulty of quantifying drug within the skin. Bioavailability is measured with different methods, depending on how the drug is administrated [56].

Despite the route of administration, to obtain a therapeutic effect, the concentration of the drug at the target site must remain in the so-called therapeutic range. To define the therapeutic range, some parameters are to take into account:

- C_{max}= it's the maximum concentration that can be reached in the blood (systemically delivery) or in the skin layers (topically delivery) and it gives indications about therapeutic and side effects of the drug;
- T_{max} = time after which maximum concentration is reached;
- $T_{1/2}$ (half-life) = time after which the concentration of the drug is reduced by 50%;
- AUC (area under the curve): indicates the total exposure of the body to the drug;

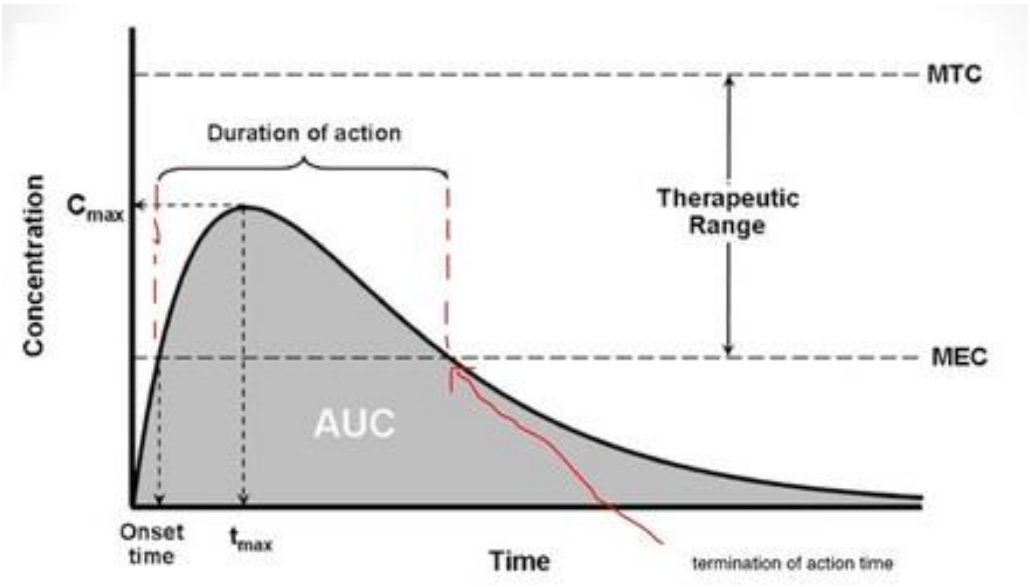

Figure 12: A typical profile of the drug concentration in the body over time [57].

Therapeutic range, as showed in Figure 12, is the window between the minimum toxic concentration (MTC) and the minimum effective concentration (MEC). Above MTC the drug can have serious side effects, under MEC, instead, the drug shall have no effects at all [58].

The route of administration (ROA) has a significant impact on both the rate and extent of bioavailability. Let's consider three systemic ROAs: oral administration, intravenous administration and respiratory administration. Each one of these is characterized by a unique ability to reach a specific drug concentration in a specific temporal arc. In the case of oral administration, the drug will meet different barriers before it is released in the systemic circulation: it goes through the stomach, the gut, the liver and eventually it reaches lungs and heart. This means that C_{max} will be lower and T_{max} will be reached later. In intravenous and respiratory administrations drugs meet fewer barriers. So, the maximum concentration will be higher and the maximum time will be reached sooner. In particular, the respiratory administration is characterized by the highest C_{max} and the lowest T_{max} because in this case, the drug goes straight to lungs and heart which pushes it into the system [56], [58], [59]. As can be seen, in systemic delivery, the pharmacokinetic profile depends mostly on the barriers drug meets in its way to the systemic circulation. In contrast, in topical delivery, the pharmacokinetic profile depends on several parameters: properties of the skin, chemical characteristics of the drug, formulation in which it is administered, etc... Moreover, it is common practice for patients to apply more than one topical medication concurrently. The coadministration may alter the formulation, changing drug absorption [60]. For these reasons, the determination of drug concentration in skin layers, after topical application, is a great, unsolved challenge. The regulatory agencies are considering different possible approaches since the protocols followed in systemic formulations could not be applied for topical ones, but as of now, a definitive protocol has not been achieved yet [53].

If the drug is applied systemically, bioavailability is evaluated by measuring the drug concentration in urine or plasma. If the drug is applied topically the concentration in plasma or urine is not the parameter to measure, because of the extremely low concentrations achieved. Hence, in this case, other methods are used and the most common is known as tape stripping. Tape stripping consists of sequentially removing microscopic layers (typically 0.5–1 μm) of stratum corneum to assess cutaneous drug or excipient levels in the skin [55], [61].

2 Materials and methods

Rational drug design is one of the greatest challenges researchers are focused on nowadays. Humanity finds itself constantly fighting life-threatening diseases and the demand for efficacious drugs is always increasing. In this context, rational design finds great growth opportunities; as the transition from traditional pharmacology towards computational modeling has enabled to enormously reduce time, costs and errors in developing new compounds which can quickly be modeled by software [62].
Mathematical methods provide extremely useful tools because they can predict two aspects: pharmacokinetics of the compound, typically described by a set of differential equations, broadly representing the physical compartments where different effects take place, and pharmacodynamics, describing the relationship between the drug concentration and its killing efficacy. By combining these two models, predictions of the likely efficacy and decay over time of the novel drug can be achieved. In this way, mathematical models make it possible to save money and time in the process, because a major part of the experiments is carried out computationally and not practically, and allows to use a reduced number of animals [63].

In drug discovery, computer-aided drug design (CADD) is one of the most effective methods for reaching these goals. CADD techniques aim to be used for the rapid evaluation of chemical libraries. In this way, the early-stage discovery of new active compounds is sped up. The typical role of CADD is to screen out large compound libraries into smaller clusters of predicted active compounds (figure 13). This enables the optimization of lead compounds by improving their biological properties [64].

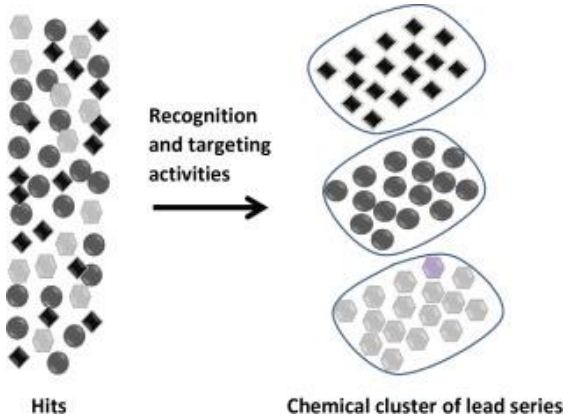

Figure 13: Screening of Drug-Like Compounds [64].

3 Results

To identify the tubulin isotype expressed specifically by neutrophils, Western Blot analysis was applied, by using isotype-specific antibodies that are commercially available. The analysis was carried on lysates of leukocytes isolated from healthy, human donors. Monocytes, macrophages, neutrophils, platelets and lymphocytes express distinct quantities of α and β-tubulin isotypes. To determine the isotype overexpressed by neutrophils, a comparison of the intensities of the bands of each isotype was performed by immunodetection [18].

Once the isotype of interest is found, its genomic expression can be analyzed. This step is fundamental to identify the binding sites with the drug. Homologous models of β-tubulins were then realized using a common software; the basic structures were provided by research databases. The software builds the models using an alignment of the sequences already known. Missing regions are predicted by simulated annealing using a molecular mechanics model. Once β-tubulins models are available, complex models representing the bond with Colchicine are provided.

A total of 29 residues of β -tubulin where identified within the 6 °A cut-off from the bound colchicine, namely: 235-240, 246-257, 312-316 and 347-352. Of these 29 residues, seven positions show differences between the β-tubulin isoforms [65], [66].

Knowing the genetic expression of the target, the basic structure of Colchicine (Figure 3) was modified by adding different groups such as an alkane, alkene, ester, ether, aromatic in different positions. Molecular Dynamics simulation (MD) makes it possible to anticipate conformational and structural 3D-changes when molecules interact. An initial model of the system is required and it is obtained from either experimental structures or comparative modeling. Once the system was built, forces acting on every atom are obtained by deriving equations, and potential energy was computed from the molecular structure [67].

In previous studies, the basic structure of colchicine was computationally designed by adding different groups such as alkanes, alkenes, esters, ethers, aromatics in different positions [65],[68]-[89].

In this way, many derivatives were generated and each one of them was characterized by different affinities in the bond between the drug and its protein target, which is tubulin.

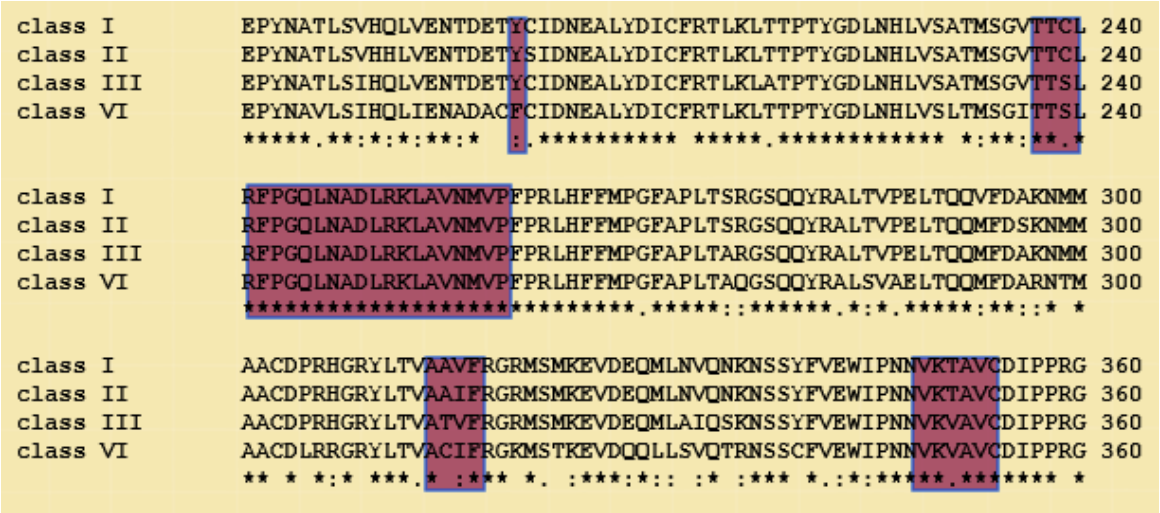

Figure 14: Residues which make direct contact with the ligand [107]

4 Topical formulation of a novel drug to treat gout

The central goal of structure-based CADD is to design compounds that bind tightly to the target, with a large reduction in free energy, improved drug metabolism and pharmacokinetics.

Absorption, distribution, metabolism and excretion (DMPK/ADMET) properties are also increased. The successful application of these methods will result in a compound that has been validated in vitro and in vivo and its binding location has been confirmed, ideally through a cocrystal structure.

The objective of the next part of this thesis is, thus, to achieve the ultimate goal: the development of a proper formulation, that guarantees the correct delivery of the new compound to the target site of gout-related inflammation.

Introduction

4.1 Drug delivery system

Drug delivery systems can be described as engineered technologies used to administer a pharmaceutical compound into the body. The concept of drug delivery has changed a lot in the past few decades and there have been many important breakthroughs in this field. The study of drug delivery dynamics is important also to minimize, if not eliminate, possible side effects. For this reason, attention is focused on new and improved local delivery systems able to guarantee the control of the release in time and space [90], [91].

Control in space is based on the concept of targeting. In the targeting phase, the delivery system recognizes the pathologic cells. Only when the target-has been identified the release of the drug is triggered. In the study case, the targeting phase is somehow carried out by the drug itself. In this way, we can be reasonably sure that at the target site the released concentration is within the therapeutic window (blu lines in figure 15), while at the non-target sites the released concentration is within the so-called systemic window (yellow lines in figure 15). Values exceeding systemic window cause side effects in the non-target sites [58].

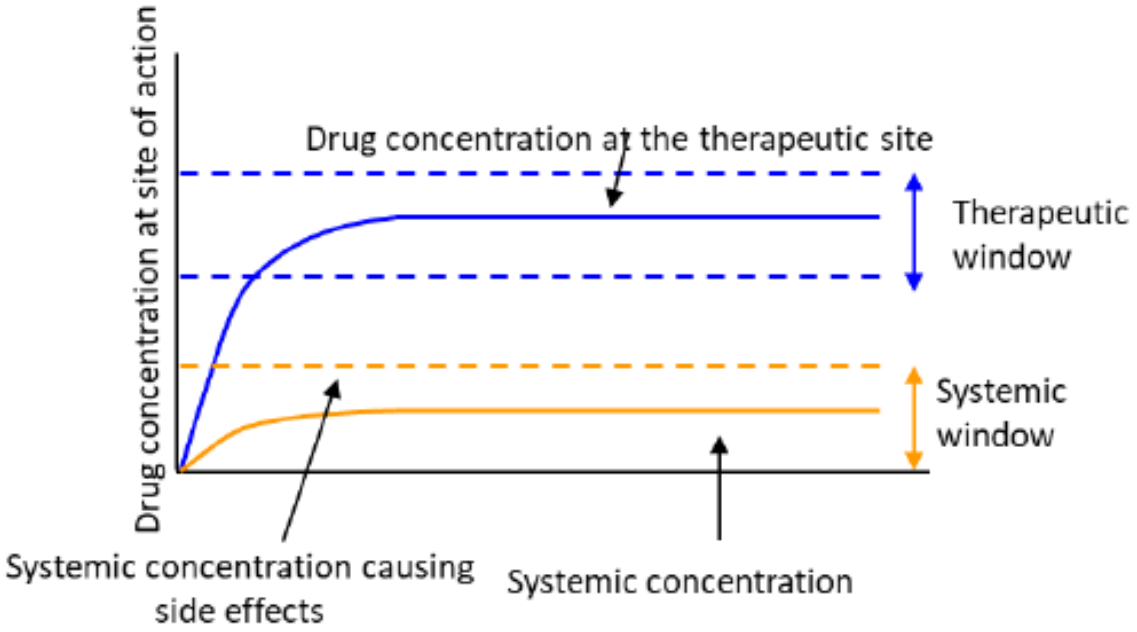

Figure 15: control in space of the release [58]

In contrast, control in time is about the ability of the local delivery system (for example polymeric nanoparticles) to release the drug with a concentration that remains constant in time. As previously stated, every drug is characterized by its therapeutic window. This consists in a range of concentrations. Values exceeding the range indicate that the drug is toxic, while values below the range imply that the drug has no therapeutic effect at all. So, for the drug to be most effective, it must be administrated with a concentration that remains constant and always included in the therapeutic window (blue line in Figure 16). With classic parenteral systems, this control in time is not guaranteed, because the administration consists of consequent injections. After every single injection, concentration follows a parabolic trend, reaching peaks out of the window (red lines in Figure 16). Giving only one injection won't work because the drug does not remain in the organism long enough to produce any effect, while with multiple injections high and unneeded dose of drug is introduced in the body, which may result in detrimental side effects [58].

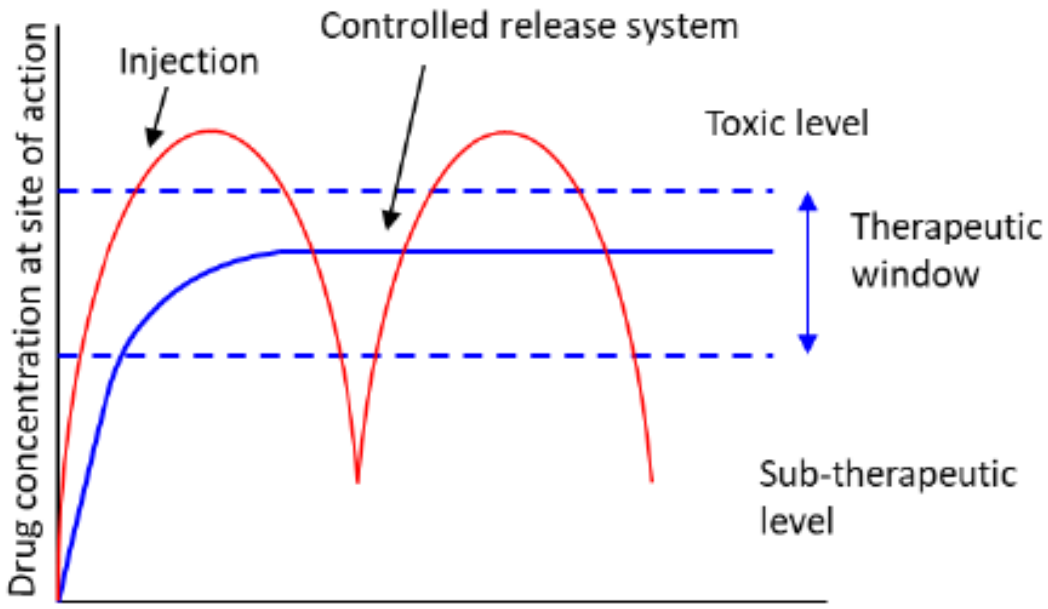

Figure 16: Control in time of the release [58]

4.2 Gel formulation

Gel formulation is one of the most common topical delivery systems along with cream, pastes and ointment. Gels are systems characterized by a semisolid composition. They exhibit an external solvent phase, which is fluid, while the minority component forms a semisolid matrix. This matrix consists of a gelling agent such as carbomer or natural gumis, and when it is dispersed in purified water or in organic solvent forms a uniform dispersion. Gel's consistency and rheological properties derive from polymers that interact with water to thicken and increase viscosity. Polymers may interact physically, by chain entanglement, or chemically, by ionic or hydrophobic/hydrophilic interactions.

Chemical gels are associated with permanent covalent bonding while physical gels result from relatively weaker and reversible secondary intermolecular forces, such as hydrogen bonding, electrostatic interactions, dipole-dipole interactions, Van der Waals forces and hydrophobic-interactions [92]. Polymer matrix in gels provides physical stabilization and prevention of migration of suspended crystals, maintenance of product homogeneity throughout the shelf life, clean application, easy spreading and acceptable aesthetics [93].

Gels' high content of water permits greater dissolution of drugs and facilitates their migration through the vesicle. Moreover, by their high water content, they can hydrate the skin retaining a significant amount of transdermal water. Gels can be divided into two categories, according to the nature of their liquid phase: hydrogels contain water and organogels contain organic solvent [94]. Gels should fulfill some indispensable properties when they are intended to be used for medical applications. Ideally, the gelling agent for pharmaceutical use should be safe, and inert to reactions with other formulation components. The gelling agent included in the preparation should also produce solid-like structure during storage that can be easily broken when subjected to shear forces generated during topical application. It also should possess suitable anti-microbial properties to prevent a microbial attack. Finally, the topical gel should not be tacky and should be sterile [92].

5 Material and methods

5.1 Chemicals

The compound CCI-001 ($C_{23}H_{27}NO_6S$) in the amount of 126 mg was provided by the Department of Oncology, University of Alberta, Edmonton, Canada.
Carbopol 980® (Lubrizol Advanced Materials, Inc. Cleveland, OH), Methyl Paraben (Cooper Pharma, Casablanca, Morocco), Propylene Glycol (Cooper Pharma, Casablanca, Marocco), Triethanolamine (Cooper Pharma, Casablanca, Marocco), Ethanol and Acetonitrile were kindly provided by University of Lyon 1 Claude Bernard, Laboratory of Automatic Control, Chemical and Pharmaceutical Engineering (LAGEPP).

As a gelling agent, Carbopol 980 (C-980) was chosen among other carbomers because of its capacity to form gels in aqueous solution, compatibility with many active ingredients, and good patient acceptance. C-980 is a hydrophilic polyacrylic acid polymer and it is used as a pH-sensitive gelling thickener agent [95]. Carbopol 980 can be used only after neutralization with TEA (triethanolamine) or other bases because the unneutralized dispersions of Carbopol have very low viscosities and bad applicability. Neutralization of the polymer carboxylate groups using an alkaline substance makes them highly-ionized to form rigid gels. TEA, particularly, neutralizes the carboxylic groups of the polymer and facilitates the formation of cross-links between the polymeric chains [96]. The typical use level of Carbopol as gelling agent is between 05% -2% (w/w) depending on the desired viscosity. The direct dispersion method can be used since it is effective with concentration up to 1.5 % (w/w) and we are using 0.5% [97], [98].

Therefore, once a neutralizer is added to the dispersion, thickening gradually occurs. Optimum viscosity is typically achieved at a pH of 6.5-7.5 [99]. Before making contact with water C-980 cross-linked polyacrylic acid is tightly coiled (Figure 17). Once dispersed in water, C-980 begins to hydrate and the carboxyl groups of the acrylic acid backbone will ionize the hydrion. Thanks to the electrostatic repulsion force and the effect of hydrogen bonding, cross-linked polyacrylic acid will partially uncoil, forming a colloidal dispersion (Figure 18). When the dispersion is finally neutralized, the molecule is ionized and generates negative charges along the backbone of the molecule. In this way, the electrostatic repulsion

force of the carboxylate ionization of the polymer backbone will be much stronger, so the neutralized mucilage will swell (Figure 19) [100].

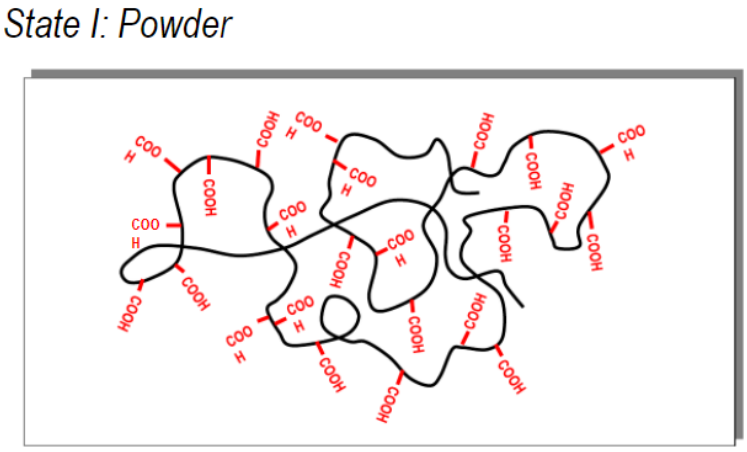

Figure 17: C-980 before hydration [101]

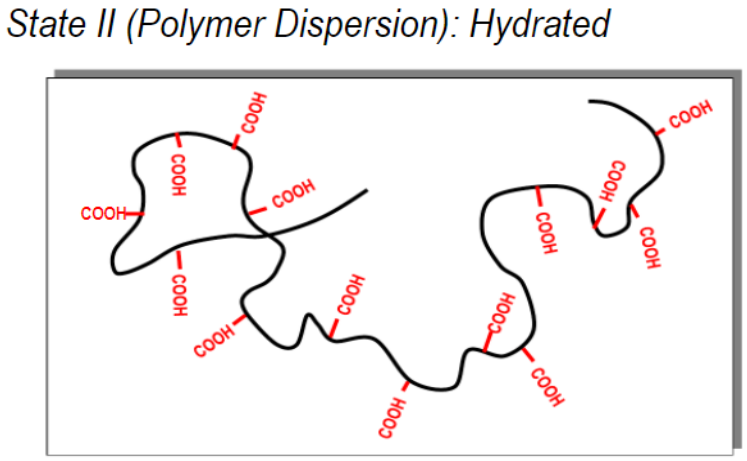

Figure 18: Hydrated C-980 [101]

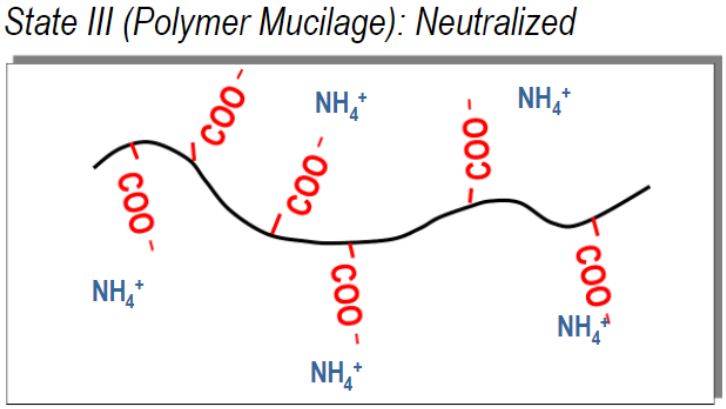

Figure 19: C-980 after neutralization [101]

Methylparaben is used as a preservative agent. It is preferred to other parabens, because it is characterized by a short chain so it dissolves easily in hot water. The high temperature of 65-70°C is reached to prevent Methylparaben re-crystallization [102].

Propylene glycol is used as a permeation enhancer, together with Ethanol. Their presence modifies the partitioning of the drug between the gel and the skin. It has been shown that the presence of Propylene Glycol and Ethanol enhances the permeability the most. It is also expected that Propylene Glycol will increase both viscosity and pH of the gel because, at the pH conditions reached in the preparation, Propylene Glycol tends to be more alkalized than C-980, easing up carbomer swelling. The swelling increases pH and polymer cross-links. Polymer cross-links increase viscosity [103]. As will be shown later, Propylene Glycol effects on pH and viscosity are expected [104].

5.2 Instruments

Instruments	*Use*
Scale	Weight all the components
magnetic hot plate stirrer	Heat the distilled water and to mix the different solutions
overhead stirrer	Mix major amounts of gel
pH meter	Evaluate pH
viscosimeter	Evaluate viscosity
HPLC machine	Evaluate the exact concentration of the compound in the gel.

Table 1: List of instruments and their use

5.3 Formulation of Carbopol 980 based topical gel

The protocol implemented to prepare the topical gel includes a list of reagents with quantities and conditions of reaction. In the beginning, four different formulations were implemented without adding the CCI-001, to define the best one in terms of pH and viscosity. To prepare the aforementioned samples of Carbopol 980 based gel, direct dispersion method was used and the following steps were followed:

- Methylparaben was dissolved in distilled water at 65-70°C, then the temperature was lowered at 40°C and the Carbopol 980 was added to the solution.
- When the Carbopol 980 was completely hydrated, the solution was stirred with moderate rate agitation of approximately 150-200 rpm [97], [105].
- Propylene glycol and Ethanol were added.
- The pH of the resulting solution was adjusted to 6.5-7, adding diluted Triethanolamine (dTEA) and the stirring was persued till the gel was formed.

The dTEA is a solution containing 33% (w/w) of TEA with a pH of 11.10.

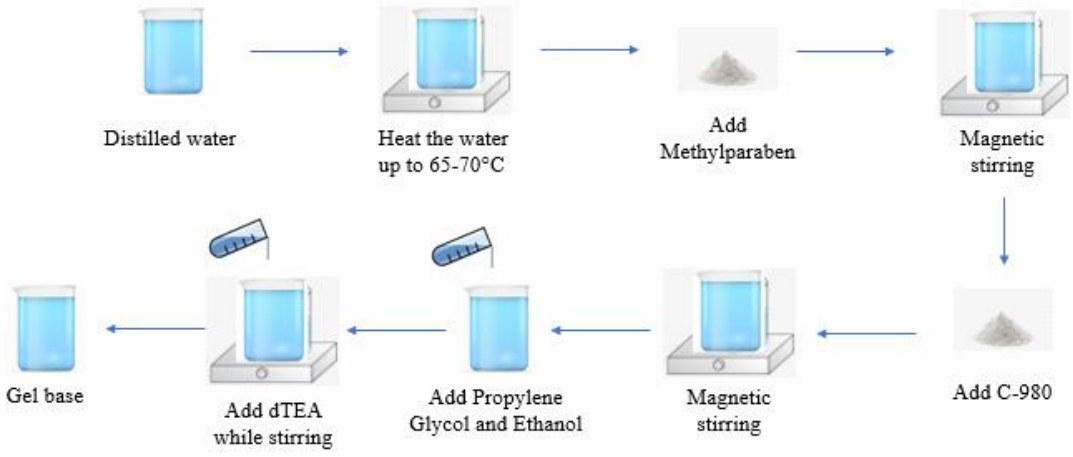

Figure 20: Formulation of C- 980 based topical gel protocol

In Table 2, the list of all reagents and respective amounts to prepare 200 g of each formulation can be found (this great amount of product is necessary to carry out the rheological analysis).

	F1	*F2*	*F3*	*F4*
Distilled Water (g)	186.8	185.8	185.222	196.8
Carbopol 980 (g)	1	2	1	1
Methyl Paraben (g)	0.2	0.2	0.2	0.2
Propylene Glycol (g)	10	10	10	/
Ethanol (ml)	/	/	2	/
Diluited TEA (ml)	2	2	2	2

Table 2: Reagents and amounts for each formulation without CCI-001

In Table 3, instead, the percentage of every reagent, for each formulation, is reported.

	F1	*F2*	*F3*	*F4*
Carbopol 980	0.5 %	1%	0.5%	0.5%
Methyl Paraben	0.1%	0.1%	0.1%	0.1%
Propylene Glycol	5%	5%	5%	/
Ethanol[1]	/	/	0.79%	/
Diluited TEA	1%	1%	1%	1%

Table 3: Concentration (w/w) of every reagent for each formulation without CCI-001

[1] [1] ρ_{et}=789 $\frac{kg}{m^3}$ a T=20°C [106].

5.4 Formulation of Carbopol 980 based CCI-001 topical gel

At this point, the CCI-001 was added, in three different concentrations. Since the amount of compound was limited, only 20 g of final product for each concentration was prepared.
To produce 20 g of Carbopol 980 based gel, direct dispersion method was used and the following steps were implemented:

- Methyparaben was dissolved in distilled water at 65-70°C, then the temperature was lowered to 40°C and Carbopol 980 was added to the solution. The solution was stirred with moderate rate agitation of approximately 150-200 rpm.
- Separately, CCI-001 was added in Ethanol
- CCI-001 solution was incorporated in the Carbopol solution, with continuous stirring.
- Propylene glycol was added.
- The pH of the resulting solution was adjusted to 6.5-7, adding diluted Triethanolamine (dTEA) and the stirring was pursued till the gel was formed.

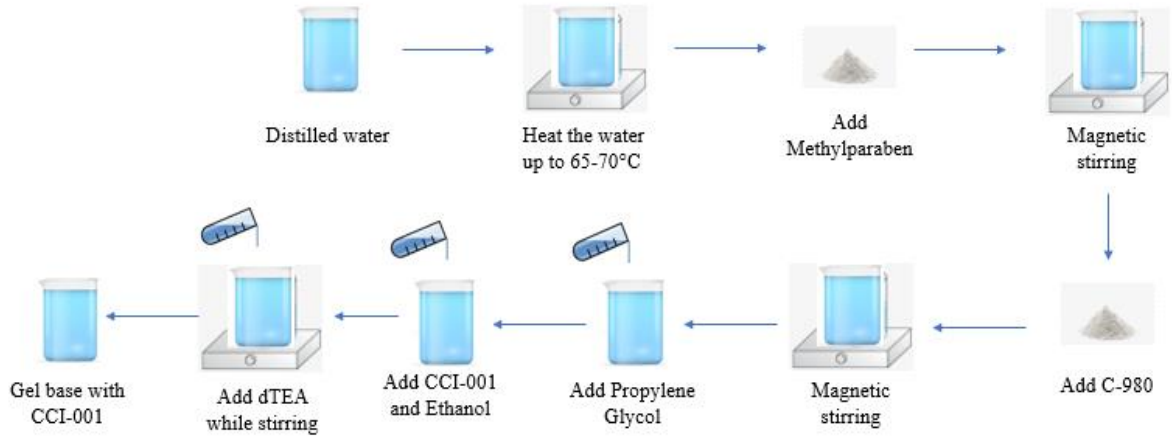

Figure 21: Formulation of Carbopol 980 based CCI-001 topical gel protocol

Table 4 lists all reagents and respective amounts used to prepare 20 g of each formulation.

	F4	*F5*	*F6*
Distilled Water (g)	18,5022	18,4822	18,4622
Carbopol 980 (g)	0.1	0.1	0.1
Methyl Paraben (g)	0.02	0.02	0.02
Propylene Glycol (g)	1	1	1
Ethanol (ml)	0,2	0,2	0,2
Diluited TEA (ml)	0.2	0.2	0.2
CCI-001 (mg)	20	40	60

Table 4: Reagents and amounts for each formulation with CCI-001

In Table 5 the percentage of every reagent, for each formulation, is reported.

	F4	*F5*	*F6*
Carbopol 980	0.5%	0.5%	0.5%
Methyl Paraben	0.1%	0.1%	0.1%
Propylene Glycol	5%	5%	5%
Ethanol	0,79%	0,79%	0,79%
Diluited TEA	0.2%	0.2%	0.2%
CCI-001	0.1%	0.2%	0.3%

Table 5: Concentration (w/w) of every reagent for each formulation with CCI-001

5.5 pH evaluation method

To evaluate pH a HANNA HI 8424 pH meter was used. The measurements were performed once a day, for six days, starting from the day of preparation. The measurement of pH was carried out on samples without CCI-001 to choose the best one. For each one of the formulations, six samples were prepared. At the end of the sixth day, the averages between values corresponding to samples of the same formulation for every day were reported.

5.6 Viscosity evaluation method

Viscosity evaluation was performed with a RM 100 Touch viscometer and it has been carried out for six days, starting from the day after the preparation. The evaluation of viscosity immediately after the preparation would have given an unfaithful result, since the structure wouldn't have assessed yet.

In about 24 h the gel had time to stabilize. All the evaluations were performed at room temperature.

Following the same protocol implemented for pH evaluation, six samples for every formulation were evaluated every day. On the sixth day, the average between the viscosity values corresponding to different samples of the same formulation was performed and plotted against time. Note that F2 was not evaluated.

A cup and bob system was used. Viscosity changes between shear rates of 5 and 1000 s^{-1}, both in the ascendent and descendent phase, were measured. The final comparison between different formulations was performed taking into account the viscosity value corresponding to shear rate 100 s^{-1} in the descendent phase. All gel formulations showed pseudoplastic behavior: viscosity decreases with increasing shear rate.

5.7 HPLC analysis of Carbopol 980 based CCI-001 topical gel

A Waters Arquity Arc HPLC system was used to evaluate the concentration of CCI-001 in the samples. Mobile phase was an isocratic mixture of water and acetonitrile (50:50). The separation was obtained by a Cortex 2.7 µm C18 column (4.6 x 50 mm). The flow rate was set at 0.5 mL/min at room temperature and the detection wavelength was 386 nm. The assay was found linear over the examined range of 5-70 µg/mL in the mobile phase, with a calibration curve of equation y = 22181x + 11538, shown in Figure 22. The correlation coefficient is 0.983237. The calibration curve was obtained by analyzing six samples as shown in Table 6. To validate the repeatability of the method, five different samples of solution (50 µg/mL) from the same sample were analyzed and the RSD % was found to be 0.22%. The concentrations of CCI-001 in the samples were determined from the aforementioned regression equation.

Samples	*CCI-001 Concentration (µg/mL)*
Sample 1	0
Sample 2	10
Sample 3	25
Sample 4	50
Sample 5	70

Table 6: Samples used to plot the calibration curve

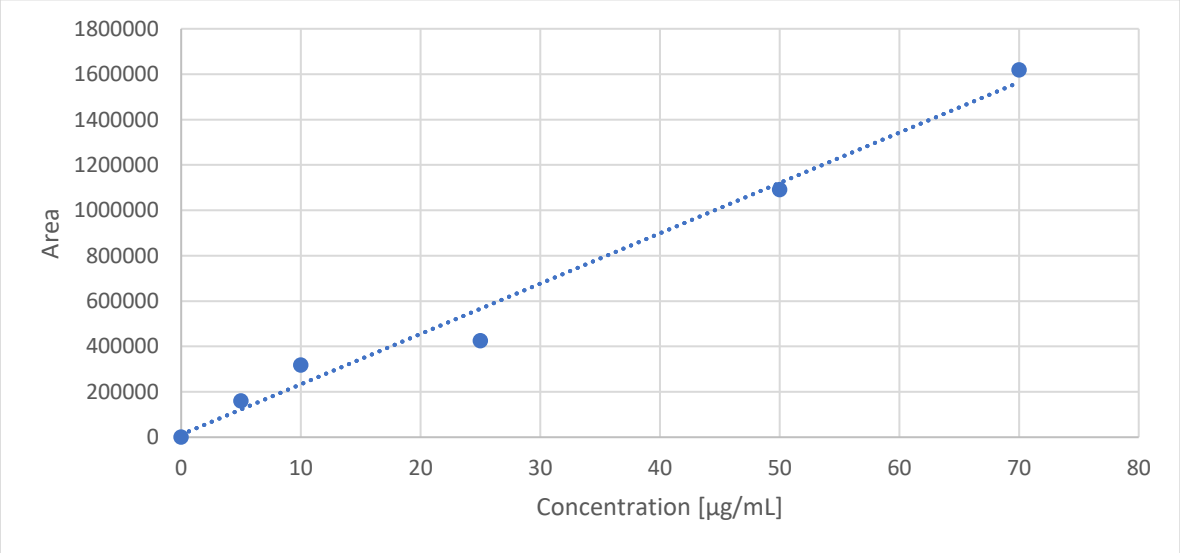

Figure 22: Calibration curve

6 Results

6.1 pH evaluation

In Figure 23, pH trends of all the formulations tested are plotted against time, showing a recurrent trend: on the first day the pH is higher but starting from the second day, it decreases and reaches a value that remains almost constant. The only exception to this trend is given by F4. The comparison between F1 and F2 is plotted in Figure 24, showing that F2 reaches lower pH values. On the other hand, Figure 25 shows the plot of F1 and F4. The observation reveals that F1 trend is more stable, while F4 trend tends to be more irregular. Formulations F1 and F3, shown in Figure 26 have similar trends except for day six, in which F3 pH is lower.

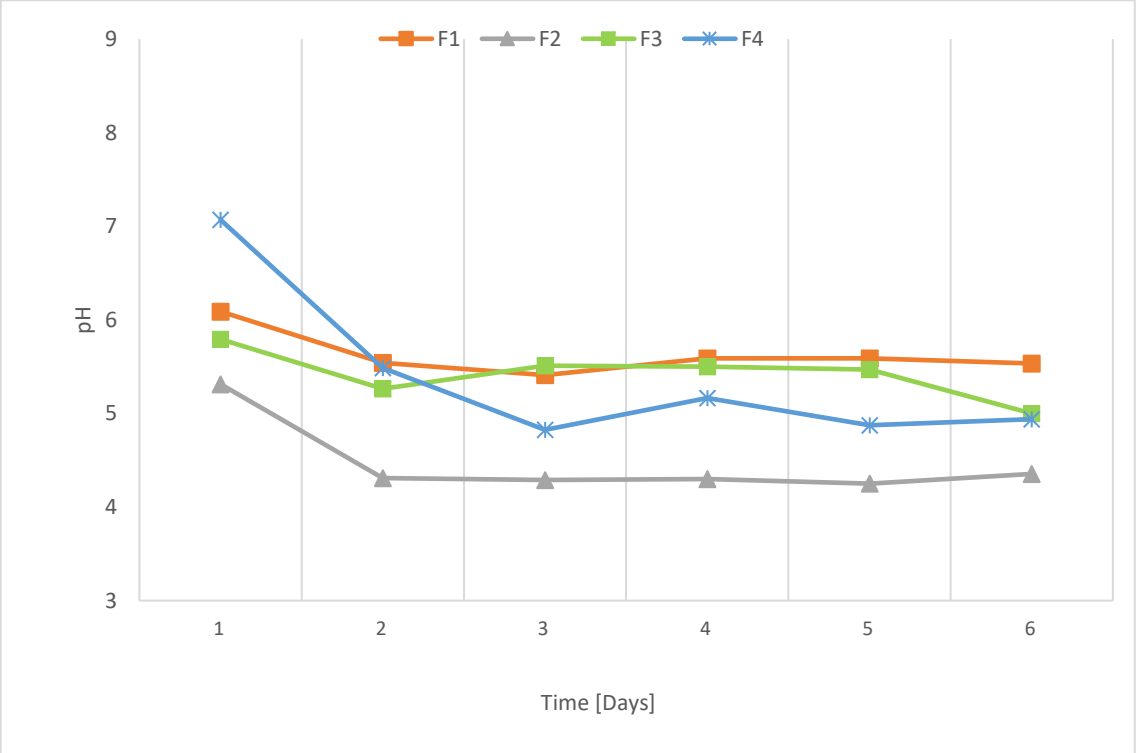

Figure 23: pH against time for F1, F2, F3 and F4

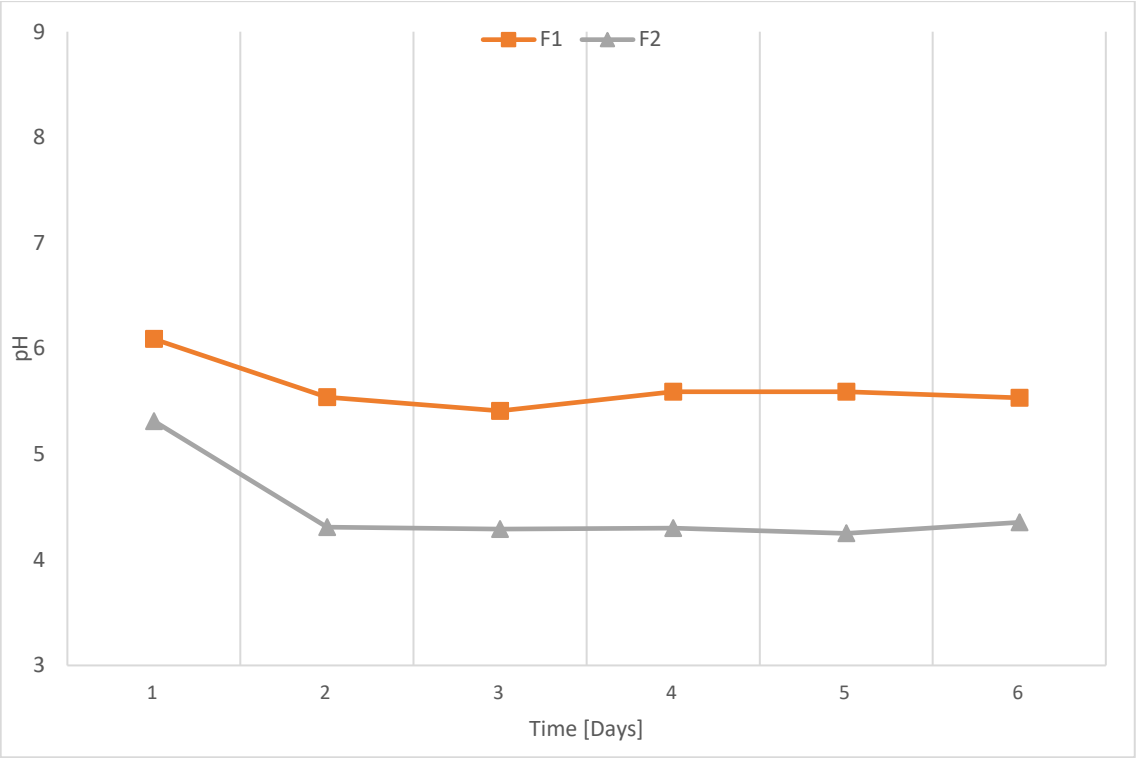

Figure 24: pH against time for F1 and F2

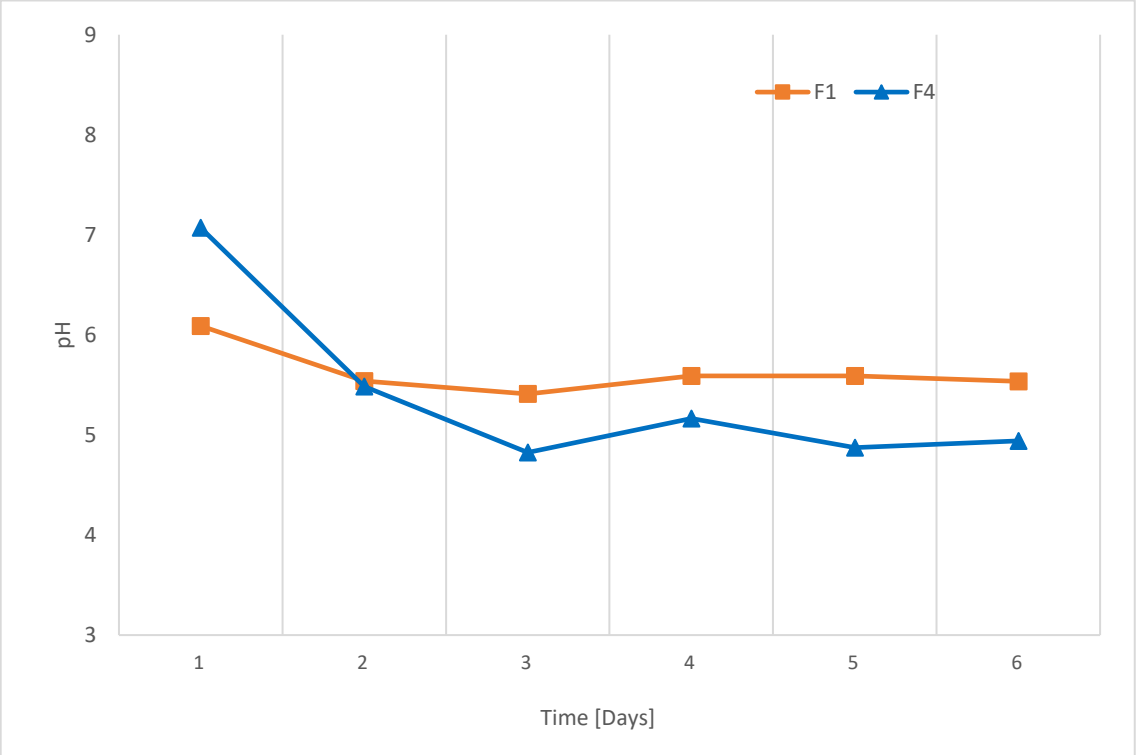

Figure 25: pH against time for F1 and F4

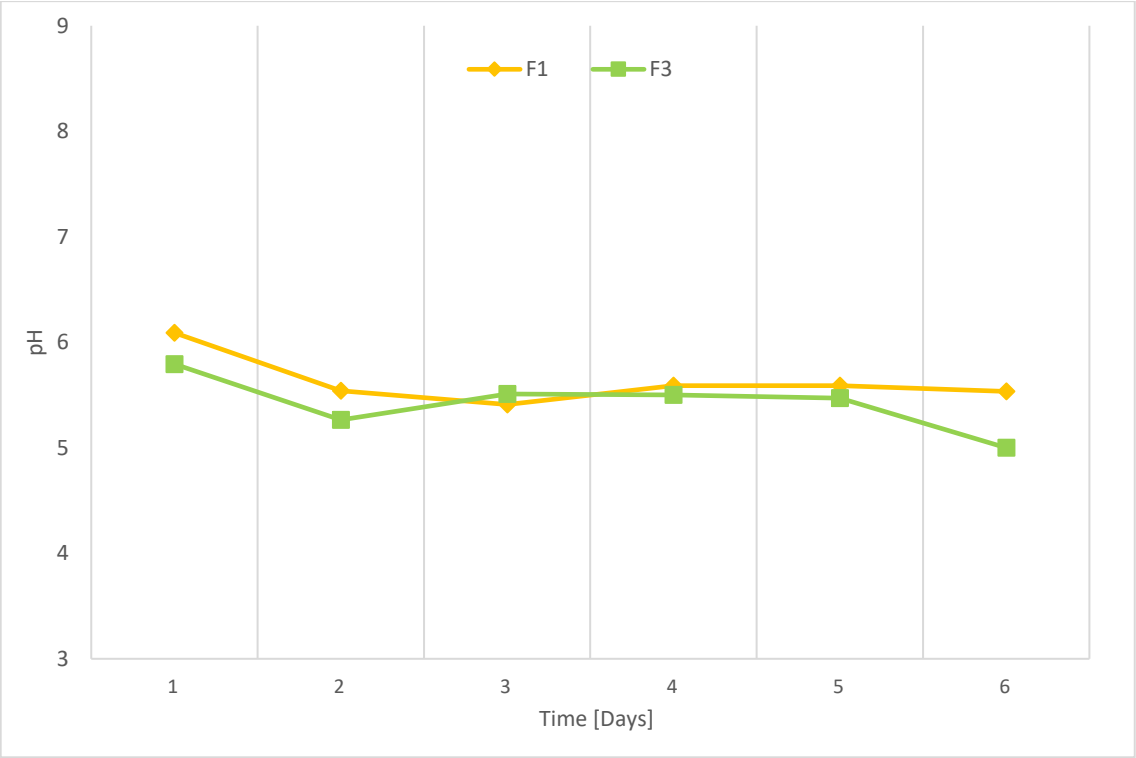

Figure 26: pH against time for F1 and F3

6.2 Viscosity evaluation

In Figure 27, a comparison between F1, F3 and F4 viscosity trends against time is plotted. F2 was not included in the viscosity evaluation. Observing the three trends together, it is clear how F4 has the most stable trend among all, while F1 and F3 tend to have a slightly more unstable course.

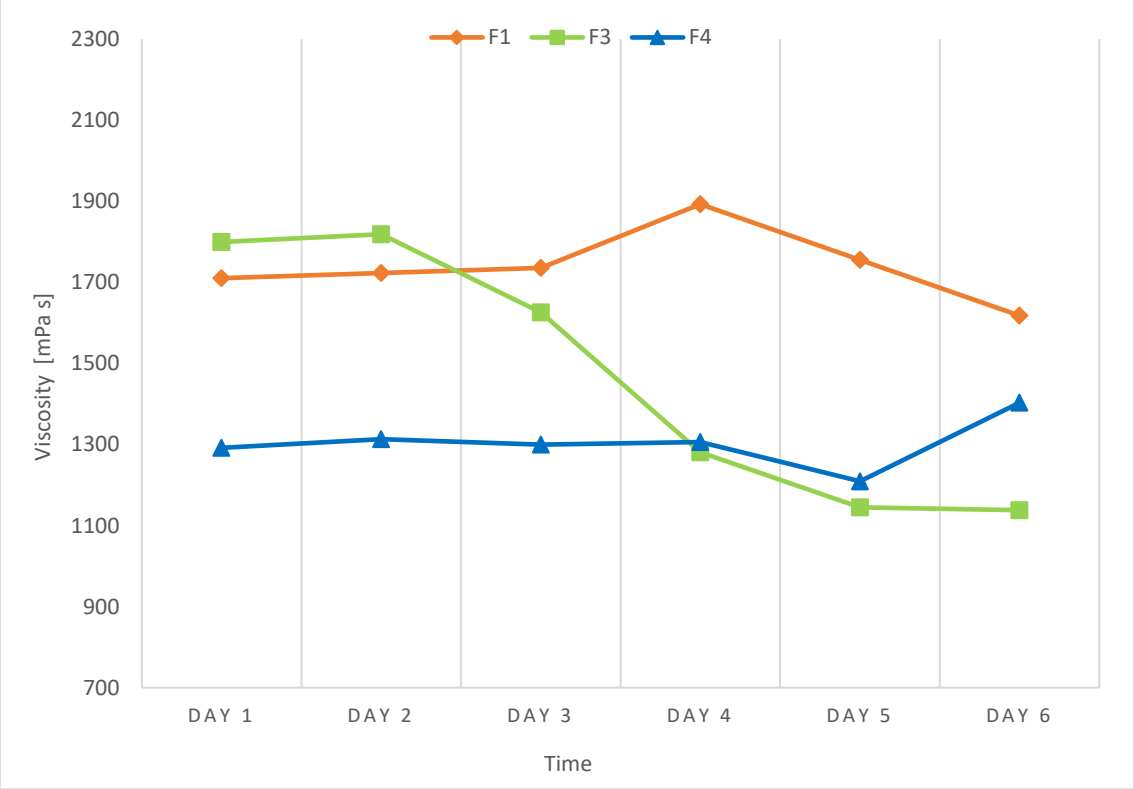

Figure 27: Plots of viscosity against time for F1, F3 and F4

6.3 HPLC analysis of Carbopol 980 based CCI-001 topical gel

HPLC analysis was carried out preparing three solutions (50μg/mL) in the mobile phase of F5, F6, and F7. The resulting chromatograms are shown in Figures 30, 31 and 32, while retention times and concentrations of CCI-001 are reported in Table 7.

Sample	*Retention time (min)*	*Concentration (μg/mL)*
F5	2,234	36,625
F6	2,231	53,625
F7	2,214	59,918

Table 7: Retention times and concentrations for F5, F6 and F7

7 Discussion

As stated above, all the formulations show a recurrent trend of pH in time, in which pH value is higher the first day and then it decreases till reaching a value of plateau. The higher pH of the first day can be explained by the fact that a) the structure of the gel is not completely assessed yet, b) the first evaluation was carried out immediately after the preparation and the temperature of the gel did not cool down to room temperature yet. Nevertheless, observing closely, F2 and F4 present behaviors that do not fulfill the criteria that the best formulation should respect. F2, even following a very regular trend with no peaks, reaches pH values far too low with respect to the other formulations, while F4 presents quite an irregular pH trend in time.

The gel should have preferably a pH of around 5. F2, containing 1% of C-980 (w/w), is the only formulation that reaches pH values under this threshold, and, for this reason, F2 is excluded. The lower pH values shown by F2 are explained by the bigger concentration of C-980, which is an acid polymer [95]. The only exception to the recurrent regular trend of pH in time is given by F4, in which neither Propylene glycol and Ethanol are present. The irregular trend characterizing F4 leads to the conclusion that either Propylene Glycol or Ethanol can stabilize the pH. To assess which one of them is the stabilizer factor, the comparison between F1 and F4 alone against time comes in handy. F1 contains Propylene glycol, F4 does not contain Propylene Glycol, while neither of them contains Ethanol. The observation reveals that the F1 trend is more stable: after the second day, a plateau is reached and the pH value remains almost constant, with a percentage variation between maximum and minimum values of 3%. F4 trend, instead, is more irregular: the pH does not stabilize until the third day, and various oscillations can be observed in the following days, with a percentage variation between maximum and minimum values of 14%. The observation of F1 and F4 alone confirms the role of Propylene Glycol in stabilizing the pH, since, neither of the samples contains Ethanol.

So far, it can be assessed that the formulation should contain 0,5 % (w/w) of C-980 and Propylene Glycol, since it has been demonstrated that both of them have a better influence on pH. At this point, it is important to understand how Ethanol would influence pH, since it is necessary as solvent for CCI-001. To do so, F1 and F3 are plotted alone against time and

the comparison shows that they follow practically the same trend, indicating that Ethanol does not influence pH.
Simultaneously to pH, viscosity was evaluated for F1, F3 and F4. Observing the plots, it is worth noting that F4 presents the most stable course: viscosity values remain almost constant, except for the last two days, in which slight peaks are present. Moreover, F4 shows the lowest viscosity values among all the formulations tested. It was expected that Propylene Glycol would increase viscosity and this hypothesis has been confirmed. F1 and F3 trends, instead, have a different development in time: F1 viscosity remains almost constant for the first three days, then it reaches a peak in day 4 followed by a very slow descendent phase. F3 viscosity, instead, remains constant for the first two days and, starting from day 3, it decreases quite quickly. On the sixth day, F3 viscosity reaches even a lower value than F4. The fact that F3 reaches low values of viscosity, even though it contains Propylene Glycol, can be probably explained by the presence of Ethanol, which compensates for Propylene Glycol influence.

As mentioned above, topical systems characterized by lower viscosities are known to be more effective in the release of the drug, so F1 would not be the best choice. At this point, considering both pH and viscosity evaluations, it is quite obvious that the formulation that presents the best behavior is F3.

After the preparation of the samples containing CCI-001 in different concentrations, HPLC analysis has been carried out. For each one of the samples, the retention time is around two minutes. The expectation was to find a concentration of about 50 μg/mL in every sample but the results failed this assumption. For F6 the resulting concentration is near to the one expected, while for F5 and F7 the results are not that satisfying. Most probably, these results are due to the fact that CCI-001 was not properly dispersed in the gel.

8 Conclusions

In the last two decades, gout incidence increased significantly around the world and the co-existence, in gout patients, of other conditions has been widely recognized. Therefore, new and improved treatments for this pathology are needed, since the currently available ones present important side effects that limit their use in patients with co-morbidities, such as CKD and CVD. In this scenario, rational drug design showed its potential in developing new compounds with enhanced efficiency and selectivity and reduced side effects. The important role of RDD has been proved by developing the CCI-001 compound, which showed great potential in downregulating neutrophils' action in inflammation response during gout attacks, by binding specifically the tubulin isotype differentially expressed by neutrophils themselves. The algorithm used was able to perform the design of a new compound by combining the high affinity with the target tubulin isotype (beta VI) and the lowest affinity with off-target tubulin isotypes. The result is a more potent and more selective drug for gout inflammation.

To achieve the best performance of the new drug, a topical formulation was implemented by laboratory experimentation. Different protocols have been tried and the best formulation was chosen among the others, observing their behavior in terms of pH, viscosity and stability in time.
F3 has been chosen, eventually, because its characteristics combine the necessity for low viscosity and pH around 5, including Ethanol in the formulation that works as a solvent for CCI-001.
The results of the HPLC analysis are not completely satisfying, due to the incorrect dispersion of the drug in the gel. This limitation, although, can be easily overstepped by increasing the stirring time and decreasing the rate of agitation. Further improvement can be reached by stabilizing the viscosity trend in time.

Samples of the formulations prepared in this project were sent to the lab of Prof. Maria Fernandes at the University of Laval, Quebec, Canada. They will be shortly tested both on artificial human skin and in animal models of gout.

9 Other figures

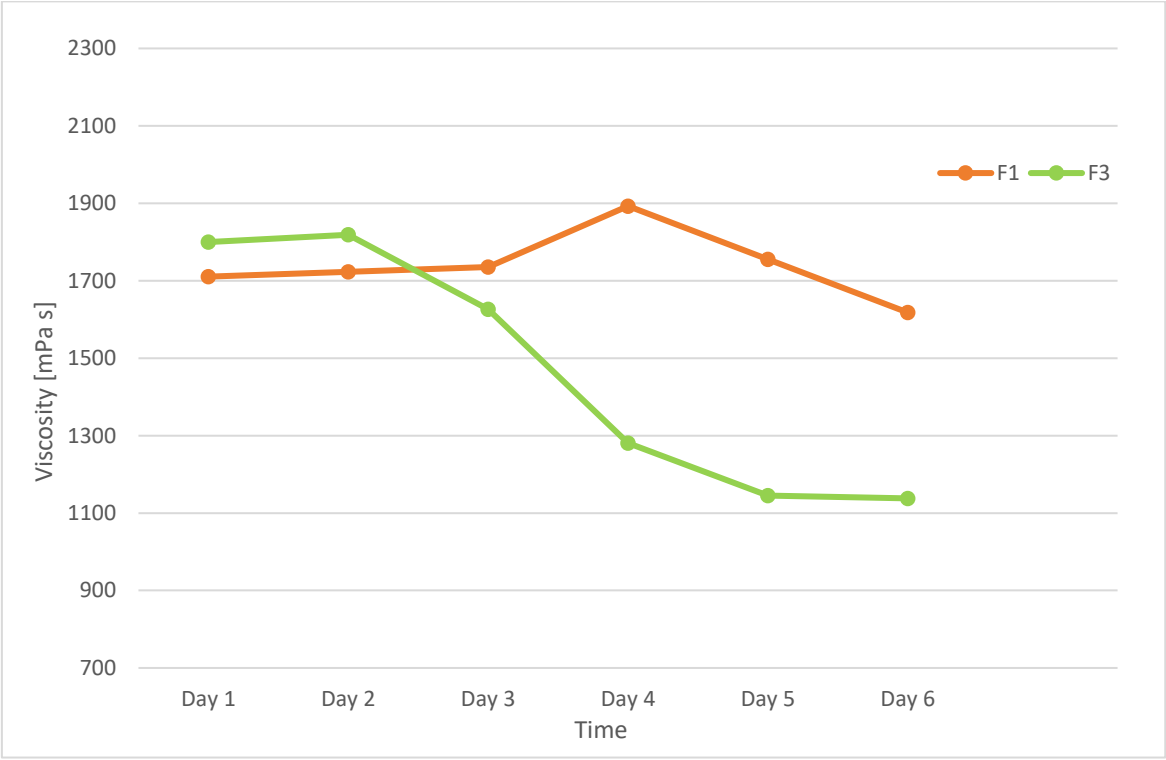

Figure 28: Viscosity against time for F1 and F3

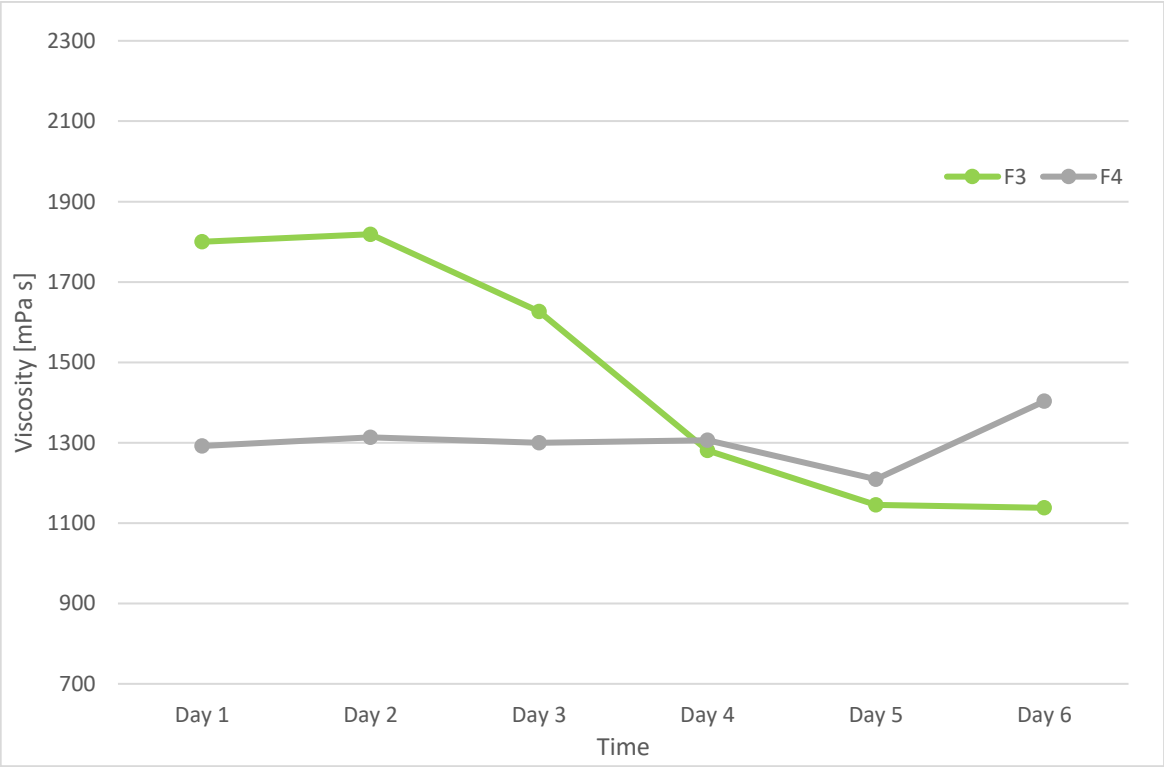

Figure 29: Viscosity against time for F1 and F4

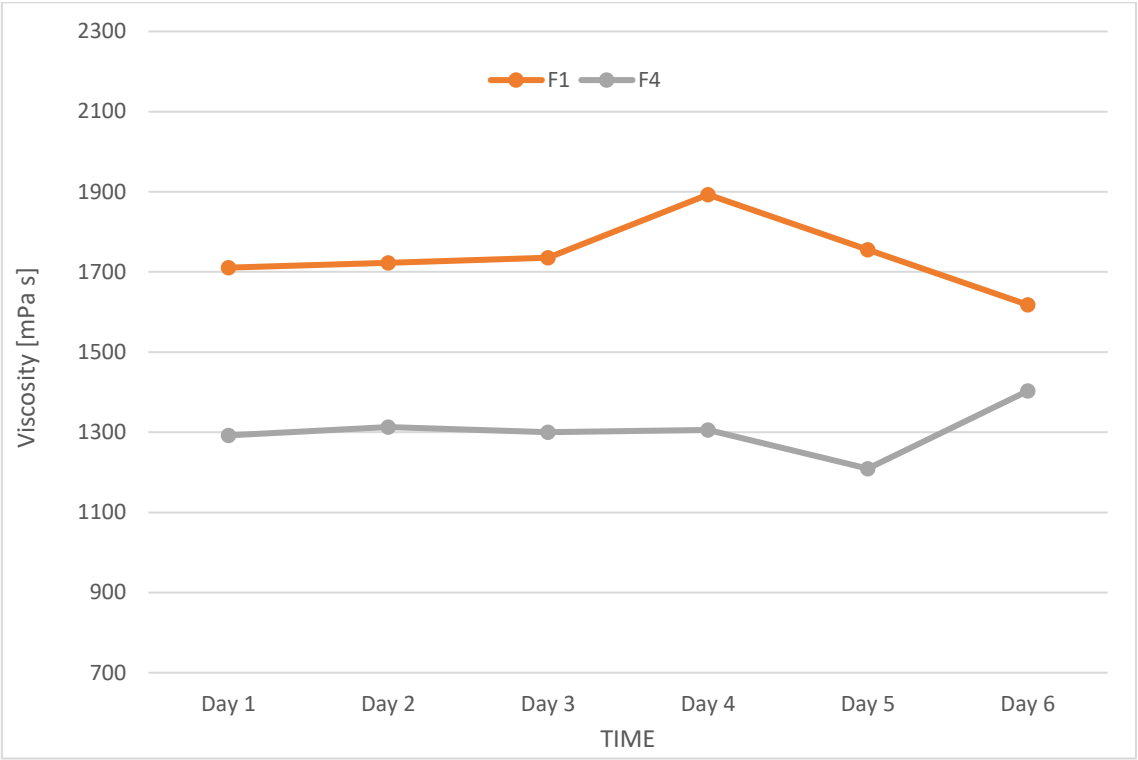

Figure 30: Viscosity against time for F1 and F4

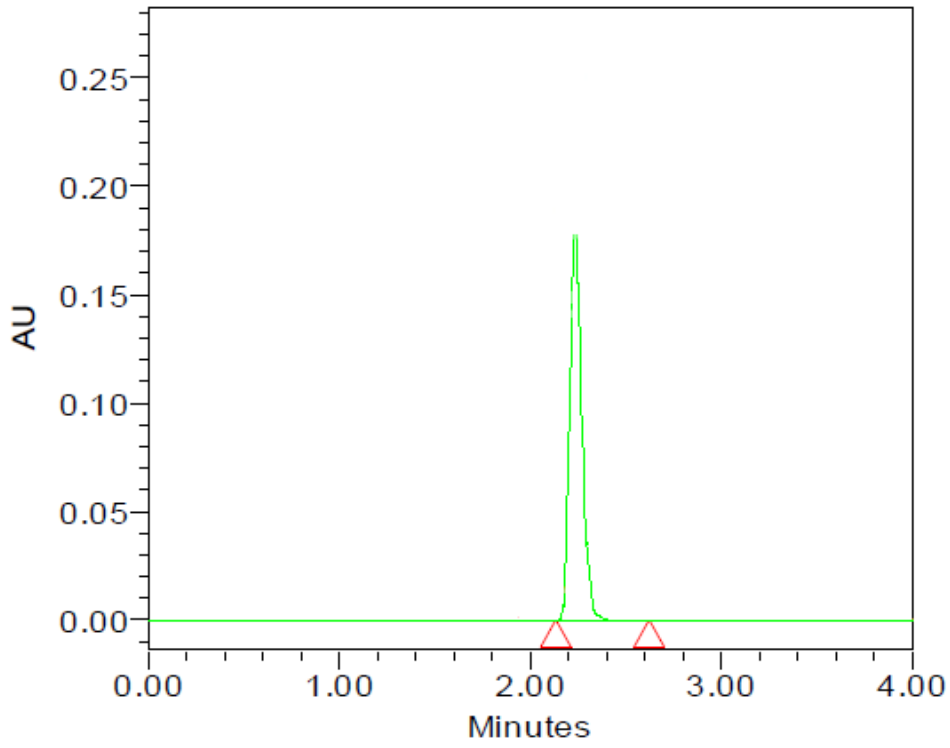

Figure 31: Chromatogram of F5

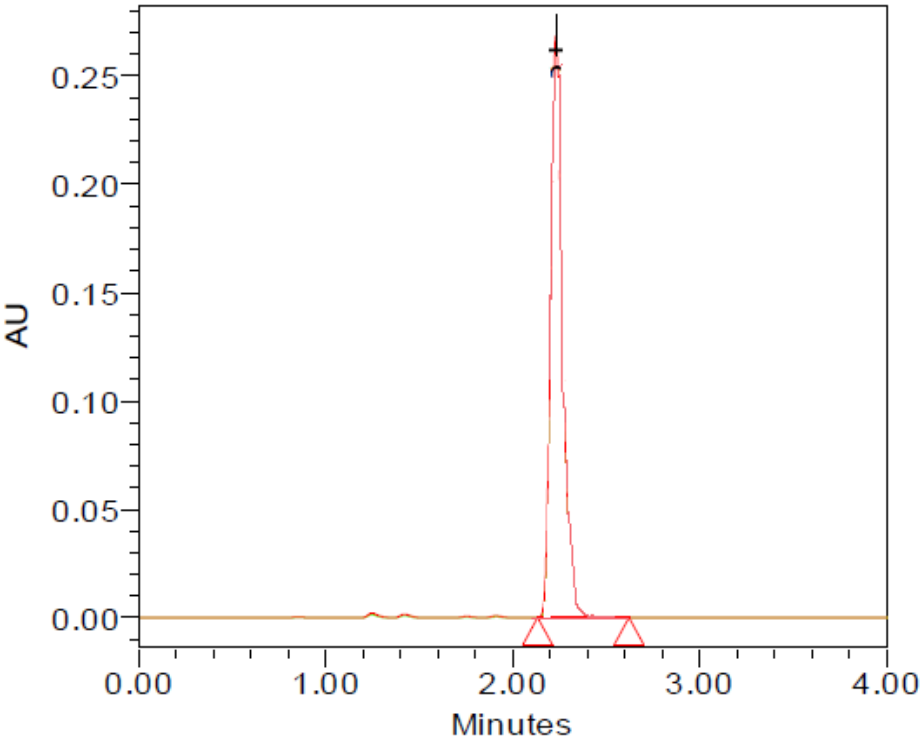

Figure 32:Chromatogram of F6

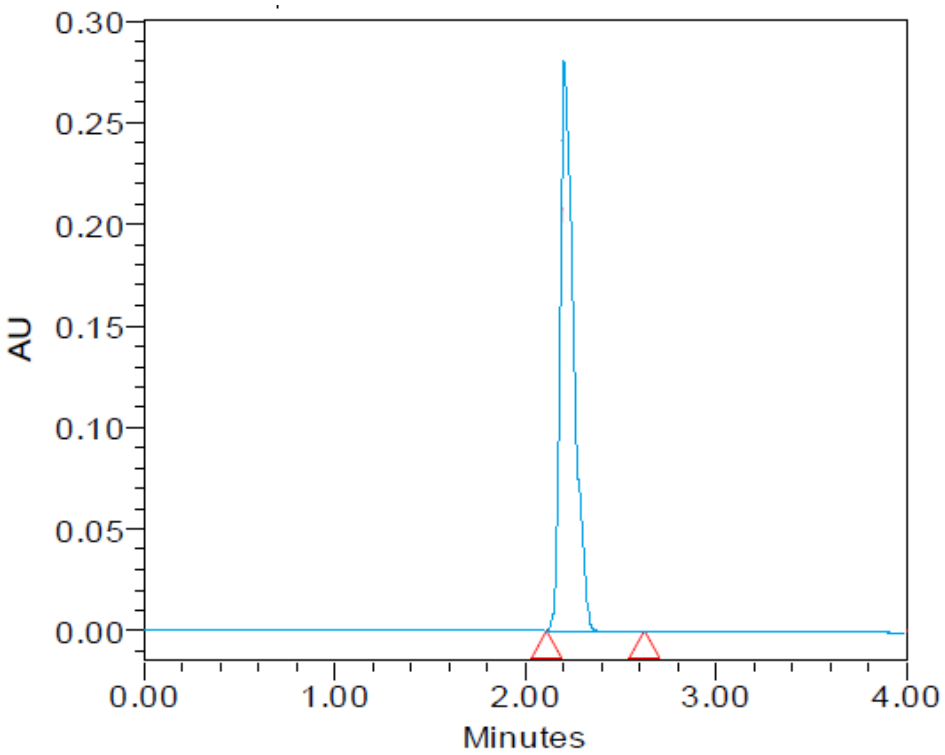

Figure 33:Chromatogram of F7

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