UNIVERSITÀ DEGLI STUDI DI PAVIA

DOTTORATO IN SCIENZE CHIMICHE E FARMACEUTICHE
XXXII CICLO
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BIOAVAILABILITY ENHANCEMENT OF POORLY WATER SOLUBLE DRUGS

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Anno Accademico 2018/2019

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ABSTRACT

The number of newly developed drug molecules with greater lipophilicity, higher molecular weight, and poor water solubility has increased over the last few decades due to the emerging trends in combinatorial chemistry and drug design [1–3]. About 40% of drugs with market approval and nearly 90% of molecules in the discovery pipeline are poorly water-soluble [4]. The majority of failures in new drug development have been attributed to poor water solubility of the drug. It is well-known that poor solubility and slow dissolution can lead to suboptimal drug delivery, resulting in low bioavailability [5, 6]. Commonly used approaches for enhancing the dissolution rate and/or absorption of these molecules include nanoparticle-based formulations [7, 8], lipid-based drug delivery systems [9, 10], pro-drugs [11, 12], amorphous solid dispersions [13, 14], salt formation [15, 16], co-crystals [17, 18], and cyclodextrin complexes [19, 20].

During the first year, the aim of the research project was focused on the development of a pharmaceutical system able to improve the solubility and/or the permeability of a poorly water soluble drug and, consequently, its bioavailability, since the poor bioavailability of the drug could be either attributed to permeability or solubility limits.

Different formulation strategies and pharmaceutical forms were evaluated in order to improve the bioavailability of the candidate drug molecule.

Two different routes of administration were considered: oral and parenteral.

Regarding the oral route, different formulations were developed based on Active Pharmaceutical Ingredient (API) solubility, compatibility and ability of the formulation to maintain API solubilization after dilution/dissolution in biorelevant media. An *in vitro* dissolution test of the prototype formulations was performed in a screening phase; absorption enhancement properties of the selected formulations were tested *in vitro* on Intestinal Epithelial Cells (Caco-2) to evaluate how the different excipients can influence the permeability of the loaded API. With some of the new formulations developed the increase in bioavailability could result from both the increased solubility/dissolution rate and the improved permeability.

Two different prototype formulations, with API concentration of 8% w/w and 10% w/w respectively, were selected. Based on preliminary stability results and dissolution profile, the prototype formulation containing 10% w/w of API has been selected.

In parallel, the solubility behavior of the API in several parenteral vehicles, normally used in preclinical studies, was evaluated. The results obtained directed the formulation development to the use of cyclodextrins in order to achieve the API target dose.

Cyclodextrin type and concentration were identified. A formulation characterized by 0.36% w/w API concentration and by osmolality and pH compatibility with a parenteral administration was obtained.

During the second year, the aim of the research project was focused on three main objectives: preparation of the batches for preclinical studies, process parameters optimization and oral formulation optimization and characterization.

Three main different preclinical studies have been carried out during the second year in order to evaluate bioavailability, Maximum Tolerated Dose (MTD) and efficacy of the two prototype formulations. For these studies both active and placebo formulations have been tested. Since the preclinical studies required quite high quantities of product, an optimization of the process parameters has been necessary in order to increase the batch size. The process temperature and time have been studied in order to optimize the solubilisation of the API reducing the impurities formation.

For one preclinical study it was necessary to develop an oral formulation containing 30 mg/mL of API in order to administer to the animals the same volume of formulation containing 10%w/w of API but at different dose. Based on Lipid Formulation Classification System, a new oral formulation has been developed and characterized in order to obtain a Self Emulsifying Drug Delivery System (SEDDS).

During the third year the results of the preclinical studies were obtained. It has been demonstrated that the API is able to provide a tumor growth suppression via inhibition of the Androgenic Receptor (AR) and Glucocorticoid Receptor (GR).

The API induces cell death in various tumor cell lines grown in vitro and shows a marked inhibition of tumor growth in experimental animal models of cancer at well-tolerated dose levels.

Both the oral formulations tested in vivo showed good safety profile. However, in both cases it was evidenced that the excipients selected to grant the drug substance solubility were also characterized by an awful taste that could not be masked with flavours and could jeopardize the compliance of the oral administration in humans.

For this reason, it was decided to use capsules to administer the API solubilized in an appropriate formulation for human use.

The aim of the third year was to optimize the oral prototype formulation for the first clinical trial because the concentration of the API into 10% oral formulation corresponds to its solubility, therefore a restricted storage condition 20-25°C should be respected to avoid any API precipitation that can occur at a temperature below 20°C.

On the contrary, the concentration of the formulation containing 3% (30 mg/mL) of API is too low to obtain the desired dose in human clinical study because of the elevate number of capsules.

The main tests used for the characterization of the formulations developed during the third year are *in vitro* dissolution test and *in vivo* bioavailability study.

Three different prototype formulations were developed, one liquid and two semisolids. The liquid formulation was developed based on the liquid formulation containing 3% w/V of API but increasing this concentration at 8%, instead the semisolid formulations (containing 8% V/w of API) was developed in order to facilitate the filling procedures in hard gelatin capsules without the use of sealing or banding, that are required with liquid formulations.

All the developed formulations are classified as Self Emulsifying Drug Delivery System, and analyzed in terms of dissolution profile compared with the liquid formulation containing 10% w/w of API and used during preclinical studies.

All the new formulations showed a higher released value after one hour and a lower variability respect to the formulation used during pre-clinical studies.

The three new oral prototype formulations and the formulation at 10% w/w used during pre-clinical studies were used in a bioavailability study in comparison with IV administration.

The lead semisolid formulation filled in capsules was presented in IND application and it obtained the FDA allowance.

Based on the obtained results the formulation submitted in the IND will be tested in phase I clinical trial in tumor bearing patients in order to evaluate safety, pharmacokinetics and pharmacodynamic activity.

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INTRODUCTION

1.1 Bioavailability

Bioavailability is defined as the rate and extent (amount) of drug absorption into the systemic circulation where it can be considered available for its activity. The concentration of drug in plasma depend upon the bioavailability of drug from its dosage form. Any alteration in the drug's bioavailability is reflected in its pharmacologic effects. The measurement of bioavailability gives the net result of the effect of drug release into solution in physiological fluids at the site of absorption, its stability in those physiological fluids, its permeability, and its presystemic metabolism on the rate and extent of drug absorption [1].

The rate and extent of drug absorption from gastrointestinal (GI) tract are very intricate and affected by many factors. These include physicochemical factors, physiological factors, and factors related to dosage form. Despite this complexity, the Biopharmaceutics Classification System (BCS) developed by Amidon et al. revealed that the essential key parameters controlling oral drug absorption are the solubility/dissolution of the drug dose in the GI milieu and the permeability of the drug through the GI membrane (Figure 1.1). These important parameters are characterized in the BCS as one of the most significant tools in modern pharmaceutics and biopharmaceutics of oral drug products [2].

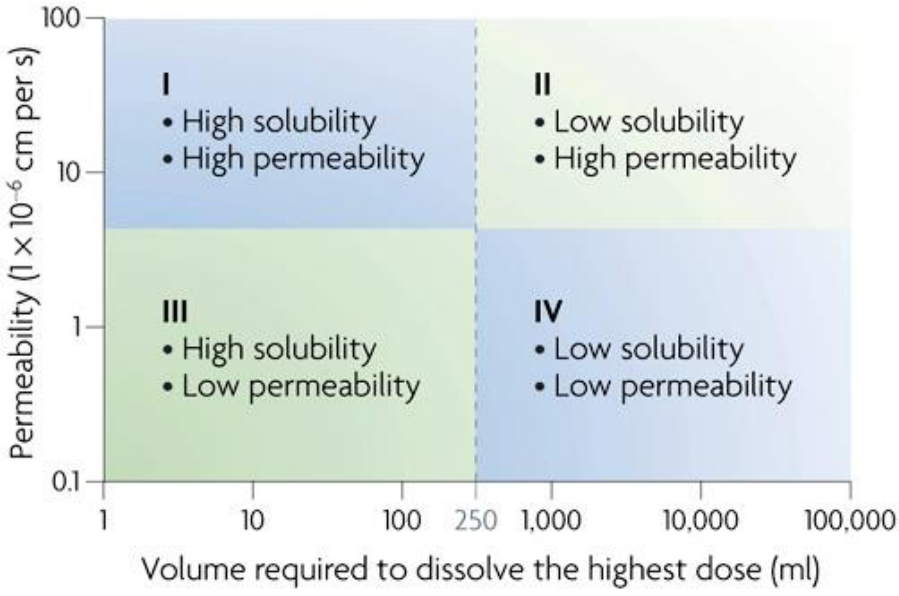

Figure 1.1 Biopharmaceutical classification system (BCS) characterization of drugs based on solubility and permeability measures [3].

1.2 Poorly water-soluble drugs

It is estimated that between 40% and 70% of all new chemical entities identified in drug discovery programs are insufficiently soluble in aqueous media. The increase in the proportion of poorly soluble candidates is frequently attributed to improvements in synthesis technology, which has enabled the design of very complicated compounds, and a change in discovery strategy from a so-called phenotypic approach to a target-based approach. Various physicochemical properties which contribute to the poor solubility of various drugs include their complex structure, size, high molecular weight, high lipophilicity, compound H-bonding to solvent, intramolecular H-bonding, intermolecular H-bonding (crystal packing), crystallinity, polymorphic forms, ionic charge status, pH, and salt form. Lipinski's rule of five has been widely proposed as a qualitative predictive model for the assessment of absorption of poorly absorbed compounds. In the discovery setting "the rule of 5" predicts that poor absorption or permeation is more likely when there are more than 5 H-bond donors, 10H-bond acceptors, the molecular weight is greater than 500, and the calculated LogP is greater than 5. The rule of five only holds for compounds that are not substrates for active transporters and efflux mechanisms. Thus, in vivo assessment of new drug candidates in animal model is performed to assess the absorption of drug. Poorly absorbed drugs pose a challenge to the formulation scientists to develop suitable dosage form which can enhance their bioavailability [4].

1.3 Strategies for formulating poorly water-soluble drug

Poorly soluble drugs can be formulated using different approaches such as pH adjustment, prodrug design, modification of the solid state, micro/nanonization of the API, use of co-solvents, use of solubility and/or permeability enhancers, use of lipid formulations, use of cyclodextrins [4-7].

1.3.1 pH adjustment

Nearly 70% of drugs are reported to be ionizable, of which a majority are weakly basic. A pH-dependent solubility is exhibited by ionizable drugs, wherein weakly acidic drugs are

more soluble at pH>p*K*a (ionization constant) and weakly basic drugs are soluble at pH<p*K*a. This pH dependent solubility was explored extensively to formulate insoluble drugs [6]. However, drugs are generally neutral at physiological pH. Thus, the pH of the formulation can be adjusted with buffering excipients to ensure the presence of the most soluble form of the poorly water-soluble drug. For solid dosage forms, the buffering excipients control the pH of the microenvironment surrounding drug particles during *in vivo* dissolution [8].

The pH adjustment is a simple approach and represents a first-line strategy for the formulation of insoluble drugs. It is frequently combined with other solubilizing approaches such as surfactants, cyclodextrins or cosolvents. The pH of the final formulation is selected not only according to drug solubility, but also considering its tolerance, bioavailability, efficacy and stability, which can strongly depend on the pH. In addition, the potential risk of drug precipitation after administration needs to be considered [5].

1.3.2 Prodrug design

Prodrugs are bioreversible derivatives of drug molecules that undergo an enzymatic and/or chemical transformation in vivo to release the active parent drug, which can then exert the desired pharmacological effect. In both drug discovery and development, prodrugs have become an established tool for improving physicochemical, biopharmaceutical or pharmacokinetic properties of pharmacologically active agents [9].

Two main prodrug design categories can be identified: i) carrier-linked prodrugs where the parent drug backbone is covalently linked to a prodrug moiety and ii) bioprecursor prodrugs which are modified parent drugs with functional groups requiring hydration or redox reactions. In addition, pre-prodrugs or double prodrugs combine two prodrug design approaches in their design (carrier-linked and/or bioprecursor) [5].

Prodrug design represents a versatile and powerful approach that can solve a large variety of issues related to drug solubility, absorption, distribution, metabolism, toxicity or stability, among others [5].

1.3.3 Modification of the solid state

The ability of metastable forms of drug substances to enhance solubility over their thermodynamically stable counterparts has been well documented in a number of reviews

[10-12]. Metastable systems utilized for the purpose of increasing the apparent solubility of drug substances with poor aqueous solubility include crystalline polymorphs, solvates, desolvates, amorphous forms, co-crystal and salts (Figure 1.2) [5], [13].

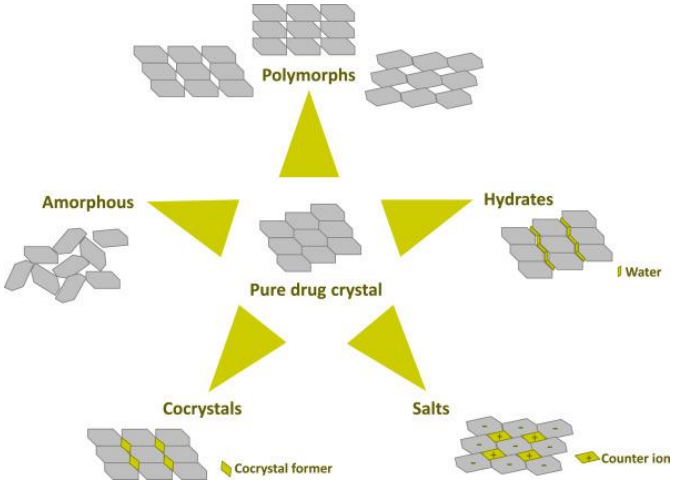

Figure 1.2 Different drug solid forms that can be used in formulations [5].

Polymorphs have been studied extensively since the early reports of their existence. These substances are defined as having identical chemical compositions, but differ in internal structure, including unit cell dimensions and crystal packing which can affect pharmaceutical performance. These effects include solubility, rates of dissolution, bioavailability, processability, physical stability, and chemical stability.

Pseudopolymorphs, also known as hydrates or solvates, contain molecules from the crystallization solvent within the crystal lattice. These solvent molecules are often located in discrete crystal sites and bound within the lattice (e.g., hydrogen bonding). It is possible to have both stoichiometric and nonstoichiometric forms of these substances, with the stoichiometric form being most common. Solvates, which contain a solvent other than water, are mostly nonpractical from a pharmaceutical standpoint due to potential toxicity. While hydrates are normally more stable than their anhydrous counterparts, they rarely provide any solubility advantage and, in most cases, exhibit a lower solubility. However, hydrates and solvates may be desolvated to provide solubility enhancement, albeit in a relatively thermodynamically unstable form.

Amorphous forms of drug substances are yet another physical modification of the solid-state which provide the greatest solubility potential. They differ from their crystalline polymorph counterparts by having low packing efficiency and lack of long-range crystalline order. This

results in a very high free energy and, thus, the capability to provide a substantial improvement in solubility [13].

Cocrystals contain a drug and a cocrystal former, linked by H-bonds (different from salts in which a proton transfer takes place). These H-bonds decrease the strength of the drug–drug interactions compared to the pure drug crystal structure. The cocrystal approach has been successfully used for drug dissolution and bioavailability enhancement of poorly water soluble drugs. This approach does not require the presence of ionisable groups on the drug [5].

Crystalline salts are based on a proton transfer between an ionizable group of the drug and a counter ion species. The resulting salt modifies the pH in the thin diffusion layer surrounding the drug, resulting in an increased solubility compared to the corresponding free form. The counter ion species highly influences the solubility and must be carefully chosen [5]. Compared to solvates, co-crystals exhibit a higher-degree physical stability and may incorporate innocuous co-crystal formers, the number of which is numerous. While co-crystal systems have been studied for many years, their ability to improve solubility and bioavailability of poorly water-soluble drug substances has only recently been fully realized [13].

1.3.4 Micro/nanonization of the API

A decrease in particle size of poorly water-soluble drugs allows to i) increase of the drug surface area and its dissolution rate, ii) improve its bioavailability and iii) reduce toxicity. This approach can be combined with any of the modified solid states detailed above, cumulating the advantages of both strategies [5].

Micronization/nanonization reduces respectively the particle down to micrometer or nanometer dimension which increases the bioavailability of poorly soluble active ingredient. A wide range of drug types which includes injectable, medicated drops, inhaled products, solid dosage are benefited from micronization. Drug having high dose number, defined as the mass divided by the product of uptake volume (250 mL that is initial gastric volume) and solubility of drug [14], is not suitable for micronization as it does not change the saturation solubility of the drug [15]. Micronization is usually achieved by spray-drying, freeze-drying, crystallization or milling processes. Ultrasonic energy in the range of 20kHz-5MHz is also used to increase nucleation rate, which not only induces the crystallization, but also provide reduced particle size with

narrow size distribution. As it uses ultrasonic energy, this process is called sono crystallization [7].

Nanonization is the reduction of particle size to the submicron range (100 to 200nm), which provide further enhancement in the dissolution rates. Supercritical fluid (SCF) technology is increasingly used for this purpose. In this technique, Drug particles are first dissolved in the SCF like carbon dioxide, nitrous oxide, ethylene, propylene, propane, n-pentane, ethanol, ammonia, and water, followed by bring rapid expansion of supercritical solutions either by moderate changes in temperature and/or pressure. Nano-suspensions with particle diameter between 5-2,000nm diameters are generated using SCF. The nano-suspension provides higher dissolution rates of particles compared to conventional suspensions. Several enhancements in SCF technology such as supercritical anti-solvents processes (SAS), impregnation or infusion of drug with polymers, solution enhanced dispersion by SCF (SEDS), and aerosol supercritical extraction system (ASES) have been recently proposed [7].

1.3.5 Use of co-solvents

A cosolvent is a water-miscible organic solvent used to increase the solubility of a drug in water. This approach is based on the theory that the dissolution is enhanced when the solute and solvent have similar physicochemical characteristics. The most important factor to be considered is the polarity of the mixture (i.e. its dielectric constant). A large variety of cosolvents such as ethanol, polyethylene glycol (PEG), propylene glycol (PG), glycerin, dimethylacetamide (DMA), N-methyl-2-pyrrolidone (NMP), dimethylsolfoxide (DMSO), as well as a number of oils (e.g. peanut, corn, sesame, olive or peppermint) can be used [16, 17].

This cosolvent strategy presents some limitations linked to i) cosolvent taste and stability, ii) adverse physiological effects, iii) potential modification of the pharmacokinetic profile of the drug and iv) potential drug precipitation after administration. This strategy remains a simple option frequently used in combination with other solubilizing strategies for the formulation of poorly water-soluble drugs. Nevertheless, the risks of drug instability, drug precipitation and modification of the pharmacokinetic profile need to be considered [5].

1.3.6 Use of solubility and/or permeation enhancers

Oral ingestion is the most convenient and commonly employed route of drug delivery due to its ease of administration, high patient compliance, cost-effectiveness, least sterility

constraints, and flexibility in the design of dosage form. As a result, many of the generic drug companies are inclined more to produce bioequivalent oral drug products [18].

However, the major challenge with the design of oral dosage forms lies with their poor bioavailability. The oral bioavailability depends on several factors including aqueous solubility, drug permeability, dissolution rate, first-pass metabolism, pre-systemic metabolism, and susceptibility to efflux mechanisms. The most frequent causes of low oral bioavailability are attributed to poor solubility and low permeability.

Poorly water-soluble drugs often require high doses in order to reach therapeutic plasma concentrations after oral administration. Low aqueous solubility is the major problem encountered with formulation development of new chemical entities as well as generic development. Any drug to be absorbed must be present in the form of an aqueous solution at the site of absorption. Water is the solvent of choice for liquid pharmaceutical formulations. Most of the drugs are either weakly acidic or weakly basic having poor aqueous solubility [19].

A strategy to improve the bioavailability of poorly soluble drugs is the use of solubility enhancers in order to allow the solubilisation of the drug up to the site of absorption and, consequently, to reach their therapeutic plasma concentration.

Meanwhile, a strategy to improve the bioavailability of BCS Class III and Class IV active ingredients, characterized by low permeability, consists in the use of permeation enhancers. Permeation enhancers are functional excipients included in formulations to improve the absorption of a pharmacologically active drug. The term permeation enhancer usually refers to an agent whose function is to increase absorption by enhancing membrane permeation [20].

Chemical permeation enhancers (CPEs) aid oral drug absorption by altering the structure of the cellular membrane (transcellular route) and/or the tight junctions between cells (paracellular route) of the intestinal epithelium [21, 22]. Unfortunately, many reports indicate that enhancer efficacy is often linked to toxicity [23, 24], and as a result, permeation enhancers are not widely used in oral formulations. However, the full potential of CPEs for oral delivery remains unclear since there is no fundamental understanding of the principles that govern enhancer behaviour [25].

1.3.7 Lipid formulations

Lipid systems have the advantage that they can present the drug as a stable liquid solution, but the term "lipid formulation" has come to mean one of large group of formulations which share some common features. Lipid systems may include triglycerides, mono and diglycerides, lipophilic surfactants, hydrophilic surfactants and cosolvents; excipients with a wide variety of physicochemical properties. A classification system was introduced in 2000 to help identify the critical performance characteristics of lipid systems [26].
Table 1.1 is an updated version of what could reasonably be called lipid formulation classification System (LFCS).

Table 1.1 The proposed Lipid Formulation Classification System (LFCS) showing typical composition of various type of lipid formulations [27].

Excipients in formulation	Content of formulation (%, w/w)				
	Type I	Type II	Type IIIA	Type IIIB	Type IV
Oils: triglycerides or mixed mono and diglycerides	100	40–80	40–80	<20	–
Water-insoluble surfactants (HLB < 12)	–	20–60	–	–	0–20
Water-soluble surfactants (HLB > 12)	–	–	20–40	20–50	30–80
Hydrophilic cosolvents (e.g. PEG, proylene glycol, transcutol)	–	–	0–40	20–50	0–50

Type I formulations are oils which require to be digested, Type II formulations are water-insoluble self-emulsifying drug delivery system (SEDDS), Type III systems are SEDDS or self-microemulsifying drug delivery systems (SMEDDS) which contain some water-soluble surfactants and/or cosolvents (Type IIIA) or a greater proportion of water-soluble components (Type IIIB).

Table 1.1 includes an additional category (Type IV) to represent the recent trend towards formulations which contain predominantly hydrophilic surfactants and cosolvents. Type IV formulations contain no oils and represent the most extremely hydrophilic formulations. The advantage of blending a surfactant with a cosolvent to give a Type IV formulation is that the surfactant offers much greater good solvent capacity on dilution (as a micellar solution) that the cosolvent alone. The cosolvent is useful to facilitate the dispersion of the surfactant, which is likely to reduce variability and irritancy caused by the high local concentration of surfactant. Type IV formulation is useful for drugs which are hydrophobic but not lipophilic [27].

The general characteristics, advantages and disadvantages of each type of lipid formulation are shown in Table 1.2.

Table 1.2 Characteristic features, advantages and disadvantages of the various types of "lipid" formulations [26].

LFCS type	Characteristics	Advantages	Disadvantages
Type I	Non-dispersing; requires digestion	GRAS status; simple; excellent capsule compatibility	Formulation has poor solvent capacity unless drug is highly lipophilic
Type II	SEDDS without water-soluble components	Unlikely to lose solvent capacity on dispersion	Turbid o/w dispersion (particle size 0.25–2 μm)
Type IIIA	SEDDS/SMEDDS with water-soluble components	Clear or almost clear dispersion; drug absorption without digestion	Possible loss of solvent capacity on dispersion; less easily digested
Type IIIB	SMEDDS with water-soluble components and low oil content	Clear dispersion; drug absorption without digestion	Likely loss of solvent capacity on dispersion
Type IV	Oil-free formulation based on surfactants and cosolvents	Good solvent capacity for many drugs; disperses to micellar solution	Loss of solvent capacity on dispersion; may not be digestible

The main common advantages of Lipid based drug delivery systems (LBDDS) are reported in the list below [28]:

1) Drug release in controlled and targeted way.
2) Pharmaceutical stability.
3) High and enhanced drug content (compared to other carriers).
4) Feasibilities of carrying both lipophilic and hydrophilic drugs.
5) Biodegradable and biocompatible.
6) Excipients versatility.
7) Formulation versatility.
8) Low risk profile.
9) Passive, non-invasive formation of vesicular system which is available for immediate commercialization.

1.3.8 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides of a glucopyranose, containing a relatively hydrophobic central cavity and hydrophilic outer surface. Owing to the lack of free rotation around the bonds connecting the glucopyranose units, the CDs are not perfectly cylindrical molecules but are toroidal or cone shaped [29]. As a result of their molecular structure and shape, they possess a unique ability to act as molecular containers by entrapping guest molecules in their internal cavity. No covalent bonds are formed or broken during drug CD complex formation, and in aqueous solution, the complexes readily dissociate, and free drug molecules remain in equilibrium with the molecules bound within the CD cavity. The parent or natural CDs consist of 6, 7 or 8 glucopyranose units and are referred to as alpha, beta and gamma CD, respectively. CDs containing 9, 10, 11, 12 and 13 glucopyranose units have also

been reported. Hundreds of modified CDs have been prepared and shown to have research applications, but only a few of these derivatives, those containing the hydroxypropyl (HP), methyl (M) and sulfobutylether (SBE) substituents have been commercially used as new pharmaceutical excipients.

CDs have mainly been used as complexing agents to increase the aqueous solubility of poorly water-soluble drugs and to increase their bioavailability and stability. In addition, CDs have been used to reduce or prevent gastrointestinal or ocular irritation, reduce or eliminate unpleasant smells or tastes, prevent drug-drug or drug-additive interactions, or even to convert oils and liquid drugs into microcrystalline or amorphous powders [30].

CDs have been playing a very important role in formulation of poorly water-soluble drugs by improving apparent drug solubility and/or dissolution through inclusion complexation or solid dispersion, by acting as hydrophilic carriers for drugs with inadequate molecular characteristics for complexation, or as tablet dissolution enhancers for drugs with high dose, with which use of a drug/CD complex is difficult [30].

CDs enhance the bioavailability of insoluble drugs by increasing the drug solubility, dissolution and/or drug permeability. CDs increase the permeability of insoluble, hydrophobic drugs by making the drug available at the surface of the biological barrier, e.g., skin, mucosa or the eye cornea, from where it partitions into the membrane without disrupting the lipid layers of the barrier. In such cases it is important to use just enough CD to solubilize the drug in the aqueous vehicle since excess may decrease the drug availability. In the case of water-soluble drugs, CDs increase drug permeability by direct action on mucosal membranes and enhance drug absorption and/or bioavailability [31].

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CHAPTER 1

Oral Formulations Pharmaceutical Development

2.1 Aim

Aim of the study is to develop an oral delivery system able to increase the bioavailability of the cortexolone 17α-valerate-21-propionate (CB-03-10) thanks to an increment in its solubility and/or permeability, in order to ameliorate its clinical efficacy.

CB-03-10 (cortexolone 17α-valerate-21-propionate) is a steroidal new chemical entity (NCE) cortexolone derivative synthesized by Cosmo Pharmaceuticals. It has shown to be able to provide a tumor growth suppression through the induction of both extrinsic and intrinsic apoptotic pathways via inhibition of the androgen receptor (AR) and glucocorticoid (GR). CB-03-10 induces cell death in various tumor cell lines grown in vitro and shows a marked inhibition of tumor growth in experimental animal models of cancer at well-tolerated dose levels. AR and GR receptors are expressed in several solid epithelial tumors including pancreas, colon, prostate, breast, and ovarian carcinomas. GR inhibition may also potentially increase sensitivity to chemotherapy in cancer therapy and could compensate the lack of activity after some time of exposure recorded for other cell receptors [1].

The development has been articulated in several phases and different formulation strategies and pharmaceutical forms have been evaluated.

The lead approach has been the evaluation of different kinds of excipients (in particular lipid excipients) to improve the solubility/permeability of the drug.

The goal of the oral formulations was to obtain a Self-emulsifying system able to form emulsion or microemulsion when in contact with Gastro-Intestinal Tract fluids without the need of mechanical or thermal energy. Self-emulsifying systems are formulated without the hydrophilic phase and they consist of mixture of drug, surfactants and oil.

Self-emulsifying systems include Self Emulsifying Drug Delivery System (SEDDS) and Self Micro-Emulsifying Drug Delivery System (SMEDDS).

2.2 Quality Target Product Profile

Based on the clinical purpose, a Quality Target Product Profile for the oral formulation was defined in Table 2.1.

Table 2.1 Quality Target Product Profile (QTPP) for the oral formulation.

QTPP Elements		Target
Route of administration		Oral
Dosage form		Liquid/Semisolid
Characteristics		Improved solubility and bioavailability both *in vitro* and *in vivo*
Product quality attributes	Physical attributes	Functional aim requirement: Must meet the same compendial or other applicable (quality) standards (i.e., identity, assay, purity, and quality).
	API Assay	
	API Purity	
	Viscosity	
	Density	
	Microbial limits	
Stability		At least 36 month shelf-life at room temperature or in ICH conditions
Container closure system		Container closure system qualified as suitable for this product

2.3 Materials and methods

Materials and methods related to the preparation, characterization and analysis of the oral formulations are reported here below.

2.3.1 Materials

2.3.1.1 Drug substance: CB-03-10

Chemical name: Cortexolone 17α-valerate-21-propionate

Other name: 3,20-dioxopregn-4-en-17-yl valerate 21-yl propionate

Product code: CB-03-10

CAS Number: 1902166-22

Structure

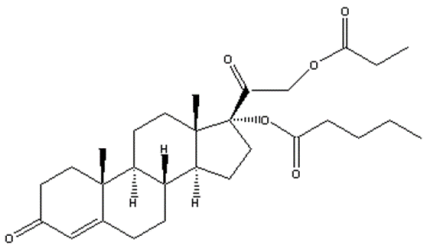


Figure 2.1 Cortexolone 17α-valerate-21-propionate structure.

Manufacturer	FARMABIOS S.p.A. Via Pavia, 1 27027 Gropello Cairoli (PV) Italy

General properties of CB-03-10 are reported in Table 2.2.

Table 2.2 General properties of CB-03-10.

Appearance	White crystalline powder
Molecular formula	$C_{29}H_{42}O_6$
Molecular weight	486.64
Stereochemistry	8R, 9S, 10R, 14S, 13S and 17R
Melting point	109.2°C
LogP (octanol/water calculated)	5.7
Solubility	practically insoluble in water; DMSO=263mg/ml; Acetone=632mg/ml

2.3.1.2 Excipients

2.3.1.2.1 Surfactants

Surfactants are compounds that lower the surface tension (or interfacial tension) between two liquids or between a liquid and a solid. Surfactants may act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants [2].

Caprylocaproyl polyoxyl-8 glycerides (Labrasol® ALF)

Mixtures of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols with mean relative molecular mass between 200 and 400. They are obtained by partial alcoholysis of medium-chain triglycerides using polyethylene glycol or by esterification of glycerin and polyethylene glycol with caprylic (octanoic) acid and capric (decanoic) acid or a mixture of glycerin esters and condensates of ethylene oxide with caprylic acid and capric acid. They may contain free polyethylene glycols [3].
Supplier: Gattefossè Italia S.r.l.

Oleoyl polyoxyl-6 glycerides (Labrafil® M1944CS)

Mixtures of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. They are obtained by partial alcoholysis of an unsaturated oil mainly

containing triglycerides of oleic (cis-9- octadecenoic) acid, using polyethylene glycol with mean relative molecular mass between 300 and 400, or by esterification of glycerol and polyethylene glycol with unsaturated fatty acids, or by mixing glycerol esters and condensates of ethylene oxide with the fatty acids of this unsaturated oil [3].
Supplier: Gattefossè Italia S.r.l.

Stearoyl polyoxyl-32 glycerides (Gelucire® 50/13)

It is a non-ionic, water dispersible surfactant composed of well-characterized PEG-esters, a small glyceride fraction and free PEG. Its chemical name is Stearoyl macrogol-32 glycerides. It is able to self-emulsify on contact with aqueous media forming a fine dispersion i.e. microemulsion [4]. It has excellent surfactive power to enhance the solubility and wettability of active pharmaceutical ingredients both *In-vitro* and *In-vivo*. It enhances the *In-vivo* drug solubilization and facilitates absorption. It has good thermoplasticity for use as binder in melt/fusion processes. It has HLB value 11 and melting point 50 °C. It is used in melt granulation or agglomeration techniques. It is also suitable for hard gelatin capsule molding and adsorption onto neutral carrier powders for use in tabletting, capsule filling and sachets [5].
Supplier: Gattefossè Italia S.r.l.

Lauroyl polyoxyl-32 glycerides (Gelucire® 44/14)

It is composed of PEG-esters, a small glyceride fraction and free PEG. Its chemical name is Lauroyl polyoxyl-32 glycerides. It is able to form microemulsion i.e. emulsify on contact with aqueous media forming a fine dispersion. It is a non-ionic water dispersible surfactant. It has excellent surfactive property that enhances the solubility and wettability of active pharmaceutical ingredients *In-vitro* and *In-vivo*. Its bioavailability enhancement can be attributed to improve *In-vivo* drug solubilization which facilitates absorption. It can be used as binder in melt processes, associated with rapid formation of stable crystalline phase because of its thermo plasticity behaviour. It can be formulated by melt granulation or melt extrusion techniques for capsule filling, tabletting, sachets etc. It has HLB value 11 and melting point 44 °C. It is available in the form of semi solid blocks. I can be used in the field of human pharmaceutical products, veterinary products excluding food producing animals [5].
Supplier: Gattefossè Italia S.r.l.

Polyethylene glycol monostearate (Gelucire® 48/16)

Gelucire 48/16 is a non-ionic surfactant recommended for use in Lipid-Based Formulations (LBFs) to increase aqueous solubility and oral bioavailability of poorly soluble active ingredients.

It is a PEG 32 ester lipid excipient comprising a mixture of stearic and palmitic acid monoesters and diesters.

Its high Hydrophilic-Lipophilic Balance (HLB) enhances the solubility of hydrophobic and lipophobic active ingredients.

It is solid at ambient temperature with a melting point of 48°C, making it suitable for capsule filling and melt processes. [4]

Supplier: Gattefossè Italia S.r.l.

2.3.1.2.2 Oils

Medium-chain triglycerides (Labrafac™ lipophile WL1349)

Medium-chain triglycerides have been used in a variety of pharmaceutical formulations including oral, parenteral, and topical preparations. In oral formulations, medium-chain triglycerides are used as the base for the preparation of oral emulsions, microemulsions, selfemulsifying systems, solutions, or suspensions of drugs that are unstable or insoluble in aqueous media, e.g. calciferol. Mediumchain triglycerides have also been investigated as intestinalabsorption enhancers and have additionally been used as a filler in capsules and sugar-coated tablets, and as a lubricant or antiadhesion agent in tablets. In parenteral formulations, medium-chain triglycerides have similarly been used in the production of emulsions, solutions, or suspensions intended for intravenous administration. In rectal formulations, medium-chain triglycerides have been used in the preparation of suppositories containing labile materials. In cosmetics and topical pharmaceutical preparations, medium-chain triglycerides are used as a component of ointments, creams, and liquid emulsions. Therapeutically, medium-chain triglycerides have been used as nutritional agents. Diets containing medium-chain triglycerides are used in conditions associated with the malabsorption of fat, such as cystic fibrosis, since medium-chain triglycerides are more readily digested than long-chain triglycerides. Medium-chain triglycerides have been particularly investigated for their use in total parenteral nutrition (TPN) regimens in combination with longchain triglycerides.

Although similar to long-chain triglycerides, medium-chain triglycerides have a number of advantages in pharmaceutical formulations, which include better spreading properties on the skin; no impedance of skin respiration; good penetration properties; good emollient and cosmetic properties; no visible film on the skin surface; good compatibility; good solvent properties; and good stability against oxidation [3].
Supplier: Gattefossè Italia S.r.l.

Soybean oil

A typical analysis of refined soybean oil indicates the composition of the acids, present as glycerides, to be: linoleic acid 50–57%; linolenic acid 5–10%; oleic acid 17–26%; palmitic acid 9–13%; and stearic acid 3–6%. Other acids are present in trace quantities.
In pharmaceutical preparations, soybean oil emulsions are primarily used as a fat source in total parenteral nutrition (TPN) regimens. Although other oils, such as peanut oil, have been used for this purpose, soybean oil is now preferred because it is associated with fewer adverse reactions. Emulsions containing soybean oil have also been used as vehicles for the oral and intravenous administration of drugs; drug substances that have been incorporated into such emulsions include amphotericin, diazepam, retinoids, vitamins, poorly water-soluble steroids, fluorocarbons, ibuprofen, and insulin. In addition, soybean oil has been used in the formulation of many drug delivery systems such as liposomes, microspheres, dry emulsions, self-emulsifying systems, microemulsions, nanoemulsions and nanocapsules, solid-in-oil suspensions, and multiple emulsions. Soybean oil may also be used in cosmetics and is consumed as an edible oil [3].
Supplier: Croda.

Sesame oil

A typical analysis of refined sesame oil indicates the composition of the acids, present as glycerides, to be: arachidic acid 0.8%; linoleic acid 40.4%; oleic acid 45.4%; palmitic acid 9.1%; and stearic acid 4.3%. Sesamin, a complex cyclic ether, and sesamolin, a glycoside, are also present in small amounts. Note that other reported analyses may vary slightly from that above. The monographs for Sesame Oil in the USP32–NF27 and Refined Sesame Oil in the PhEur 6.3 specify the acceptable range of eight triglycerides found in sesame oil.
Sesame oil has been included in self-microemulsifying drug delivery systems, and fast-disintegrating lyophilized dry emulsion tablets for oral administration [3].
Supplier: Croda.

2.3.1.2.3 Solubilizing solvents

Diethylene glycol monoethyl ether (Transcutol® HP)

Transcutol® (Diethylene glycol monoethyl ether, DEGEE) is commonly used as a vehicle in the formulation or manufacturing process of pharmaceuticals, cosmetics, and food additives [6]. It is a solubilizing agent and absorption enhancer. It is an excipient used in injectable products. It has been used in human therapeutics in cosmetics and other topical formulations and in oral and sublingual preparations [4].

Supplier: Gattefossè Italia S.r.l.

Polyethylene glycols (PEG)

Polyethylene glycols are widely used in a variety of pharmaceutical formulations. Generally, they are regarded as nontoxic and nonirritant materials. Adverse reactions to polyethylene glycols have been reported, the greatest toxicity being with glycols of low molecular weight. However, the toxicity of glycols is relatively low. [3].

The polyethylene glycols tested was PEG 400 (Lipoxol 400 med) and PEG 300 (Lipoxol 300 med).

Supplier: EIGENMANN & VERONELLI S.p.A.

Ethanol (Alcohol)

Ethanol and aqueous ethanol solutions of various concentrations are widely used in pharmaceutical formulations and cosmetics. Although ethanol is primarily used as a solvent, it is also employed as a disinfectant, and in solutions as an antimicrobial preservative. Topical ethanol solutions are used in the development of transdermal drug delivery systems as penetration enhancers. Ethanol has also been used in the development of transdermal preparations as a co-surfactant [3].

Supplier: Silcompa SpA

2.3.1.2.4 Preservatives and antioxidants

Butylated Hydroxyanisole (BHA)

Butylated hydroxyanisole is an antioxidant with some antimicrobial properties. It is used in a wide range of cosmetics, foods, and pharmaceuticals. When used in foods, it is used to delay or prevent oxidative rancidity of fats and oils and to prevent loss of activity of oil-

soluble vitamins. Butylated hydroxyanisole is frequently used in combination with other antioxidants, particularly butylated hydroxytoluene and alkyl gallates, and with sequestrants or synergists such as citric acid. FDA regulations direct that the total content of antioxidant in vegetable oils and direct food additives shall not exceed 0.02% w/w (200 ppm) of fat or oil content or essential (volatile) oil content of food. USDA regulations require that the total content of antioxidant shall not exceed 0.01% w/w (100 ppm) of any one antioxidant or 0.02% w/w combined total of any antioxidant combination in animal fats. Japanese regulations allow up to 1 g/kg in animal fats [3].

Supplier: C.F.M. Co. Farmaceutica Milanese.

Vitamin E Polyethylene Glycol Succinate (Vitamin E TPGS)

Vitamin E polyethylene glycol succinate is an esterified vitamin E (tocopherol) derivative primarily used as a solubilizer or emulsifying agent because of its surfactant properties. Structurally, it is amphipathic and hydrophilic, unlike the tocopherols, and therefore it is a water-soluble derivative that can be used in pharmaceutical formulations such as capsules, tablets, hot-melt extrusion, microemulsions, topical products, and parenterals. One of the most important applications is its use as a vehicle for lipid-based drug delivery formulations. It can also be used as a source of vitamin E. Vitamin E polyethylene glycol succinate has been characterized with respect to its mechanism of action and studied as a Pglycoprotein inhibitor [3].

Supplier: BASF

D, L-Alpha Tocopherol

Alpha tocopherol is primarily recognized as a source of vitamin E, and the commercially available materials and specifications reflect this purpose. While alpha tocopherol also exhibits antioxidant properties, the beta, delta, and gamma tocopherols are considered to be more effective as antioxidants. Alpha-tocopherol is a highly lipophilic compound and is an excellent solvent for many poorly soluble drugs [3].

Supplier: BASF

2.3.1.3 Analytical materials

Purified Water, HPLC grade; Acetonitrile, HPLC grade; Cortexolone 17α-valerate-21-propionate W.S.; DMEM, Sigma D5671; Foetal bovine serum, Sigma F6178; Penicillin/Streptomycin, Sigma PO781; Glutamine, Sigma G7513; Trypsin-EDTA 10X, Sigma 59418C; HBSS, Sigma 55037C; PBS, Sigma D8537; Tween-80, Sigma P4780; Hepes, Sigma H0887; HBSS + 0.05% Tween 80 + 25 mM HEPES.

2.3.2 Methods

2.3.2.1 API solubility in different media

The solubility of CB-03-10 in different media was evaluated adding, under stirring, 25 g the API in 1000 mL of each medium, the dispersion has been maintaining under stirring for 24h at 37°C.

Analytical conditions

Instrumentation: USP dissolution apparatus II complying with USP <711>;
Stirrer speed: 100 rpm;
Volume: 1000 ml;
Medium temperature: 37 ± 0.5°C;

Spectrophotometric analytical conditions

Instrumentation: UV- Visible spectrophotometer connected to a PC for data recording;
Wavelength: 240nm;
Quartz glass cell: 0.2 cm.

2.3.2.2 Morphological characteristics and dissolution behavior of the API

The influence of API particle size on its dissolution profile was verified. Particle size distribution (PSD) affects the dissolution behavior because the surface area influences the dissolution rate. In order to define and select the optimal PSD suitable to obtain an API particle size able to amelioration of the dissolution profile, 100g of CB-03-10 has been sieved through 450, 250 and 125 microns stainless sieve. A portion has been then analyzed

per dissolution profile and another portion has been analyzed with Microscope to define the PDS profile.

2.3.2.2.1 API particle size distribution

A portion of the powder was analyzed by an optical microscope (Morphologi G3S), equipped with internal camera and PC The sample, about 3 mm^3, was transferred to the disperser on the instrument, and automatically dispersed onto a glass plate.
Analysis parameters:

- Injection pressure 1 bar;
- Injection time 20 mms;
- Setting time 60s.

The powder was analysed under diascopic light at 80% power with optics 5x and 50x.
Filter out particles with:

- Circularity <0.4;
- CE diameter <0.50µm;
- Solidity <0.9;
- Convexity <0.95;

A separate picture was recorded of each particle. The following diameters were recorded: D[V,0.10]; D[V,0.25]; D[V,0.50]; D[V,0.75]; D[V,0.90].

2.3.2.2.2 API Dissolution profile

Analytical conditions

Dissolution test conditions

Instrumentation:	USP dissolution apparatus II complying with USP <711>;
Stirrer speed:	100 rpm;
Dissolution medium:	HCl 0.1M + 3% Macrogol Cetostearyl Ether;
Volume:	1000 ml;
Medium temperature:	37 ± 0.5°C;
Sampling time:	10, 20, 30, 40, 50, 60, 90, 120 minutes.

Spectrophotometric analytical conditions

Instrumentation: UV- Visible spectrophotometer connected to a PC for data recording;

Wavelength: 240nm;

Quartz glass cell: 0.2 cm.

2.3.2.3 Solubility studies

As Cortexolone 17α-valerate-21-propionate is a poorly soluble drug, the main focus of the development was the evaluation of a wide range of excipients, in particular lipid excipients, with various molecular structures and HLBs (hydrophilic-lipophilic balance) to improve the solubility/permeability of the drug.

The main excipients used during the pharmaceutical development are reported in Table 2.3.

Table 2.3 Main excipients used during the pharmaceutical development.

Function	**Excipient**
Surfactants	Caprylocaproyl polyoxyl-8 glycerides (Labrasol)
	Oleoyl polyoxyl-6 glycerides (Labrafil M1944CS)
	Stearoyl polyoxyl-32 glycerides (Gelucire 50/13)
	Lauroyl polyoxyl-32 glycerides (Gelucire 44/14)
	Polyethylene glycol monostearate (Gelucire 48/16)
Oil phase	Medium-chain triglycerides (Labrafac Lipophile WL1349)
	Soybean oil
	Sesame oil
Solubilizing solvents	Diethylene glycol monoethyl ether (Transcutol)
	Polyethylene glycol 400 (PEG 400)
	Polyethylene glycol 300 (PEG 300)
	Ethanol (Alcohol)
Preservatives and antioxidants	Butylated Hydroxyanisole (BHA)
	Vitamin E Polyethylene Glycol Succinate (Vitamin E TPGS)
	D, L-Alpha Tocopherol

As first screening a preliminary solubility test has been carried out adding, under stirring, 500 mg of API in 2 g of each excipient, the dispersion has been maintaining under stirring for 3 h at 70°C. The experiment has been conducted at 70°C due to the physical status of some of the excipient under evaluation that are semisolid or solid at 25°C but liquid at 70°C. The samples were analyzed by HPLC method.

2.3.2.3.1 HPLC Analytical Conditions for solubility studies

Reagents

Purified Water, HPLC grade;

Acetonitrile, HPLC grade;

Chromatographic conditions

Instrumentation:	High Performance Liquid Chromatograph equipped with autosampler, pump, UV detector, and PC workstation for data acquisition and analysis
Column:	Zorbax XBD C18 1.8 µm, 50 x 2.1 mm
Detector wavelength:	240 nm
Flow rate:	0.6 mL/minute
Injection volume:	2 µl
Column temperature:	35° C
Mobile phase:	Phase A: water Phase B: acetonitrile Mixed according to gradient program reported in Table 2.4:

Table 2.4 Gradient program.

Time	Phase A	Phase B
0	60	40
0.5	60	40
7	20	80
8	20	80
8.1	60	40
10	60	40

2.3.2.4 Solubility test

In the second phase drug solubility with the excipients selected in the previous phase were evaluated either to verify the compatibility between drug and excipients and to evaluate if some excipients have a synergic effect.

The solubility of the API into the different excipients was evaluated by testing by HPLC method purity and assay.

Saturated solutions have been prepared and maintained at 25°C for 24 h, then the samples were analyzed for Assay and Purity by HPLC method.

2.3.2.4.1 HPLC Analytical Conditions

Reagents

Purified Water, HPLC grade;

Acetonitrile, HPLC grade;

Chromatographic conditions

Instrumentation:	High Performance Liquid Chromatograph equipped with autosampler, pump, UV detector, and PC workstation for data acquisition and analysis
Column:	Zorbax XBD C18 1.8 µm, 50 x 2.1 mm
Detector wavelength:	240 nm
Flow rate:	0.6 mL/minute
Injection volume:	2 µl
Column temperature:	35° C
Mobile phase:	Phase A: water Phase B: acetonitrile Mixed according to gradient program reported in Table 2.5:

Table 2.5 Gradient program.

Time	Phase A	Phase B
0	60	40
0.5	60	40
7	20	80
8	20	80
8.1	60	40
10	60	40

2.3.2.5 Permeability study

The Caco-2 monolayer is widely used across the pharmaceutical industry as an *in vitro* model of the human small intestinal mucosa to predict the absorption of orally administered drugs. The correlation between the *in vitro* apparent permeability (Papp) across Caco-2 monolayers and the *in vivo* absorbed fraction is well established.

2.3.2.5.1 Aim

This study phase aims to assess permeability of API as is and of API contained in six different formulations. The permeability of the API though Caco-2 monolayer was assessed after 30 and 120 minutes.

The Caco-2 used to perform this test was human colorectal adenocarcinoma cells purchased from ATCC (Lgc standards) at passage number 38.

2.3.2.5.2 Materials and methods

Analyte

The following formulations were assessed:

- API as is
- Formulation 1

Table 2.6 Quali-quantitative composition of Formulation 1.

Component(s)	**% w/w (g/100g)**
API	10.00
Phosphatidylcholine	20.00
Mannitol	63.70
Croscarmellose Sodium	6.30

In a suitable tank, pour Alcohol and heat up to 40°C. Add Phosphatidylcholine and stir until solubilization. Add API and stir at 40°C.

Add a mixture of Mannitol and Croscarmellose Sodium, mix manually at 40°C. Switch off the heating and continue to mix until the bulk reach room temperature.

Dry at 40°C overnight. Sieve the granulate by 20 µm sieve.

- Formulation 2

Table 2.7 Quali-quantitative composition of Formulation 2.

Component(s)	**% w/w (g/100g)**
API	6.00
Butylated hydroxyanisole	0.02
Caprylocaproyl Polyoxyl-8 glycerides	93.98

In a suitable tank, pour Caprylocaproyl Polyoxyl-8 glycerides. Add Butylated hydroxyanisole and stir until solubilization.
Add API and stir for 60 minutes. Heat up to 50°C and stir until solubilization. Cool up to room temperature maintaining the stirring.

- Formulation 3

Table 2.8 Quali-quantitative composition of Formulation 3.

Component(s)	**% w/w (g/100g)**
API	48.00
Croscarmellose Sodium	16.00
Mannitol	36.00

In a suitable tank, mix API and Croscarmellose Sodium. Mill and sieve though 20 µm sieve. Add mannitol, mix and sieve though 20 µm sieve.

- Formulation 4

Table 2.9 Quali-quantitative composition of Formulation 4.

Component(s)	**% w/w (g/100g)**
API	8.00
Butylated hydroxyanisole	0.02
Ethanol 96%	5.00
Caprylocaproyl polyoxyl-8 glycerides	86.98

In a suitable tank, pour Caprylocaproyl Polyoxyl-8 glycerides. Add Butylated hydroxyanisole and Ethanol and stir until solubilization.
Add API and stir for 60 minutes. Heat up to 50°C and stir until solubilization. Cool up to room temperature maintaining the stirring. Add ethanol up to final weight.

- Formulation 5

Table 2.10 Quali-quantitative composition of Formulation 5.

Component(s)	**% w/w (g/100g)**
API	10.00
Oleoyl polyoxyl-6 glycerides	56.00
D,L-α-tocopherol	5.00
Ethanol 96%	5.00
Polyethylene glycol 400	3.00
Medium-chain triglycerides	21.00

In a suitable tank, pour Oleoyl polyoxyl-6 glycerides, add Medium-chain triglycerides and stir for 5 minutes. Add Polyethylene glycol 400 and stir for 5 minutes then add D,L-α-tocopherol and ethanol and stir until homogenization. Add API and stir for 60 minutes. Heat up to 50°C and stir until solubilization. Cool up to room temperature maintaining the stirring. Add ethanol up to final weight.

- Formulation 6

Table 2.11 Quali-quantitative composition of Formulation 6.

Component(s)	**% w/w (g/100g)**
API	7.00
Lauroyl Polyoxyl-32 glycerides	21.52
Stearoyl polyoxyl-32 glycerides	21.52
Cellulose microcrystalline	22.30
Mannitol	22.30
Croscarmellose Sodium	5.36

In a suitable tank, pour Lauroyl Polyoxyl-32 glycerides and Stearoyl polyoxyl-32 glycerides and heat up to 70°C. Stir until completely melted.

Mix Cellulose microcrystalline, Mannitol and Croscarmellose Sodium. Add the melted phase to the powders mix kneading and heating up to 70°C. Cool up to to room temperature maintaining the kneading.

Sieve the obtained granules though 20 µm sieve.

Chemicals

- DMEM, Sigma D5671 or equivalent
- Foetal bovine serum, Sigma F6178 or equivalent
- Penicillin/Streptomycin, Sigma PO781 or equivalent
- Glutamine, Sigma G7513 or equivalent
- Trypsin-EDTA 10X, Sigma 59418C or equivalent
- HBSS, Sigma 55037C or equivalent
- PBS, Sigma D8537 or equivalent
- Tween-80, Sigma P4780 or equivalent
- Hepes, Sigma H0887 or equivalent
- HBSS + 0.05% Tween 80 + 25 mM HEPES (Solvent A)

Disposables

- 24-well polystyrene plates, DB 353047 or equivalent
- 6.5 mm Transwell with 0.4 µm pore polycarbonate membrane insert, Costar 3413 or equivalent

Equipments

- Analytical balance, Sartorius CP225D-0CE
- Laminar flow hood, Labosystem BSB-4
- CO2 incubator, Heraeus BB16
- Orbital shaker, Heidolph Unimax 1010DT
- Thermomixer, Eppendorf Comfort
- Micropipettes, Gilson
- Centrifuge, Thermo Heraeus Fresco 21
- Mass Spectrometer, AB Sciex API 4000 ESI-Triple Quadrupole Mass Spectrometer
- Agilent HP1100 pump system
- Millipore Millicell ERS-2

Caco-2 cell culture conditions

Caco-2 cells were maintained in DMEM containing 10% FBS, 2 mM Penicillin-streptomycin, and 2 mM glutamine, at 37°C, 5% CO2, in a humidified atmosphere and the medium was changed every two to three days. Cells were sub-cultured at 70-80% confluence by splitting them with trypsin-EDTA.

For cultivation of Caco-2 cell monolayers on permeable support, the needed number of filters was placed in cell culture cluster, filters were pre-wet with medium for at least 1 hour, followed by seeding cells, at passage number 38, at 100,000 cells per insert by dispensing 200 µL of re-suspended cell solution. Basolateral was filled with 850 µL medium. The plate was incubated at 37°C, 5% CO2, in a humidified atmosphere and medium was changed every two to three days.

The schematic representation of the Caco-2 cell monolayers on permeable support is reported in Figure 2.2.

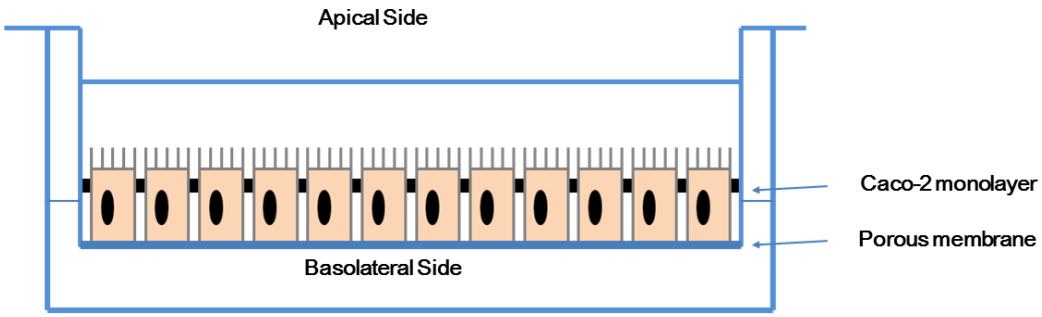


Figure 2.2 Schematic representation of the Caco-2 cell monolayers on permeable support.

Monolayer integrity

TEER measurements were used as indicator of monolayer integrity, using a Millipore Millicell ERS-2 instrument. The electrical resistance across the monolayer was measured by positioning the probes one into the apical chamber and one into basolateral chamber of the well. The resultant value in Ω was multiplied by the membrane area (0.33 cm^2) to calculate transepithelial electrical resistance (TEER (Ω cm^2)) values for each monolayer.

TEER value was recorded before cell treatment. If the resistance was < 200 Ω cm^2, monolayer was not used for permeability assessment.

TEER value was also recorded after the last time point of cell treatment, after substitution of the donor volume with HBSS.

API and formulations

API compound as is, was suspended in HBSS containing 0.05% Tween 80 and 25 mM HEPES (solvent A) to obtain a 10 mM stock. The 10 mM stock was used to prepare a 50 µM suspension in solvent A. The preparation was vortexed for 30 seconds, incubated at 37°C for 20 minutes and vortexed again.

The six formulations containing the API ware stored at room temperature.

Stock solutions (1 mM) were prepared the day of cell treatment, by addition of solvent A to powder or liquid. Resulting pH was between 6.5-7.5. the obtained preparations were diluted to 50 µM suspensions/emulsions in Solvent A. The preparations were vortexed for 30 seconds, incubated at 37°C for 20 minutes and vortexed again.

Preparation of API and formulations were prepared in glass vials and pre-warmed at 37°C before use.

Placebo formulations were diluted in the same way as active formulations, for use in the receiver compartment, to avoid the precipitation of API compound after cell permeation.

Assay performance

Three controls (low permeability, Atenolol; Medium Permeability, Famotidine; High Permeability, Metaprolol), at 100 µM were assessed in five replicates to verify assay performance.

Apical-to-basolateral permeability assessment

At the time of the experiment, the medium was removed, the monolayers rinsed with HBSS, then 200 µL HBSS was added into the apical chambers of the monolayers and 850 µL of HBSS into the basolateral chambers, and TEER values recorded at 37°C.

Then, the donor volume was removed and 250 µL of test compound, prepared at 50 µM, were added into the apical chambers. Receiver chambers contained 850 µL of respective placebo. Five replicates were assessed for each compound formulation.

Two additional replicates for each compound, were plated on transwells without cells for assessment of non-specific binding. Plates were incubated for 2 hours at 37°C at 100 rpm on an orbital shaker, in a CO_2 incubator humidified atmosphere. Immediately after addition (T_0), after 30 minutes (T_{30}) and after 2 hours (T_{120}), 50 µL was collected from donor and receiver compartments for LC-MS/MS analysis. After the 30 minutes withdrawal, volume was restored with additional of solvent A. After the permeability experiment, donor and receiver volumes were removed and 200µL of HBSS was added into the apical chambers and 850 µL of HBSS into the basolateral chambers, and TEER values were recorded to assess the integrity of the monolayer during the experiment.

LC-MS/MS analysis

The samples collected from donor and receiver compartment, were appropriately diluted in acetonitrile containing 0.1% trifluoroacetic acid, then internal standard (glafenine for controls, a mixture of API and its metabolite for the API) were added.

Samples were centrifugated at 14,000 rpm for 10 minutes at 4°C, transferred to glass vials and injected for LC-MS/MS analysis.

Calibration curve samples, prepared in 20% HBSS – 80% acetonitrile containing 0.1% trifluoroacetic acid, with 5 concentrations of each analyte (API, its metabolite and controls) were analysed after addition of the appropriate internal standard. Standard concentrations were 6.25, 12.5, 25.0, 50, and 100 nM, for both the analytes.

For quantification at T_0, samples were diluted before LC-MS/MS analysis (LLOQ = 3125 for both analytes). For all other time points, samples were diluted from 1:5 to 1:100 (LLOQ = 31.25 for both analytes). The determination of the concentrations of the analytes was performed through a linear regression model.

The saples were analyzed under the following analytical conditions:

HPLC separation of injected samples (5 µL) was performed using a Gemini 5µ C18 RP, 50 x 2.00 mm (Phenomenex).

For the samples containing the API the following gradient (Table 2.12) of acetonitrile (mobile phase B) in 0.1% formic acid in water (mobile phase A) was used:

Table 2.12 Sample gradient program.

Flow (mL/min)	Time (min)	% mobile phase A	% mobile phase B
0.4	0.0	70	30
0.4	0.3	70	30
0.4	1.8	5	95
0.4	2.3	5	95
0.4	2.4	70	30
0.4	3.4	70	30

For controls the following gradient (Table 2.13) of acetonitrile (mobile phase B) in 0.1% formic acid in water (mobile phase A) was used:

Table 2.13 Control gradient program.

Flow (mL/min)	Time (min)	% mobile phase A	% mobile phase B
0.4	0.0	95	5
0.4	0.3	95	5
0.4	1.8	5	95
0.4	2.3	5	95
0.4	2.4	95	5
0.4	3.4	95	5

Data Analysis

All estimated concentrations were obtained by Analyst software (AB Sciex, version 1.5.1) directly from chromatograms.

The permeability coefficient (expressed as cm/sec) was calculated as follows:

$$P_{app} = (C_r.V_r)/(t.A.C_0)$$

Where:

V_r = receiver volume (0.850 mL)

C_r = measured receiver concentration (nM)

t = time (sec)

A = filter surface area (0.33 cm^2)

C_0 = measured concentration of donor solution (nM)

Mass balance was calculated as follows: $((C_d \times V_d) + (C_r \times V_r))/(C_i.V_i)$

Where:

C_d = measured donor concentration (nM)

V_d = donor volume (0.200 mL)

C_r = measured receiver concentration (nM)

V_r = receiver volume (0.850 mL)

C_i = measured initial donor concentration (nM)

V_i = donor volume (0.200 mL)

P_{app} and mass balance were calculated for both time points (30 minutes and 2 hours).

For permeability assessment and mass balance of API, amounts of API and its metabolite were measured.

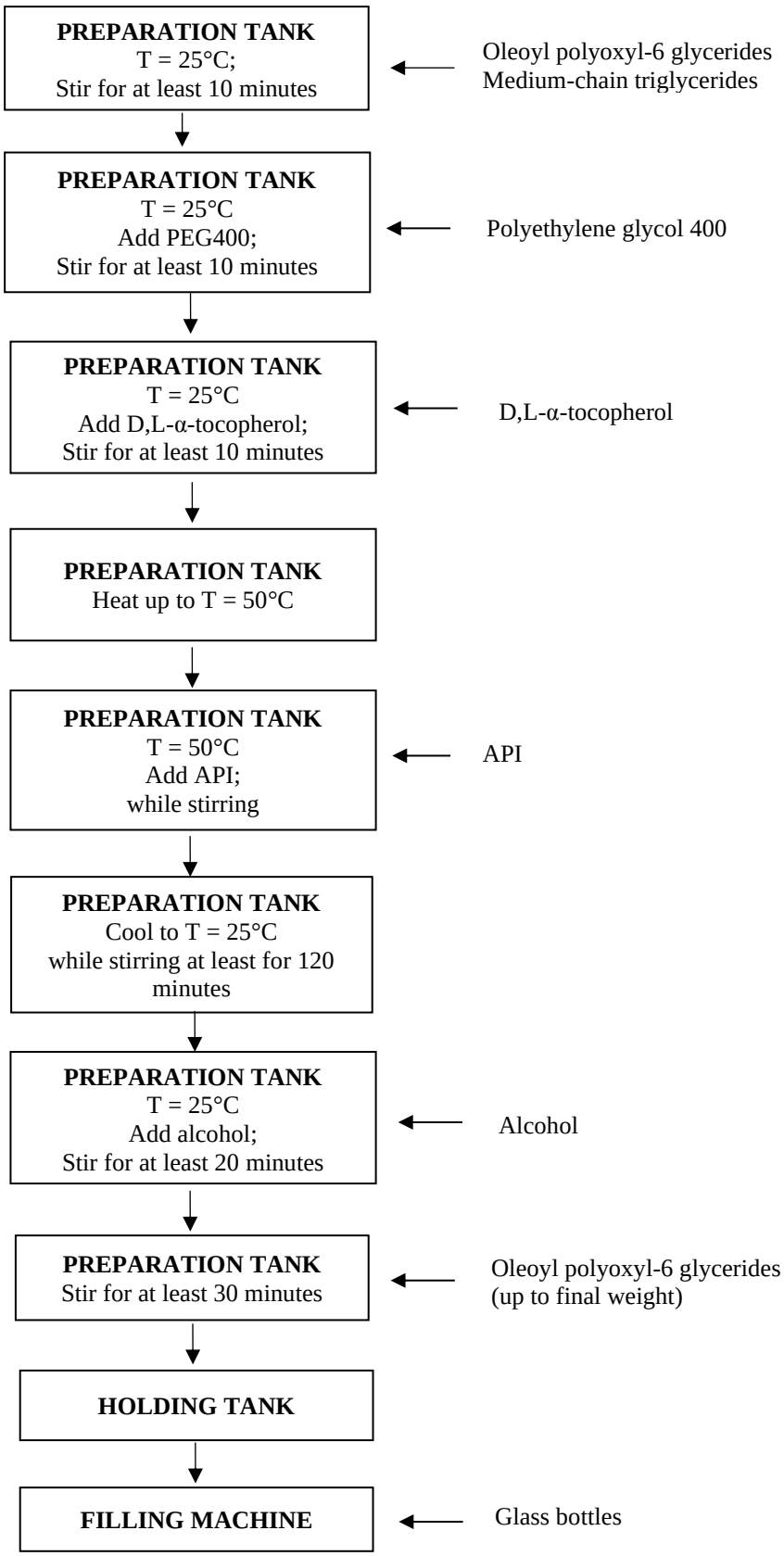
2.3.2.6 Preparation of the selected 10% oral formulation

Table 2.14 Composition of the selected 10% oral formulation.

Component(s)	**Specification**	**Function**	**% w/w (g/100g)**
CB-03-10 (cortexolone 17α-valerate-21 propionate)	Internal monograph	Active ingredient	10.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	EP, USP	Surfactant	56.00
D, L-α-tocopherol	EP, USP	Antioxidant/solubilizer	5.00
Ethanol 96%	EP, USP	Co-surfactant/solubilizer	5.00
Polyethylene glycol 400 (PEG 400)	EP, USP	Co-surfactant	3.00
Medium-chain triglycerides NF (Labrafac Lipophile WL1349)	EP, USP, JPE	Oil	21.00

2.3.2.6.1 Manufacturing process

The manufacturing process is summarized in the following flow-chart:



2.3.2.6.2 Brief description of the manufacturing process

PHASE I (Labrafil and Labrafac mixture)

In the Preparation Tank pour the weighed amount of Oleoyl polyoxyl-6 glycerides. Then add slowly under high speed stirring Medium-chain triglycerides. Stir for at least 10 minutes maintaining the temperature around 25°C.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE II (Addition of Polyethylene glycol 400)

Add slowly under high speed stirring Polyethylene glycol 400. Stir for at least 10 minutes maintaining the temperature around 25°C.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE III (Addition of D, L-α-tocopherol)

Add slowly under high speed stirring D, L-α-tocopherol. Stir for at least 10 minutes maintaining the temperature around 25°C.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE IV (Heating and CB-03-10 solubilization)

Heat up to 50°C and then add slowly under high speed stirring, CB-03-10.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE V (cooling phase)

Cool to 25°C while stirring. Stir for at least 120 minutes.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE VI (Addition of Ethanol)

Add under high speed stirring Ethanol. Stir for at least 20 minutes maintaining the temperature around 25°C.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

FINAL WEIGHT

Add Oleoyl polyoxyl-6 glycerides up to final weight.

PRIMARY PACKAGING

Container: type III amber glass bottle, 65 mL +/- 3 mL

Polypropylene child proof capsule

Filling weight: 50 g

2.3.2.7 Analytical procedures for the selected 10% oral formulation

2.3.2.7.1 CB-03-10 identification (HPLC)

Perform the test during the assay, using the same sample preparation.

The retention time of the main peak in the sample solution chromatogram corresponds to that of the standard solution.

2.3.2.7.2 CB-03-10 assay (HPLC)

Analytical Conditions

Reagents

Purified Water, HPLC grade;

Acetonitrile, HPLC grade;

Chromatographic condition

Instrumentation:	High Performance Liquid Chromatograph equipped with autosampler, pump, UV detector and PC workstation for data acquisition and analysis.

Column:	Zorbax Extend C18 1.8 µm, 100 x 2.1 mm or equivalent
Detector wavelength:	240 nm
Flow rate:	0.6 mL/minute
Injection volume:	2 µl
Column temperature:	35° C
Mobile phase:	Phase A: water
	Phase B: acetonitrile
	Mixed according to gradient program reported in Table 2.15:

Table 2.15 Gradient program.

Time	Phase A	Phase B
0	60	40
0.5	60	40
7	30	70
8	30	70
8.1	60	40
10	60	40

Standard Solution and Control Standard Solution

In a 50 mL volumetric flask weigh accurately 50 mg of Cortexolone 17α-valerate-21-propionate W.S., dissolve in acetonitrile and bring to volume with the same solvent.

Filter through a 0.45 µm membrane filter before injection. (Standard Solution: Cortexolone 17α-valerate-21-propionate theoretical concentration: 1.0 mg/mL).

Sample preparation

Prepare a Sample Solution containing Cortexolone 17α-valerate-21-propionate 1.0 mg/mL on the basis of the theoretical concentration of the formulation.

Stir on a magnetic stirrer and filter through a 0.45 µm cellulose membrane filter prior to injection.

System suitability test

Analyse the Standard solution and record the chromatograms.

Verify that the System Suitability Test criteria are fulfilled:

1) The USP tailing of Cortexolone 17α-valerate-21-propionate, calculated by the following formula, is not more than 1.5.

$$T = \frac{W_{0.05}}{2f}$$

where:

$w_{0.05}$ = width of the peak at 5% height;

f = distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

2) The Relative Standard deviation (RSD) of Cortexolone 17α-valerate-21-propionate peak areas, calculated on 5 replicate injections of the *Standard Solution*, is not more than 2.0%.

3) Analyse the *Control Standard Solution* at least after the injections of the Standard Solution (n=5) and at the end of HPLC sequence.
Cortexolone 17α-valerate-21-propionate recovery must range between 98.0% and 102.0%.

Calculation

Inject the Sample Solution. Record the chromatograms and calculate the peak areas.
Calculate the Cortexolone 17α-valerate-21-propionate amount in the sample, as % w/w using the following formula:

$$\text{Cortexolone 17α-valerate-21-propionate (\% w/w)} = \frac{A_{SMP} \times C_S \times D_{SMP} \times 100}{A_S \times W_{SMP}}$$

where:

A_{SP} = Cortexolone 17α-valerate-21-propionate peak area in Sample Solution chromatogram;

C_S = concentration (mg/mL) of Cortexolone 17α-valerate-21-propionate in Standard solution, corrected by its potency %;

D_{SMP} = sample dilution (mL);

A_S = mean peak area (n=5) of Cortexolone 17α-valerate-21-propionate in Standard Solution chromatograms;

W_{SMP} = sample weight (mg);

2.3.2.7.3 Related Substances

Analytical conditions

Reagents

Purified Water, HPLC grade;

Acetonitrile, HPLC grade;

Chromatographic conditions

Instrumentation:	High Performance Liquid Chromatograph equipped with autosampler, pump, UV detector and PC workstation for data acquisition and analysis.
Column:	Zorbax Extend C18 1.8 μm, 100 x 2.1 mm or equivalent
Detector wavelength:	240 nm
Flow rate:	0.6 ml/minute
Injection volume:	2 μl
Column temperature:	35° C
Mobile phase:	Phase A: water
	Phase B: acetonitrile
	Mixed according to gradient program reported in Table 2.16:

Table 2.16 Gradient program.

Time	Phase A	Phase B
0	65	35
1	65	35
18	30	70
21	30	70
22	20	80
24	20	80
26	65	35
28	65	35

Resolution Solution

In a 25 ml volumetric flask weigh accurately 5 mg of Cortexolone, 5 mg of Cortexolone 17α-propionate W.S, 5 mg of Cortexolone 21-acetate, 5 mg of Cortexolone 21-propionate, 5 mg of Cortexolone17α-valerate, 5 mg of Cortexolone 21-valerate and 5 mg of Cortexolone 17α-valerate-21-propionate then dissolve and dilute to final volume with acetonitrile.

Transfer 1.0 ml of this solution into a 10 ml volumetric flask, dilute to volume with acetonitrile (Resolution Solution).

Filter the solution by a 0.2 μm membrane filter.

Standard Solution and Control Standard Solution

In a 50 ml volumetric flask weigh accurately 50 mg of Cortexolone 17α-valerate-21-propionate W.S., then dissolve and bring to volume with acetonitrile.

Transfer 1.0 ml of this solution in a 100 ml volumetric flask, bring to volume with acetonitrile (Standard Solution, Cortexolone 17α- valerate-21-propionate theoretical concentration: 0.01 mg/ml).

Filter the solution by a 0.2 μm membrane filter.

Sample preparation

Prepare a Sample Solution containing Cortexolone 17α-valerate-21-propionate 1.0 mg/mL on the basis of the theoretical concentration of the formulation.

Stir on a magnetic stirrer and filter through a 0.2 μm cellulose membrane filter prior to injection.

System Suitability Test

Chromatograph the Resolution Solution and the Standard Solution (6 replicate injections) and record the chromatograms.

Verify that the System Suitability Test criteria are fulfilled:

1) The resolution (R) between Cortexolone 21-acetate and Cortexolone 17α-propionate peaks in the chromatogram of the *Resolution Solution* is not less than (NLT) 2.0. Peak resolution is calculated by the following formula:

$$R = \frac{1.18\,(t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

where:

t_R = retention time measured from time of injection to time of elution of peak maximum;

w_h = peak widths at half-height.

Identify the known impurities using the impurities retention time in chromatogram of the *Resolution Solution.*

2) The symmetry factor (As) of Cortexolone 17α-valerate-21-propionate peak in the chromatogram of Standard Solution is not more than (NMT) 1.5 and is calculated by the following equation:

$$A_s = \frac{w_{0.05}}{2f}$$

where:

w0.05 = width of peak at one-twentieth of the peak height;

f = distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

3) System precision: the Relative Standard Deviation (R.S.D.) of Cortexolone 17α-valerate-21-propionate peak areas calculated on 6 replicate injections of the Standard Solution is not more than 5%.

4) Analyse the Control Standard Solution at least after the injections of the Standard Solution (n=6) and at the end of HPLC sequence.

Cortexolone 17α-valerate-21-propionate recovery must range between 90.0% and 110.0%.

Calculations

Inject the Standard and the Sample Solution. Record the chromatograms and measure the peak areas.

Calculate each related substance amount as % respect to Cortexolone 17α-valerate-21-propionate content by the following formula:

$$\text{Related substance amount (\%)} = \frac{A_I \times C_S \times D_{SMP} \times 10000}{A_S \times W_{SMP} \times C}$$

where:

A_I = peak area of each related substance in the Sample Solution chromatogram;

CS = concentration of Cortexolone 17α-valerate-21-propionate W.S. in Standard Solution, corrected by its potency (mg/ml);

DSMP = sample dilution (ml);

AS = Cortexolone 17α-valerate-21-propionate mean peak area (n=6) in Standard Solution chromatograms;

WSMP = sample weight (mg);

C = labelled concentration (%w/w) of Cortexolone 17α-valerate-21-propionate in the solution.

LOD = 0.01 %

LOQ = 0.03 %

2.3.2.7.4 Viscosity

Instrument: Viscosimeter Brookfield LVDV III Ultra;
Spindle: 61;
Rotation rate: 60 rpm;
Sample temperature: 25° C.

Analyze about 50 ml of solution under exam and record three readings.
Express the result as mean of data collected.

2.3.2.7.5 Relative density

Perform the test at 25°C according to the current European Pharmacopoeia (2.2.5).

2.3.2.7.6 Microbiological Analysis

Perform the test to detect total aerobic microbial count, total combined yeasts and molds count, and to verify the absence of Escherichia Coli according to the current European Pharmacopoeia and USP.

2.3.2.8 Stability studies on CB-03-10 10% w/w oral solution

The storage conditions tested during stability for each batch of CB-03-10 10% w/w oral solution are reported in the Table 2.17:

Table 2.17 Stability storage conditions.

Batch	Storage conditions
7100	25°C/60%RH; 40°C/75%RH; 30°C/65%RH
7271	25°C/60%RH
7297	25°C/60%RH

2.3.2.9 DLS Characterization

In order to test the self-emulsify ability of the formulations, they were characterized by Zetasizer Nano S upon dilution with WFI.
Each formulation was diluted 1:200 with WFI and left under stirring 100 rpm for 30 min. After this time, the dilutions were sonicated for 5 minutes and then analysed with DLS (Zetasizer Nano S).

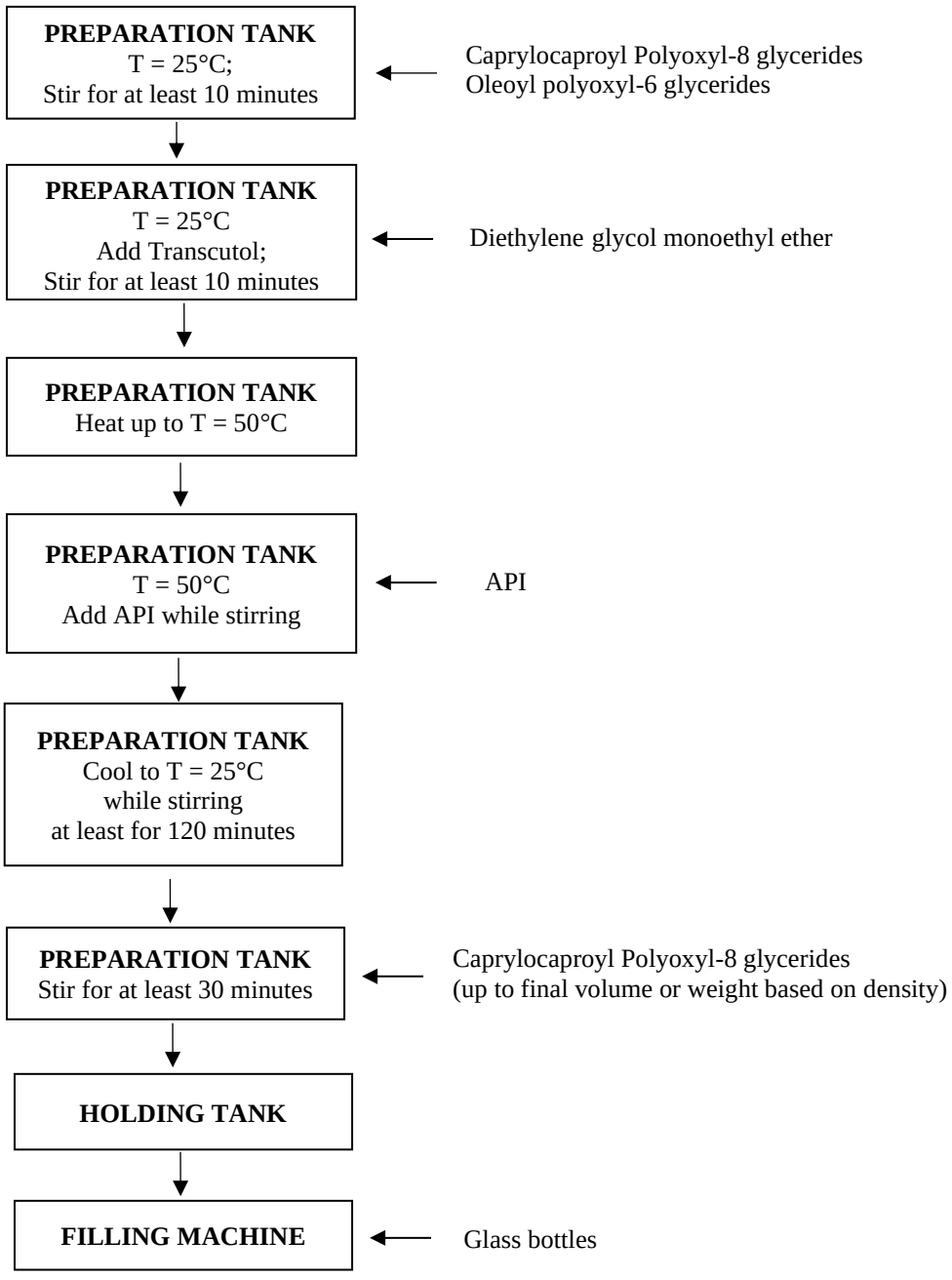
2.3.2.10 Preparation of the selected 30 mg/mL formulation

Table 2.18 Composition of the selected 30 mg/mL oral solution.

Component(s)	Specification	Function	% w/V (g/100mL)
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Internal monograph	Active ingredient	3.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	EP, USP	Surfactant	20.00
Diethylene glycol monoethyl ether (Transcutol HP)	EP, USP	Co-Solvent	20.00
Caprylocaproyl Polyoxyl-8 glycerides (Labrasol ALF)	EP, USP	Surfactant	up to 100 mL

2.3.2.10.1 Manufacturing process

The manufacturing process of CB-03-10 30 mg/mL oral solution is summarized in the following flow-chart:



2.3.2.10.2 Brief description of the manufacturing process

PHASE I (Labrasol and Labrafil mixture)

In the Preparation Tank pour Caprylocaproyl Polyoxyl-8 glycerides Then add slowly under high speed stirring Oleoyl polyoxyl-6 glycerides. Stir for at least 10 minutes maintaining the temperature around 25°C.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE II (Addition of Transcutol)

Add slowly under high speed stirring Transcutol. Stir for at least 10 minutes maintaining the temperature around 25°C.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE III (Heating and CB-03-10 solubilization)

Heat up to 50°C and then add slowly under high speed stirring, CB-03-10.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE IV (cooling phase)

Cool to 25°C while stirring. Stir for at least 120 minutes.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

FINAL VOLUME

Add Caprylocaproyl Polyoxyl-8 glycerides up to final volume (or weight, based on density).

PRIMARY PACKAGING

Container: type III amber glass bottle, 65 mL +/- 3 mL. Polypropylene child proof capsule
Filling volume: 50 mL.

2.3.2.11 Analytical procedure for 30 mg/mL formulations

The analytical methods used for the CB-03-10 30 mg/ml oral solution are the same as the analytical methods described in this section for 10% solution. The analytical methods (sample preparation) for Assay and Related Substances have been adapted and adjusted, to compensate for the reduced concentrations of active ingredient in CB-03-10 30 mg/ml oral solution.

2.3.2.11.1 Assay (HPLC)

Calculations

Inject the Sample Solution. Record the chromatograms and calculate the peak areas.

Express the content of Cortexolone 17α-valerate-21-propionate by the following calculations:

$$\text{Cortexolone 17α-valerate-21-propionate (mg/mL)} = \frac{A_{SMP} \times C_S \times D_{SMP} \times d_{SMP}}{A_S \times W_{SMP}}$$

where:

ASMP = Cortexolone 17α-valerate-21-propionate peak area in Sample Solution chromatogram;

CS = concentration (mg/mL) of Cortexolone 17α-valerate-21-propionate in Standard solution, corrected by its potency %;

DSMP = sample dilution (mL);

AS = mean peak area (n=5) of Cortexolone 17α-valerate-21-propionate in Standard Solution chromatograms;

WSMP = sample weight (g);

dSMP = sample density (g/mL at 25°)

2.3.2.11.2 Related substances

Calculations

Inject the Standard and the Sample Solution. Record the chromatograms and measure the peak areas.

Calculate each related substance amount as % respect to Cortexolone 17α-valerate-21-propionate content by the following formula:

$$\text{Related substance amount (\%)} = \frac{A_I \times C_S \times D_{SMP} \times d_{SMP} \times 100}{A_S \times W_{SMPx} C_{mg/ml}}$$

where:

AI = peak area of each related substance in the Sample Solution chromatogram;

CS = concentration of Cortexolone 17α-valerate-21-propionate W.S. in Standard Solution, corrected by its potency (mg/ml);

DSMP = sample dilution (ml);

AS = Cortexolone 17α-valerate-21-propionate mean peak area (n=6) in Standard Solution chromatograms;

WSMP = sample weight (g);

dSMP = sample density (g/mL at 25°C);

C = labelled concentration of Cortexolone 17α-valerate-21-propionate in the solution (mg/mL)

LOD = 0.01 %

LOQ = 0.03 %

2.3.2.12 Stability study on CB-03-10 30 mg/mL oral solution

The storage conditions tested during stability for CB-03-10 30 mg/mL oral solution are reported in Table 2.19:

Table 2.19 Stability storage conditions.

Batch	Storage conditions
7294	25°C/60%RH; 40°C/75%RH

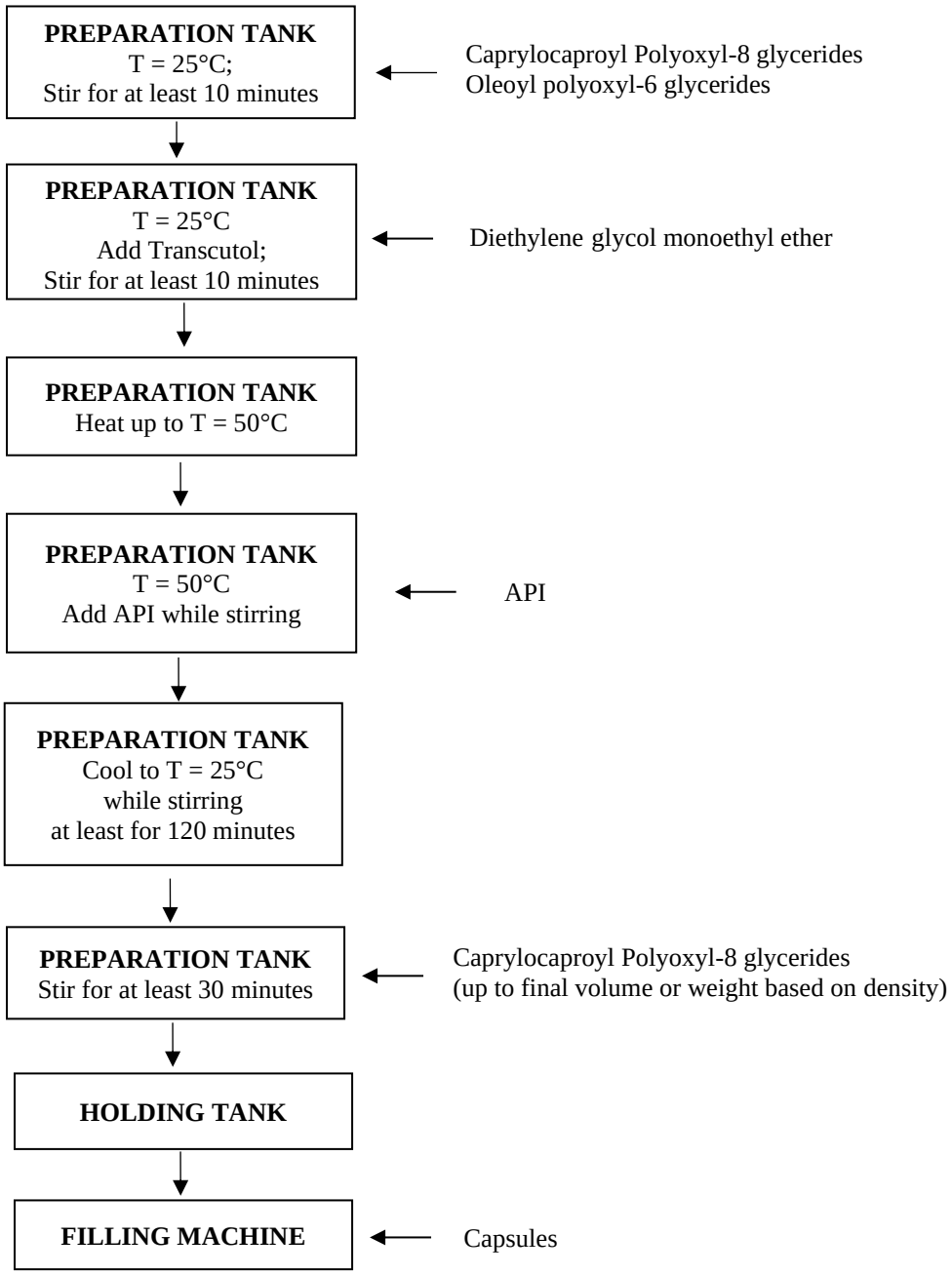
2.3.2.13 Preparation of the new liquid formulation 80 mg/mL

Table 2.20 Composition of the liquid formulation 80 mg/mL.

Component(s)	**Specification**	**Function**	**% w/V (g/100mL)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Internal monograph	Active ingredient	8.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	EP, USP	Surfactant	20.00
Diethylene glycol monoethyl ether (Transcutol HP)	EP, USP	Co-Solvent	20.00
Caprylocaproyl Polyoxyl-8 glycerides (Labrasol ALF)	EP, USP	Surfactant	up to 100 mL

2.3.2.13.1 Manufacturing process

The manufacturing process is summarized in the following flow-chart:



2.3.2.13.2 Brief description of the manufacturing process

PHASE I (Labrasol and Labrafil mixture)

In the Preparation Tank pour Caprylocaproyl Polyoxyl-8 glycerides Then add slowly under high speed stirring Oleoyl polyoxyl-6 glycerides. Stir for at least 10 minutes maintaining the temperature around 25°C.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE II (Addition of Transcutol)

Add slowly under high speed stirring Transcutol. Stir for at least 10 minutes maintaining the temperature around 25°C.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE III (Heating and CB-03-10 solubilization)

Heat up to 50°C and then add slowly under high speed stirring, API.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

PHASE IV (cooling phase)

Cool to 25°C while stirring. Stir for at least 120 minutes.

In Process Controls:

- Visual inspection: Light Yellow Clear Solution

FINAL VOLUME

Add Caprylocaproyl Polyoxyl-8 glycerides up to final volume (or weight, based on density).

2.3.2.13.3 Capsules

Based on density of the formulation (1.0277 g/mL) and the target dose of API in one capsule (100 mg), the total fill weight expected is 1.2846g/cps.

[applied formulas: 100mg:80mg/mL=1.25mL/cps

1.25mL/cps*1.0277g/mL=1.2846g/cps].

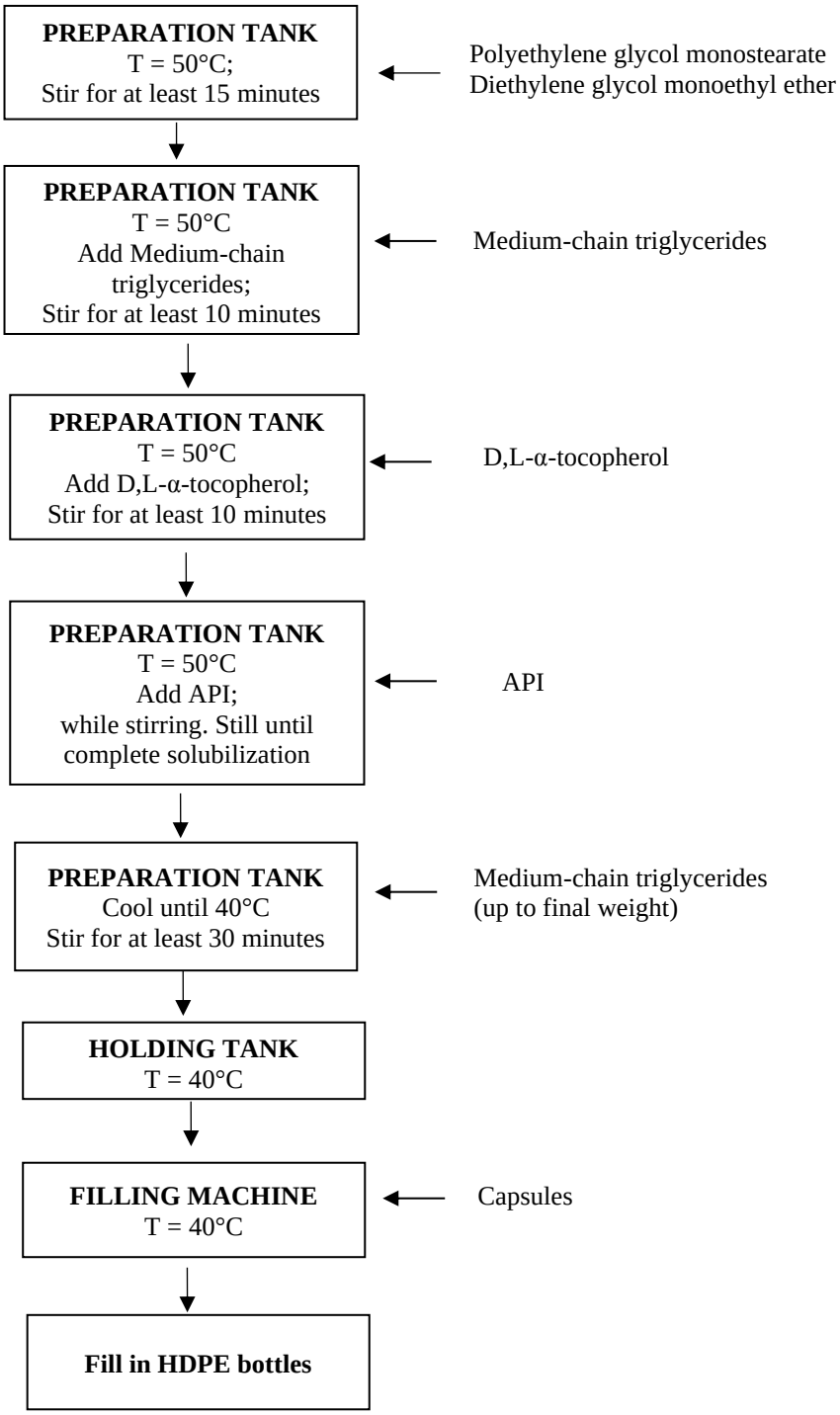
2.3.2.14 Preparation of the new semisolid formulation 8% w/w

Table 2.21 Composition of the semisolid formulation.

Component(s)	Specification	Function	% w/w (g/100g)
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Internal monograph	Active ingredient	8.00
Polyethylene glycol monostearate (Gelucire 48/16)	USP, JPE	Surfactant	50.00
Diethylene glycol monoethyl ether (Transcutol HP)	EP, USP	Co-Solvent	20.00
D,L-α-tocopherol	EP, USP	Antioxidant/solubilizer	1.50
Medium-chain triglycerides (Labrafac Lipophile WL1349)	EP, USP, JPE	Oil	20.50

2.3.2.14.1 Manufacturing process

The manufacturing process is summarized in the following flow-chart:



2.3.2.14.2 Brief description of the manufacturing process

PHASE I (Polyethylene glycol monostearate fusion and Diethylene glycol monoethyl ether mixture)

In the Preparation Tank pour the weighed amount of Polyethylene glycol monostearate. Heat up to 50°C and then add Diethylene glycol monoethyl ether. Stir for at least 15 minutes maintaining the temperature around 50°C, until the complete fusion of Polyethylene glycol monostearate is obtained with its homogeneous mixture with Diethylene glycol monoethyl ether.

In Process Controls:

- Visual inspection: Yellow Clear Solution

PHASE II (Addition of Medium-chain triglycerides)

Add slowly under high speed stirring Medium-chain triglycerides. Stir for at least 10 minutes maintaining the temperature around 50°C.

In Process Controls:

- Visual inspection: Yellow Clear Solution

PHASE III (Addition of D, L-α-tocopherol)

Add slowly under high speed stirring D, L-α-tocopherol. Stir for at least 10 minutes maintaining the temperature around 50°C.

In Process Controls:

- Visual inspection: Yellow Clear Solution

PHASE IV (API solubilization)

Add slowly under high speed stirring the API maintaining the temperature around 50°C. stir until complete solubilization of API. Then cool the solution until the solution reaches 40°C.

In Process Controls:

- Visual inspection: Yellow Clear Solution

FINAL WEIGHT

Add Medium-chain triglycerides up to final weight maintaining the temperature around 40°C. Fill-up the capsules maintaining the formulation under stirring at 40°C.

2.3.2.14.3 Capsules

Based on the target dose of API in one capsule (100 mg), the total fill weight expected is 1.25 g/cps.

[applied formula: 100mg:80mg/g= 1.25 g/cps]

2.3.2.15 Ternary diagram phase

In order to study the ratio between surfactants and oil in the semisolid formulation, a ternary diagram phase was created to evaluate the auto-emulsification properties.

The diagram was obtained using the titration method with water in order to simulate the physiological dilution with physiological fluids.

Table 2.22 Summary of the trials.

Trial	Gelucire 48/16 : Transcutol 5:2 (g)	Labrafac lipo WL1349 (g)	D,L alpha tocopherol (g)	CB-03-10 (g)
A	10	0	0.166	0.884
B	9	1	0.166	0.884
C	8	2	0.166	0.884
D	7	3	0.166	0.884
E	6	4	0.166	0.884
F	5	5	0.166	0.884
G	4	6	0.166	0.884
H	3	7	0.166	0.884
I	2	8	0.166	0.884
J	1	9	0.166	0.884
K	0	10	0.166	0.884

2.3.2.16 Preparation of the alternative semisolid formulation 8% w/w

Table 2.23 Composition of the alternative semisolid formulation.

Component(s)	Specification	Function	% w/w (g/100g)
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Internal monograph	Active ingredient	8.00
Polyethylene glycol monostearate (Gelucire 48/16)	USP, JPE	Surfactant	48.50
Diethylene glycol monoethyl ether (Transcutol HP)	EP, USP	Co-Solvent	15.00
D,L-α-tocopherol	EP, USP	Antioxidant/solubilizer	1.50
Medium-chain triglycerides (Labrafac Lipophile WL1349)	EP, USP, JPE	Oil	27.00

The manufacturing process of this alternative semisolid formulation is the same of the previous semisolid formulation (see points 2.3.2.14.1 and 2.3.2.14.2).

2.3.2.17 *In-vitro* dissolution test method

The dissolution method used is according to USP <711>. The temperature was fixed at 37°C in order to simulate physiological temperature. The test was performed using paddle apparatus at 100 rpm.
The selected dissolution medium was 900 mL HCl 0.1N with the addition of 2% Brij C20.
In order to avoid the floating of the capsules a sinker system was used.

2.3.2.18 Analytical procedures for lead semisolid formulation

2.3.2.18.1 CB-03-10 Identification (HPLC)

Perform the test during the assay, using the same sample preparation. The retention time of the major peak in the sample solution chromatogram corresponds to that of the standard solution.

2.3.2.18.2 CB-03-10 (HPLC)

Analytical Conditions

Reagents

Purified Water, HPLC grade;

Acetonitrile, HPLC grade;

Chromatographic conditions

Instrumentation:	High Performance Liquid Chromatograph equipped with autosampler, pump, UV detector, and PC workstation for data acquisition and analysis
Column:	Zorbax Extend C18 1.8 µm, 100 x 2.1 mm or equivalent
Detector wavelength:	240 nm
Flow rate:	0.6 mL/minute
Injection volume:	2 µl
Column temperature:	35° C
Mobile phase:	Phase A: water Phase B: acetonitrile Mixed according to gradient program reported in Table 2.24:

Table 2.24 Gradient program.

Time	Phase A	Phase B
0	60	40
0.5	60	40
7	30	70
8	30	70
8.1	60	40
10	60	40

Standard Solution

Prepare a solution containing CB-03-10 reference standard 1.0 mg/mL in acetonitrile.

Filter through a 0.2 µm membrane filter before injection.

Sample Solution

Prepare a sample solution containing CB-03-10 1.0 mg/mL in acetonitrile on the basis of the theoretical concentration of the formulation.
Stir on a magnetic stirrer and filter through a 0.2 μm membrane filter prior to injection.

System Suitability Test

Analyse the standard solution and record the chromatograms.
Verify that the System Suitability Test criteria are fulfilled:

1) The USP tailing of Cortexolone 17α-valerate-21-propionate, calculated by the following formula, is not more than 1.5.

$$T = \frac{W_{0.05}}{2f}$$

where:

$W_{0.05}$ = width of the peak at 5% height;

f = distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

2) The Relative Standard Deviation (RSD) of CB-03-10 peak areas, calculated on 5 replicate injections of the Standard Solution, is not more than 2.0%.

Calculations

Inject the sample solution. Record the chromatograms and calculate the peak areas.
Calculate the CB-03-10 amount in the sample, respect to the mean response factor determined on 5 injections of standard.

2.3.2.18.3 Related Substances

Analytical Conditions

Reagents

Purified Water, HPLC grade;
Acetonitrile, HPLC grade

Chromatographic conditions

Instrumentation:	High Performance Liquid Chromatograph equipped with autosampler, pump, UV detector, and PC workstation for data acquisition and analysis
Column:	Zorbax Extend C18 1.8 µm, 100 x 2.1 mm or equivalent
Detector wavelength:	240 nm
Flow rate:	0.6 mL/minute
Injection volume:	2 µl
Column temperature:	35° C
Mobile phase:	Phase A: water Phase B: acetonitrile Mixed according to gradient program reported in Table 2.25:

Table 2.25 Gradient program.

Time	Phase A	Phase B
0	65	35
1	65	35
18	30	70
21	30	70
22	20	80
24	20	80
26	65	35
28	65	35

Resolution Solution

Prepare a solution containing Cortexolone, Cortexolone 17α-propionate, Cortexolone 21-acetate, Cortexolone 21-propionate, Cortexolone17α-valerate, Cortexolone 21-valerate and Cortexolone 17α-valerate-21-propionate (CB-03-10) in acetonitrile at a concentration of 0.02 mg/mL each.

Filter the solution by a 0.2 µm membrane filter before injection.

Standard Solution and Control Standard Solution

Prepare a solution containing CB-03-10 reference standard 0.01 mg/mL in acetonitrile. Filter through a 0.2 µm membrane filter before injection.

Sample Preparation

Prepare a sample solution containing CB-03-10 1.0 mg/mL in acetonitrile on the basis of the theoretical concentration of the formulation.
Stir on a magnetic stirrer and filter through a 0.2 μm membrane filter prior to injection.

System Suitability Test

Chromatograph the resolution solution and the standard solution (6 replicate injections) and record the chromatograms.
Verify that the System Suitability Test criteria are fulfilled:

1) The resolution (R) between Cortexolone 21-acetate and Cortexolone 17α-propionate peaks in the chromatogram of the Resolution Solution is not less than (NLT) 2.0. Peak resolution is calculated by the following formula:

$$R = \frac{1.18(t_{R2} - t_{R1})}{W_{h1} - W_{h2}}$$

where:

t_R = retention time measured from time of injection to time of elution of peak maximum;

w_h = peak widths at half-height.

Identify the known impurities using the impurities retention time in chromatogram of the Resolution Solution.

2) The symmetry factor (As) of Cortexolone 17α-valerate-21-propionate peak in the chromatogram of Standard Solution is not more than (NMT) 1.5 and is calculated by the following equation:

$$A_s = \frac{W_{0.05}}{2f}$$

where:

$W_{0.05}$ = width of peak at one-twentieth of the peak height;

f = distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

3) System precision: the RSD of Cortexolone 17α-valerate-21-propionate peak areas calculated on 6 replicate injections of the Standard Solution is not more than 5%.

Calculations

Inject the sample solution. Record the chromatograms and calculate the peak areas.
Calculate the amount of each impurity in the sample, respect to the mean response factor of Cortexolone 17α-valerate-21-propionate determined on 6 injections of standard.
Express the result as %w/w of the theoretical amount of CB-03-10
LOD = 0.01 %
LOQ = 0.03 %

2.3.2.18.4 Dissolution

Perform according to USP <711>.

2.3.2.18.5 Uniformity of Dosage Unit

Perform according to USP <905> by content uniformity.

2.3.2.18.6 Microbiological Analysis

Perform the tests to detect total aerobic microbial count, total combined yeasts and molds counts, and to verify the absence of Escherichia Coli according to the current European Pharmacopoeia and USP.

2.4 Results and discussion

2.4.1 API Characterization

2.4.1.1 API solubility in different media

Table 2.26 CB-03-10 solubility results in different aqueous media.

Medium	**CB-03-10 concentration (mg/mL) Average ± sd (n=3)**
HCl 0.1N	0.00051±0.00011
USP buffer pH=4.5	0.00046±0.00009
USP buffer pH=6.8	0.00039±0.00004
HCl 0.1N+2% surfactant	0.23206±0.00212
USP buffer pH=4.5+2% surfactant	0.22412±0.00321
USP buffer pH=6.8+2% surfactant	0.22316±0.00147
HCl 0.1N+3% surfactant	0.29511±0.00133
USP buffer pH=4.5+3% surfactant	0.32106±0.00114
USP buffer pH=6.8+3% surfactant	0.27541±0.00136

The obtained results show that CB-03-10 is practically insoluble in aqueous media and that the solubility is poorly influenced by the pH.
The results suggest that the addition of surfactant improves drug solubility, which enhances as function of the concentration of the surfactant.

2.4.1.2 Morphological characteristics and dissolution behavior of the API

2.4.1.2.1 API particle size distribution

The results obtained analyzing the sieved API by Morphologi G3S are reported in Figures 2.3 and 2.4.

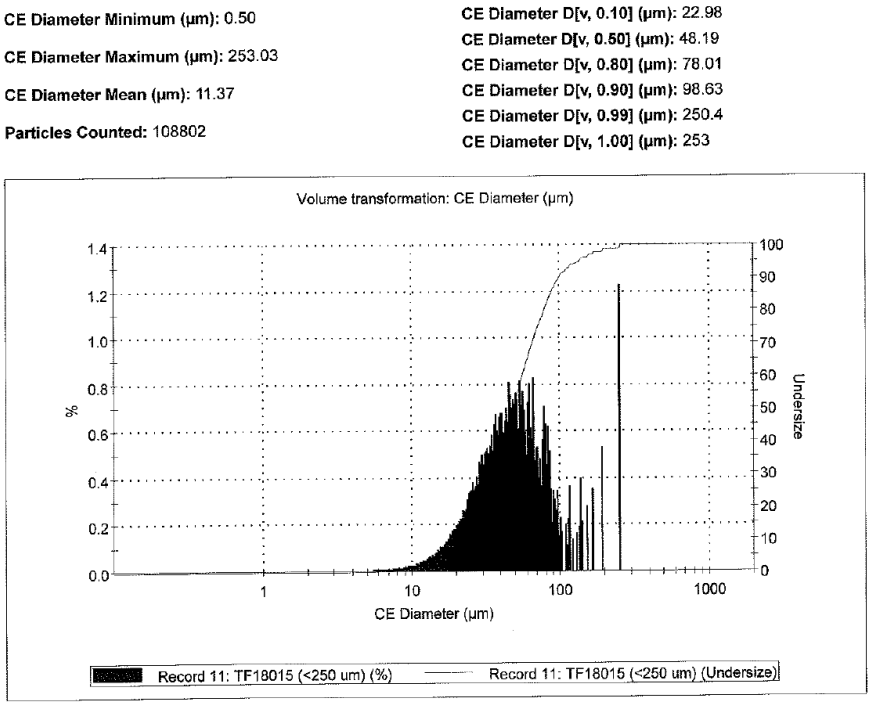


Figure 2.3 API particle size distribution obtained for the portion under 250 µm (Morphologi G3S).

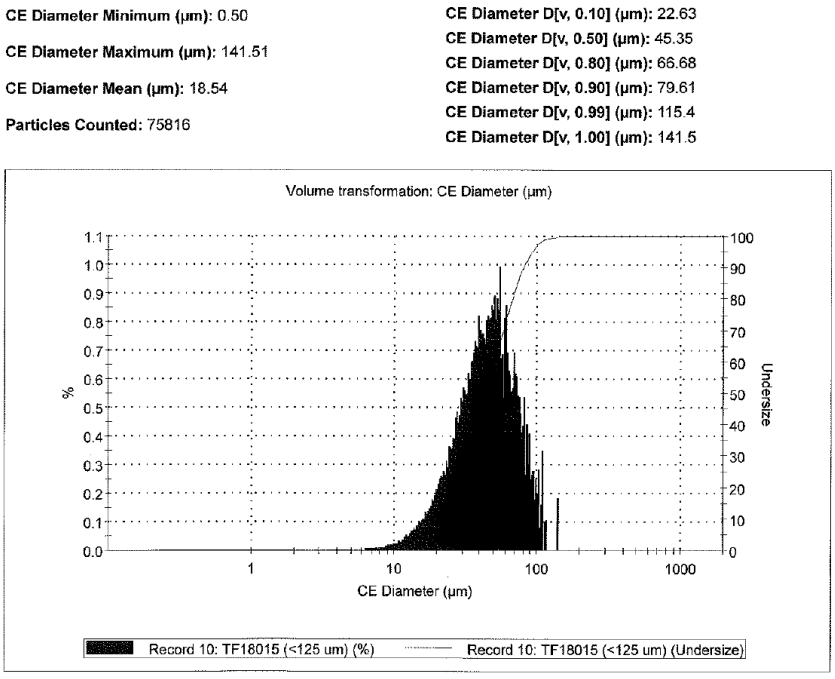


Figure 2.4 API particle size distribution obtained for the portion under 125 µm (Morphologi G3S).

The results, obtained analyzing the portions under 250 µm and under 125 µm, show that the portion under 125 µm has a narrower distribution range (0.50-141.51 µm) compared with the portion under 250 µm (0.50-253.03 µm). The value of D90 is 98.63 µm and 79.61 µm, for the portion under 250 µm and 125 µm respectively. These results suggest that a particle

size under 125 µm is recommended in order to obtain a powder characterized by better particle size distribution.

2.4.1.2.2 API Dissolution profile

The dissolution results in function of the particle size distribution are reported in the graph below (Figure 2.5).

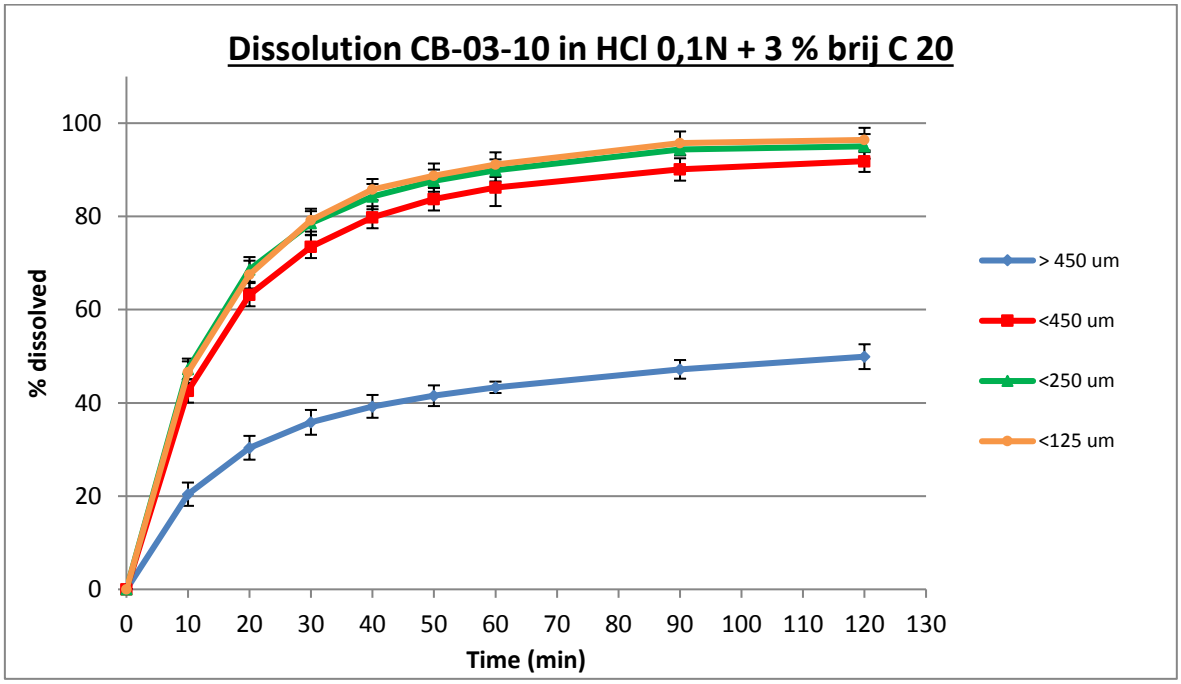


Figure 2.5 Dissolution profile vs particle size distribution (mean values ± sd; n=6).

As expected, the results of the dissolution test show that the greater difference is obtained reducing the particle size of the API under 450 µm. In fact, there is a substantial improvement in dissolution profile between the API with particle size under 450 µm compared to the particle dissolution profile of the portion with particle size above 450 µm: the percentage dissolved after 120 minutes is almost doubled and there is a faster dissolution rate for the samples with particle size under 450 µm.

Meanwhile slightly differences are observed between the samples with particle size below 450 µm, 250 µm and 125 µm, for which the dissolution curves result almost superimposable.

Moreover, for all the tested samples, the initial phase of the plateau is around 60 minutes.

2.4.2 CB-03-10 10% w/w oral solution development

2.4.2.1 Solubility results

The results obtained during the first screening a preliminary solubility are reported in the Table 2.27.

Table 2.27 Solubility results.

Excipient	**Obtained value (mg/g) Average ± sd (n=3)**
Soybean oil	100.03±0.19
Medium-chain triglycerides (Labrafac lipophile WL1349)	150.31±0.24
Caprylocaproyl polyoxyl-8 glycerides (Labrasol)	152.20±0.11
Diethylene glycol monoethyl ether (Transcutol)	200.31±0.50
Ethyl oleate	90.83±0.18
Polyethylene glycol 300 (PEG 300)	53.51±0.01
Polyethylene glycol 400 (PEG 400)	60.10±0.10
Polyoxyl castor oil (Kolliphor RH40)	83.19±0.19
Vitamin E Polyethylene Glycol Succinate (Kolliphor TPGS)	129.74±0.04
Propylene glycol	44.49±0.06
Oleoyl polyoxyl-6 glycerides (Labrafil M1944CS)	152.85±0.20
Lauroyl polyoxyl-32 glycerides (Gelucire 44/14)	196.94±0.52
Stearoyl polyoxyl-32 glycerides (Gelucire 50/13)	182.56±0.15
Sesame oil	110.01±0.06

2.4.2.2 Solubility results

- Single excipients

Table 2.28 Solubility in single excipients.

Excipient	Obtained value (% w/w)
Oleoyl polyoxyl-6 glycerides (Labrafil M1944CS)	5.1±0.2
Ethanol 96%	14.3±0.4
Polyethylene glycol 400 (PEG 400)	2.1±0.2
Medium-chain triglycerides (Labrafac lipophile WL1349)	5.9±0.2
Caprylocaproyl polyoxyl-8 glycerides (Labrasol)	6.5±0.1

- Combination of excipients

Table 2.29 Solubility in combination of excipients.

Combination n.	Excipient	Obtained value (% w/w)
1	Labrafil M1944CS (72.7%) + Labrafac lipophile WL1349 (28.3%)	5.7±0.1
2	Ethanol 96% (62.5%) + PEG 400 (37.5%)	10.2±0.2
3	Labrafil M1944CS (65.9%)+ Labrafac lipophile WL1349 (24.7%)+ Ethanol 96% (5.9%)+ PEG 400 (3.5%)	9.6±0.1
4	Labrafil M1944CS (62.2%) + Labrafac lipophile WL1349 (23.3%)+ Ethanol 96% (5.6%)+ PEG 400 (3.3%)+ D,L-α-tocopherol (5.6%)	10.1±0.1
5	Labrasol + Ethanol	8.6±0.1
6	Labrasol + Ethanol + BHA	8.6±0.1

The results obtained shown that ethanol is the best solvent. Meanwhile the two combinations that allow the solubilization of the highest amount of API are combination n. 2 and 4.
The formulation development at this stage was focused on the selection of a formulation for toxicological and pre-clinical studies.

Since the aim of the MTD study and other toxicological/pre-clinical studies is to expose the animals to the maximum amount of API, combination n. 4 has been selected. Combination n. 4 is the only combination, of the studied ones, able to solubilize the maximum amount of API. Since the concentration of the API into the formulation correspond also to its solubility, a restricted storage condition 20-25°C should be respected to avoid any API precipitation that can occur at a temperature below 20°C.

2.4.2.3 Permeability study

Monolayer integrity

Caco-2 monolayers were used for experiments at day 27 post seeding.

TEER value was recorded before cell treatment. If the resistance was < 200 Ω cm^2, monolayer was not used for permeability assessment.

TEER value was also recorded after the last time point of cell treatment, after substitution of the donor volume with HBSS. TEER value was > 200 200 Ω cm^2 in all cases, thus the compound or formulations assessed did not disrupt the Caco-2 monolayer.

Controls

The mean concentration values, mass balance and P_{app} values of the three controls (Atenolol, Famotidine and Metoprolol) are summarized in Table 2.30.

Table 2.30 Results for Atenolol, Famotidine and Metoprolol (BLLOQ: below 31.25 ng/mL).

	DONOR (nM)			**RECEIVER (nM)**	
	T_0	**T_{30}**	**T_{120}**	**T_{30}**	**T_{120}**
Atenolol	92100	96340	68420	BLLOQ	61
Famotidine	100320	99220	68820	BLLOQ	83
Metoprolol	81240	80920	39860	1644	7434

MASS BALANCE (%)	**T_0**	**T_{30}**	**T_{120}**
Atenolol	100%	105%	95%
Famotidine	100%	99%	93%
Metoprolol	100%	108%	118%

P_{app} (*10^{-6} cm/sec)	T_{30}	T_{120}
Atenolol	BLLOQ	0.2
Famotidine	BLLOQ	0.3
Metoprolol	29.0	32.9

Individual measured concentrations, mean concentration and CV% of 5 replicates at start (T_0, only donor chamber), after 30 minutes (T_{30}, donor and receiver) and 2 hours (T_{120}, donor and receiver) for controls are sown in Table 2.31.

Table 2.31 I ndividual concentrations of controls Atenolol, Famotidine and Metoprolol, measured in quintuplicate at T0, T30 and T120 (D, donor compartment; R, receiver compartment; BLLOQ: below 31.25ng/mL).

Sample Name	Measured Concentration (nM)	Mean concentration (nM)	CV%
Atenolol T0 D_01	98300	92100	8
Atenolol T0 D_02	98300		
Atenolol T0 D_03	89300		
Atenolol T0 D_04	80200		
Atenolol T0 D_05	94400		
Atenolol T30 D_01	103000	96340	6
Atenolol T30 D_02	92500		
Atenolol T30 D_03	101000		
Atenolol T30 D_04	94600		
Atenolol T30 D_05	90600		
Atenolol T30 R_01	8.75	BLLOQ	-
Atenolol T30 R_02	11.2		
Atenolol T30 R_03	8.64		
Atenolol T30 R_04	9.73		
Atenolol T30 R_05	25.8		
Atenolol T120 D_01	65400	68420	15
Atenolol T120 D_02	78200		
Atenolol T120 D_03	60000		
Atenolol T120 D_04	80300		
Atenolol T120 D_05	58200		
Atenolol T120 R_01	43.1	61.2	19
Atenolol T120 R_02	58.3		
Atenolol T120 R_03	71.7		

Sample Name	Measured Concentration (nM)	Mean concentration (nM)	CV%
Atenolol T120 R_04	64.3		
Atenolol T120 R_05	68.8		
Famotidine T0 D_01	93000	100320	7
Famotidine T0 D_02	94600		
Famotidine T0 D_03	106000		
Famotidine T0 D_04	108000		
Famotidine T0 D_05	100000		
Famotidine T30 D_01	94200	99220	11
Famotidine T30 D_02	96800		
Famotidine T30 D_03	85100		
Famotidine T30 D_04	111000		
Famotidine T30 D_05	109000		
Famotidine T30 R_01	13.65	BLLOQ	-
Famotidine T30 R_02	14.4		
Famotidine T30 R_03	5.7		
Famotidine T30 R_04	6.9		
Famotidine T30 R_05	19.4		
Famotidine T120 D_01	67000	68820	3
Famotidine T120 D_02	68400		
Famotidine T120 D_03	72000		
Famotidine T120 D_04	67300		
Famotidine T120 D_05	69400		
Famotidine T120 R_01	151	96.8	33
Famotidine T120 R_02	92.3		
Famotidine T120 R_03	72.2		
Famotidine T120 R_04	77.4		
Famotidine T120 R_05	90.9		
Metoprolol T0 D_01	88700	81240	9
Metoprolol T0 D_02	83200		
Metoprolol T0 D_03	72500		
Metoprolol T0 D_04	75100		
Metoprolol T0 D_05	86700		
Metoprolol T30 D_01	73500	80920	7
Metoprolol T30 D_02	78900		
Metoprolol T30 D_03	85300		
Metoprolol T30 D_04	78600		

Sample Name	Measured Concentration (nM)	Mean concentration (nM)	CV%
Metoprolol T30 D_05	88300		
Metoprolol T30 DR_01	2320	1644	26
Metoprolol T30 DR_02	1630		
Metoprolol T30 DR_03	1690		
Metoprolol T30 DR_04	1220		
Metoprolol T30 DR_05	1360		
Metoprolol T120 D_01	44300	39860	9
Metoprolol T120 D_02	34200		
Metoprolol T120 D_03	41700		
Metoprolol T120 D_04	39500		
Metoprolol T120 D_05	39600		
Metoprolol T120 DR_01	7410	7434	5
Metoprolol T120 DR_02	7310		
Metoprolol T120 DR_03	7820		
Metoprolol T120 DR_04	6900		
Metoprolol T120 DR_05	7730		

For high and low permeability controls (Table 2.30), expected results were obtained for mass balance (93-118%) and permeability (Atenolol, $0.2*10^{-6}$ cm/sec, Metoprolol, $32.9*10^{-6}$ cm/sec at T_{120}).

Medium permeability control Famotidine, had P_{app} value only slightly above Atenolol (Famotidine, $0.3*10^{-6}$ cm/sec at T_{120}). This could be caused by the fact that Famotidine is a P-gp substrate and therefore results for apical to basal permeability could be affected by P-gp mediated efflux. The values obtained for apical to basal permeability are similar to other published values.

API and formulations

The mean concentration values are summarized in Table 2.32, mass balance in Table 2.33 and Papp in Table 2.34 and Table 2.35.

Individual measured concentrations, mean concentrations and CV% of five replicates at start (T_0, only donor chamber), after 30 minutes (T_{30}, donor and receiver) and 2 hours (T_{120}, donor and receiver) for API and the six formulations are shown in Table 2.36.

Table 2.32 Mean concentration at T0, T30 and T120 for CB-03-10 as is and in the 6 formulations. Mean concentrations of the metabolites are also reported.

	CB-03-10 (parent)				
	DONOR (nM)			RECEIVER (nM)	
	T_0	T_{30}	T_{120}	T_{30}	T_{120}
CB-03-10	10282	690	493	BLLOQ	BLLOQ
F1	36660	4398	1784	BLLOQ	36
F2	36800	26580	7424	BLLOQ	32
F3	13724	2982	675	BLLOQ	BLLOQ
F4	3660	7352	1476	BLLOQ	BLLOQ
F5	33660	17060	8532	105	246
F6	40140	11170	1784	148	254
	CB-03-05 (M1)				
	DONOR (nM)			RECEIVER (nM)	
	T_0	T_{30}	T_{120}	T_{30}	T_{120}
CB-03-10	BLLOQ	473	1280	35	367
F1	BLLOQ	1233	5750	88	1002
F2	BLLOQ	4490	11248	159	1108
F3	BLLOQ	1216	2726	68	520
F4	BLLOQ	3474	5802	75	597
F5	BLLOQ	4262	5990	65	439
F6	BLLOQ	4172	7170	121	1134
	Unknown (M2)				
	DONOR (nM)			RECEIVER (nM)	
	T_0	T_{30}	T_{120}	T_{30}	T_{120}
CB-03-10	BLLOQ	BLLOQ	233	BLLOQ	120
F1	BLLOQ	77	696	58	359
F2	BLLOQ	56	548	55	265
F3	BLLOQ	58	486	50	255
F4	BLLOQ	54	335	33	171
F5	BLLOQ	73	350	38	170
F6	BLLOQ	85	516	53	270
	CB-03-10 + CB-03-05 + M2				
	DONOR (nM)			RECEIVER (nM)	
	T_0	T_{30}	T_{120}	T_{30}	T_{120}
CB-03-10	10282	1163	2006	35	487
F1	36660	5708	8230	146	1397
F2	36800	31126	19220	214	1405
F3	13724	4256	3887	118	775
F4	3660	10880	7613	108	768
F5	33660	21395	14872	207	854
F6	40140	15427	9470	322	1658

BLLOQ: below 3125 nM at T_0, below 31.25 ng/mL at T_{30} and T_{120}

Table 2.33 Mass balance for CB-03-10 and its 6 formulations

	Mass balance CB-03-10 + CB-03-05 + M2		
	T_0 (%)	T_{30} (% rispetto a T0)	T_{120} (% rispetto a T0)
CB-03-10	100	13	40
F1	100	17	39
F2	100	87	68
F3	100	35	52
F4	100	31	30
F5	100	66	55
F6	100	42	41

Table 2.34 Papp values of CB-03-10 as is and in the 6 formulations at T30 and T120. Papp values reflect the permeability of the sum of all the species detected.

	P_{app} (*10^{-6} cm/sec), sum of CB-03-10 and metabolites	
	T_{30}	T_{120}
CB-03-10	4.8	17.0
F1	5.7	13.6
F2	8.3	13.7
F3	12.3	20.2
F4	4.2	7.5
F5	8.8	9.1
F6	11.5	14.8

Table 2.35 Papp values of CB-03-10 as is and in the 6 formulations (without metabolites) at T30 and T120.

	P_{app} (*10^{-6} cm/sec), sum of CB-03-10 without metabolites	
	T_{30}	T_{120}
CB-03-10	-	-
F1	-	0.3
F2	-	0.3
F3	-	-
F4	-	-
F5	4.5	2.5
F6	5.3	2.1

Table 2.36 Individual concentrations of CB-03-10 and its six formulations, measured in quintuplicate at T0, T30 and T120 (D, donor compartment; R, receiver compartment; F, formulation; BLLOQ: below 3125 nM at T0; below 31.25 ng/mL at T30, T120; * outliers were removed (Dixon's Q-test).

Sample Name	Conc. CB-03-10 (nM)	Mean Conc (nM)	CV%	Conc. CB-03-05 peak 1 (nM)	Mean Conc (nM)	CV%	Conc. cortexolone-21valerate peak 2 (nM)	Mean Conc (nM)	CV %
CB-03-10 T0 D_01	2040	10282	103	358	BLLOQ	-	209	BLLOQ	-
CB-03-10 T0 D_02	15000			553			194		
CB-03-10 T0 D_03	1840			500			218		
CB-03-10 T0 D_04	26600			391			200		
CB-03-10 T0 D_05	5930			645			212		
F1 T0 S_01	27600	36660	18	970	BLLOQ	-	446	BLLOQ	-
F1 T0 S_02	39700			841			432		
F1 T0 S_03	40500			703			437		
F1 T0 S_04	31700			620			433		
F1 T0 S_05	43800			549			419		
F2 T0 S_01	40100	36800	8	1040	BLLOQ	-	408	BLLOQ	-
F2 T0 S_02	34600			928			406		
F2 T0 S_03	32800			1180			401		
F2 T0 S_04	37500			1160			451		
F2 T0 S_05	39000			853			450		
F3 T0 S_01	9640	13724	36	539	BLLOQ	-	411	BLLOQ	-
F3 T0 S_02	12400			559			444		
F3 T0 S_03	9080			623			425		
F3 T0 S_04	20900			699			406		
F3 T0 S_05	16600			455			390		
F4 T0 D_01	91900 *	22775	47	*91900	BLLOQ	-	225	BLLOQ	-

Sample Name	Conc. CB-03-10 (nM)	Mean Conc (nM)	CV%	Conc. CB-03-05 peak 1 (nM)	Mean Conc (nM)	CV%	Conc. cortexolone-21valerate peak 2 (nM)	Mean Conc (nM)	CV %
F4 T0 D_02	29400			1250			196		
F4 T0 D_03	13800			828			189		
F4 T0 D_04	34300			890			207		
F4 T0 D_05	13600			874			218		
F5 T0 D_01	31200	33660	16	819	BLLOQ	-	220	BLLOQ	-
F5 T0 D_02	33400			720			211		
F5 T0 D_03	38300			782			187		
F5 T0 D_04	26300			1260			201		
F5 T0 D_05	39100			1000			212		
F6 T0 D_01	37400	40140	37	1480	BLLOQ	-	197	BLLOQ	-
F6 T0 D_02	61400			968			218		
F6 T0 D_03	22400			940			231		
F6 T0 D_04	32600			1280			219		
F6 T0 D_05	46900			818			201		
CB-03-10 T30 D_01	772	690	12	427	473	17	25.4	BLLOQ	-
CB-03-10 T30 D_02	658			442			30.1		
CB-03-10 T30 D_03	732			482			21.4		
CB-03-10 T30 D_04	725			408			19.7		
CB-03-10 T30 D_05	564			606			33.1		
F1 T30 D_ 01	4630	4398	15	1750	1233	26	63.5	77	18
F1 T30 D_ 02	3990			1280			88.7		
F1 T30 D_ 03	3710			1110			66.3		

Sample Name	Conc. CB-03-10 (nM)	Mean Conc (nM)	CV%	Conc. CB-03-05 peak 1 (nM)	Mean Conc (nM)	CV%	Conc. cortexolone-21valerate peak 2 (nM)	Mean Conc (nM)	CV %
F1 T30 D_04	5400			1100			95		
F1 T30 D_ 05	4260			924			71.4		
F2 T30 D_ 01	16400	26580	32	4770	4490	9	63	61	23
F2 T30 D_02	18800			4530			51.9		
F2 T30 D_ 03	30900			4300			48.5		
F2 T30 D_ 04	36500			4950			58.7		
F2 T30 D_ 05	30300			3900			84		
F3 T30 D_ 01	2790	2982	16	1270	1216	5	64.5	58	10
F3 T30 D_ 02	3700			1280			57.6		
F3 T30 D_ 03	2530			1210			57.4		
F3 T30 D_ 04	3210			1200			49.5		
F3 T30 D_ 05	2680			1120			63.2		
F4 T30 D_01	5960	7352	14	3230	3474	5	58.6	54	22
F4 T30 D_02	8760			3420			37.5		
F4 T30 D_03	7550			3750			57.9		
F4 T30 D_04	6980			3480			68.3		
F4 T30 D_05	7510			3490			47.3		
F5 T30 D_01	18800	17060	11	5350	4262	18	79.7	73	10
F5 T30 D_02	19400			4190			81.8		
F5 T30 D_03	16400			3540			66.5		
F5 T30 D_04	15900			4640			70.9		
F5 T30 D_05	14800			3590			66.5		

Sample Name	Conc. CB-03-10 (nM)	Mean Conc (nM)	CV%	Conc. CB-03-05 peak 1 (nM)	Mean Conc (nM)	CV%	Conc. cortexolone-21valerate peak 2 (nM)	Mean Conc (nM)	CV %
F6 T30 D_01	8150	11170	21	5020	4172	20	93.8	85	34
F6 T30 D_02	13500			4270			125		
F6 T30 D_03	10100			3010			47.6		
F6 T30 D_04	13500			4840			73.1		
F6 T30 D_05	10600			3720			83.7		
CB-03-10 T30 R_01	< 0	BLLOQ	-	34.4	35	11	21.5	BLLOQ	-
CB-03-10 T30 R_02	0.928			38.2			20.8		
CB-03-10 T30 R_03	1.54			28.8			18.1		
CB-03-10 T30 R_04	< 0			34.1			16.7		
CB-03-10 T30 R_05	< 0			37.8			19.4		
F1 T30 R_01	33.6	BLLOQ (< 31.25 nM)	-	103	88	14	57.6	58	4
F1 T30 R_02	28.4			91.6			60.5		
F1 T30 R_03	266 *			85.2			53.6		
F1 T30 R_04	20.7			69.8			57.8		
F1 T30 R_05	18.1			90.3			58.9		
F2 T30 R_01	22.3	BLLOQ (< 31.25 nM)	-	188	159	15	40.5	55	16
F2 T30 R_02	19.2			161			61.5		
F2 T30 R_03	18.8			158			56.1		
F2 T30 R_04	16.9			121			55.6		
F2 T30 R_05	15.9			166			62.8		
F3 T30 R_01	24.6	BLLOQ (< 31.25 nM)	-	77.9	68	12	53.5	50	8
F3 T30 R_02	95.7			71.2			52.7		

Sample Name	Conc. CB-03-10 (nM)	Mean Conc (nM)	CV%	Conc. CB-03-05 peak 1 (nM)	Mean Conc (nM)	CV%	Conc. cortexolone-21valerate peak 2 (nM)	Mean Conc (nM)	CV %
F3 T30 R_03	22.7			56.3			43.6		
F3 T30 R_04	20.2			67.1			48.1		
F3 T30 R_05	21.7			65.5			52		
F4 T30 R_01	20.3	BLLOQ (< 31.25 nM)	-	86.8	75	10	36.3	33	5
F4 T30 R_02	16.2			75.7			32		
F4 T30 R_03	16.6			74.8			33.6		
F4 T30 R_04	14.9			66.4			31.7		
F4 T30 R_05	15.4			70.3			33.4		
F5 T30 R_01	87.2	105	40	77.4	65	19	42.2	38	8
F5 T30 R_02	85.3			70.8			38.6		
F5 T30 R_03	55.1			71			40.1		
F5 T30 R_04	137			50.6			34		
F5 T30 R_05	159			52.8			36.2		
F6 T30 R_01	76	148	61	120	121	6	52.2	53	9
F6 T30 R_02	296			111			50.7		
F6 T30 R_03	87.4			120			53.4		
F6 T30 R_04	109			123			48.3		
F6 T30 R_05	173			130			60.5		
CB-03-10 T120 D_01	*3650	493	7	1620	1280	17	260	233	12
CB-03-10 T120 D_02	491			1160			262		
CB-03-10 T120 D_03	459			1170			218		
CB-03-10 T120 D_04	538			1090			195		

Sample Name	Conc. CB-03-10 (nM)	Mean Conc (nM)	CV%	Conc. CB-03-05 peak 1 (nM)	Mean Conc (nM)	CV%	Conc. cortexolone-21valerate peak 2 (nM)	Mean Conc (nM)	CV %
CB-03-10 T120 D_05	484			1360			229		
F1 T120 D_01	1160	1784	29	6160	5750	10	793	696	10
F1 T120 D_02	1790			4970			683		
F1 T120 D_03	1370			6220			701		
F1 T120 D_04	2220			6010			704		
F1 T120 D_05	2380			5390			599		
F2 T120 D_01	6580	7424	17	12200	11248	8	565	548	8
F2 T120 D_02	6690			11200			486		
F2 T120 D_03	8060			11500			521		
F2 T120 D_04	6460			11600			604		
F2 T120 D_05	9330			9740			565		
F3 T120 D_01	837	675	14	2890	2726	8	321	486	22
F3 T120 D_02	628			2540			554		
F3 T120 D_03	672			2910			504		
F3 T120 D_04	595			2420			458		
F3 T120 D_05	645			2870			591		
F4 T120 D_01	1480	1476	7	5550	5802	3	318	335	16
F4 T120 D_02	1470			5770			284		
F4 T120 D_03	1520			6010			311		
F4 T120 D_04	1320			5890			423		
F4 T120 D_05	1590			5790			341		
F5 T120 D_01	9410	8532	9	7560	5990	19	410	350	11

Sample Name	Conc. CB-03-10 (nM)	Mean Conc (nM)	CV%	Conc. CB-03-05 peak 1 (nM)	Mean Conc (nM)	CV%	Conc. cortexolone-21valerate peak 2 (nM)	Mean Conc (nM)	CV %
F5 T120 D_02	8530			6260			363		
F5 T120 D_03	8410			4930			313		
F5 T120 D_04	7320			4900			330		
F5 T120 D_05	8990			6300			336		
F6 T120 D_01	1620	1784	24	6310	7170	9	458	516	16
F6 T120 D_02	1480			7880			627		
F6 T120 D_03	2530			7160			411		
F6 T120 D_04	1500			6760			522		
F6 T120 D_05	1790			7740			560		
CB-03-10 T120 R_01	34.1	BLLOQ	-	374	382	9	121	120	5
CB-03-10 T120 R_02	22.2			372			130		
CB-03-10 T120 R_03	9.89			352			121		
CB-03-10 T120 R_04	10.5			442			116		
CB-03-10 T120 R_05	5.56			371			113		
F1 T120 R_01	46.2	54	77	1050	1002	8	350	359	4
F1 T120 R_02	29.8			928			367		
F1 T120 R_03	127			954			345		
F1 T120 R_04	32			969			356		
F1 T120 R_05	34.6			1110			378		
F2 T120 R_01	38	32	14	1260	1108	8	267	265	2
F2 T120 R_02	29.7			1080			258		
F2 T120 R_03	31.6			1030			266		

Sample Name	Conc. CB-03-10 (nM)	Mean Conc (nM)	CV%	Conc. CB-03-05 peak 1 (nM)	Mean Conc (nM)	CV%	Conc. cortexolone-21valerate peak 2 (nM)	Mean Conc (nM)	CV %
F2 T120 R_04	35			1110			268		
F2 T120 R_05	26.8			1060			267		
F3 T120 R_01	23	BLLOQ	-	483	520	8	273	255	6
F3 T120 R_02	133			548			258		
F3 T120 R_03	23.2			482			234		
F3 T120 R_04	21.9			580			251		
F3 T120 R_05	21			506			258		
F4 T120 R_01	16.1	BLLOQ	-	649	597	6	174	171	2
F4 T120 R_02	16.3			545			165		
F4 T120 R_03	14.5			593			173		
F4 T120 R_04	17			600			172		
F4 T120 R_05	15.9			598			170		
F5 T120 R_01	216	246	24	488	439	11	182	170	4
F5 T120 R_02	319			477			170		
F5 T120 R_03	181			443			164		
F5 T120 R_04	214			378			164		
F5 T120 R_05	298			407			170		
F6 T120 R_01	148	254	56	1170	1134	3	254	270	6
F6 T120 R_02	493			1140			271		
F6 T120 R_03	159			1090			263		
F6 T120 R_04	197			1130			266		
F6 T120 R_05	272			1140			298		

Discussion

- CB-03-10 as such or in the formulations assessed, was converted mainly to metabolite CB-03-05. In general, while after 30 minutes, CB-03-10 was still the main component in the donor compartment, CB-03-05 was prevailing in the receiver compartment showing that mainly CB-03-05 permeates the Caco-2 monolayer.in general, after 120 minutes, concentrations of CB-03-05 were higher than CB-03-10 both in donor and receiver compartment. A second metabolite (M2) with the same molecular weight and yielding the same fragment in NMR analysis, was detected, both in donor and receiver compartment, at lower levels.
- Permeability of CB-03-10 summed to its metabolites CB-03-05 and M2 was high both as such and in all the formulations when compared with low and high permeability compounds assessed by Chen et al. [7].
- Among the formulations, the best permeability values were obtained for F3, F6, and equally, F1 and F2.
- However, considering the variability among the samples, especially at T0, and the short range of Papp estimated, the difference in term of permeability capacity of CB-03-10 either as such or in the formulations plus its metabolites, cannot be considered substantial.
- The low mass balance obtained in the majority of the experiments with cells, could be ascribed, in most cases, to aspecific binding of the compound to the materials. This means that Papp values measured could be underestimated. Further potential conversion into metabolites, not detectable in the analytical conditions applied during the experiments, and/or retention in the cellular compartment, cannot be excluded.
- The compound both as such and formulated is stable in the vehicle used, and the metabolites are generated only in the cellular compartment, where drug metabolising enzymes are present.
- The aspecific binding measured when the compound is formulated both as F3 and F5, was negligible at T120. Although the variability of CB-03-10 concentration observed for F3 was 36% at T0, the variability of the concentrations of CB-03-10 and its metabolites observed for formulations F3 and F5 was below 30% at T120, suggesting that, perhaps, the results obtained with F3 and F5 could be considered more reliable.

2.4.2.4 Selected 10% oral formulation

Based on the low variability of the concentrations of CB-03-10 and its metabolites observed during the permeability study, the liquid formulation containing 10% of CB-03-10 was selected. The qualitative-quantitative composition of the selected formulation is reported in Table 2.37.

Table 2.37 Quali-quantitative composition of the selected 10% oral formulation.

Component(s)	Specification	Function	% w/w (g/100g)
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Internal monograph	Active ingredient	10.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	EP, USP	Surfactant	56.00
D, L-α-tocopherol	EP, USP	Antioxidant/solubilizer	5.00
Ethanol 96%	EP, USP	Co-surfactant/solubilizer	5.00
Polyethylene glycol 400 (PEG 400)	EP, USP	Co-surfactant	3.00
Medium-chain triglycerides NF (Labrafac Lipophile WL1349)	EP, USP, JPE	Oil	21.00

2.4.2.5 Summary of the development batches

The summary of CB-03-10 10% w/w oral solution development batches is reported in Table 2.38.

Table 2.38 Summary of CB-03-10 10% w/w oral solution development batches.

Formulation Batch Number	Batch Size (Kg of bulk preparation)	Batch use
7085	0.15 kg	Bioavailability study in dogs 525/16
7100	6 kg	Caco-2 Permeability study Q17_023 MTD and 14-day DRF study in dogs 526/16 Efficacy Study Ephor 11-17
7271	4 kg	Preliminary Oral toxicity study in rats E0262
7297	12 kg	Toxicity Study in dogs A3251 Toxicity study in rats A3252

2.4.2.6 CB-03-10 10% oral solution batch analysis results

Table 2.39 Batch Analysis results.

Test	**Provisional Specifications**	**Batch n°**			
		7085	**7100**	**7171**	**7297**
Chemical Analysis					
Appearance	Light yellow clear solution	Light yellow clear solution	Light yellow clear solution	Light yellow clear solution	Light yellow clear solution
CB-03-10 (HPLC) Identification	Positive	Positive	Positive	Positive	Positive
CB-03-10 (HPLC) Assay	90.0 – 110.0% of labelled claim	10.07% w/w (100.7% of labelled claim)	10.12% w/w (101.2% of labelled claim)	9.91% w/w (99.1% of labelled claim)	10.01% w/w (100.1% of labelled claim)
Related Substances:					
Cortexolone	NMT 1%	<0.025%	<0.025%	<0.025%	<0.025%
Cortexolone-21-propionate	NMT 1%	0.21%	0.21%	0.39%	0.31%
Cortexolone-17α-valerate	NMT 3%	0.12%	0.12%	0.13%	0.56%
Cortexolone-21-valerate	NMT 3%	0.16%	0.14%	0.26%	0.16%
Any other related substances	NMT 0.5%	0.07%	0.08%	0.09%	0.17%
Total impurities	NMT 8%	0.60%	0.60%	1.02%	1.48%
Density at 25°C	g/mL for information only	n.a.	n.a.	0.9512 g/mL	0.9511 g/mL
Viscosity at 25°C	cP for information only	n.a.	n.a.	75.3 cP	74.1 cP
Microbiological Analysis					
Total Aerobic Microbial Count (TAMC)	NMT 1000 CFU/g	n.a.	<10 CFU/g	<10 CFU/g	<10 CFU/g
Total Yeasts and Molds Count (TYMC)	NMT 100 CFU/g	n.a.	<5 CFU/g	<5 CFU/g	<5 CFU/g
Escherichia Coli	Absent in 1 g	n.a.	Absent in 1 g	Absent in 1 g	Absent in 1 g

n.a.: data not available because the test was not in force at time of testing. NMT: not more than.

2.4.2.7 Stability studies

The stability studies were performed on the following batches of CB-03-10 10% w/w oral:

- 7100
- 7271
- 7297

The stability studies results obtained for CB-03-10 10% oral solution batch 7100 packaged in glass amber bottles are reported in the Tables 2.40, 2.41, 2.42.

Table 2.40 Stability studies results obtained for CB-03-10 10% oral solution batch 7100 stored at 25°C/60%RH.

Test	**Appearance**	**CB-03-10 identification**	**CB-03-10 Assay**		**Related substances**						**Microbiological analysis**		
					Cortexolone	**Cortexolone 21-propionate**	**Cortexolone 17α-valerate**	**Cortexolone 21-valerate**	**Any other related substance**	**Total related substances**	**TAMC**	**TYMC**	**E. Coli**
Provisional Specification	Light yellow clear solution	Positive	9.00-11.00% w/w	90.0-110.0% vs labeled	% for information only						≤1000 CFU/g	≤100 CFU/g	Absent in 1 g
					NMT 1%	NMT 1%	NMT 3%	NMT 3%	NMT 0.5%	NMT 8%			
Initial values	Complies	Complies	10.12	101.2	<0.01	0.21	0.12	0.14	0.08	0.60	<10	<5	Complies
25°C/60% RH													
03 Months	Complies	Complies	9.90	99.0	<0.01	0.21	0.14	0.09	0.07	0.63	--	--	--
06 Months	Complies	Complies	9.99	99.9	<0.01	0.22	0.17	0.08	0.07	0.60	--	--	--
09 Months	Complies	Complies	9.93	99.3	<0.01	0.21	0.18	0.09	0.07	0.60	--	--	--
12 Months	Complies	Complies	9.92	99.2	<0.01	0.21	0.21	0.09	0.08	0.64	25	<5	Complies

--: not performed per protocol;

Table 2.41 Stability studies results obtained for CB-03-10 10% oral solution batch 7100 stored at 40°C/75%RH.

Test	**Appearance**	**CB-03-10 identification**	**CB-03-10 Assay**		**Related substances**						**Microbiological analysis**		
					Cortexolone	**Cortexolone 21-propionate**	**Cortexolone 17α-valerate**	**Cortexolone 21-valerate**	**Any other related substance**	**Total related substances**	**TAMC**	**TYMC**	**E. Coli**
Provisional Specification	Light yellow clear solution	Positive	9.00-11.00% w/w	90.0-110.0% vs labeled	% for information only						≤1000 CFU/g	≤100 CFU/g	Absent in 1 g
					NMT 1%	NMT 1%	NMT 3%	NMT 3%	NMT 0.5%	NMT 8%			
Initial values	Complies	Complies	10.12	101.2	<0.01	0.21	0.12	0.14	0.08	0.60	<10	<5	Complies
40°C/75% RH													
01 Month	Complies	Complies	10.08	100.8	<0.01	0.21	0.15	0.08	0.07	0.60	--	--	--
02 Months	Complies	Complies	9.97	99.7	<0.01	0.21	0.19	0.08	0.07	0.60	--	--	--
03 Months	Complies	Complies	9.97	99.7	<0.01	0.21	0.21	0.11	0.08	0.71	--	--	--
06 Months	Complies	Complies	9.94	99.4	<0.01	0.22	0.30	0.11	0.08	0.76	<10	<5	Complies

--: not performed per protocol;

Table 2.42 Stability studies results obtained for CB-03-10 10% oral solution batch 7100 stored at 30°C/65%RH.

Test	**Appearance**	**CB-03-10 identification**	**CB-03-10 Assay**		**Related substances**						**Microbiological analysis**		
					Cortexolone	**Cortexolone 21-propionate**	**Cortexolone 17*α*-valerate**	**Cortexolone 21-valerate**	**Any other related substance**	**Total related substances**	**TAMC**	**TYMC**	**E. Coli**
Provisional Specification	Light yellow clear solution	Positive	9.00-11.00% w/w	90.0-110.0% vs labeled	% for information only						≤1000 CFU/g	≤100 CFU/g	Absent in 1 g
					NMT 1%	NMT 1%	NMT 3%	NMT 3%	NMT 0.5%	NMT 8%			
Initial values	Complies	Complies	10.12	101.2	<0.01	0.21	0.12	0.14	0.08	0.60	<10	<5	Complies
30°C/65% RH													
03 Months	Complies	Complies	9.93	99.3	<0.01	0.21	0.16	0.10	0.07	0.65	--	--	--
06 Months	Complies	Complies	9.93	99.3	<0.01	0.22	0.20	0.09	0.07	0.62	--	--	--
09 Months	Complies	Complies	9.93	99.3	<0.01	0.22	0.22	0.09	0.08	0.66	--	--	--
12 Months	Complies	Complies	9.94	99.4	<0.01	0.21	0.26	0.10	0.09	0.71	<10	<5	Complies

--: not performed per protocol;

Both chemical and microbiological results showed good stability behavior of this formulation in all the tested conditions.

The results of the assay are always within the specification limit with a maximum decrement of 2%. Impurities profile is close to the initial values and within the provisional specification. No variation in terms of aspect of the final product has been observed.

2.4.3 CB-03-10 30 mg/mL oral solution development

2.4.3.1 Aim

The aim of this study was to develop an oral formulation, with a lower API concentration, to be used for a further preclinical study. The study scheme foresaw the administration of two different dosages of CB-03-10: 100 mg/kg/day and 300 mg/kg/day.
Giving that the maximum volume administered orally to the animals was equal to 10 mL/kg and the 10% oral formulation did not allow to administer the lower dosage since the corresponding volume could not be accurately dosed by a laboratory pipette, a new oral formulation containing 30 mg/mL of CB-03-10 was developed.. This concentration allowed the administration respectively of 10 mL/kg (as the previous study with CB-03-10 10% oral formulation) for the higher dosage, and 3.3 mL/kg for the lower dosage.
The goal was to obtain a formulation with the ability to self-emulsify upon dilution with physiological fluids.

2.4.3.2 Prototype Formulations

Based on Lipid Formulation Classification System (LFCS) and on the previous developed formulation, different prototype formulations were developed using different excipients.

A) The first prototype formulation was developed based on the oral formulation used in the previous studies reducing the concentration of CB-03-10 from 10% w/w (100mg/g) to 30 mg/mL. the ratio between API and D, L-α-tocopherol was maintained at 2:1, the concentration of ethanol and PEG400 was not changed. The concentrations of Labrafil and labrafac were modified in order to compensate the amount of API.

Table 2.43 Quali-quantitative composition of the first 30 mg/mL formulation.

Component(s)	**Function**	**% w/V (g/100mL)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Active ingredient	3.00
D, L-α-tocopherol	Antioxidant/solubilizer	1.50
Ethanol 96%	Co-surfactant/ solubilizer	5.00
Polyethylene glycol 400 (PEG 400)	Co-surfactant	3.00
Medium-chain triglycerides NF (Labrafac Lipophile WL1349)	Oil	24.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	Water insoluble surfactant	up to 100 mL

Aspect: clear solution

B) The second prototype formulation was developed based on LFCS in order to obtain a type IV formulation (according to LFCS reported in Table 1.1).

Table 2.44 Quali-quantitative composition of the second 30 mg/mL formulation.

Component(s)	**Function**	**% w/v (g/100ml)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Active ingredient	3.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	Water insoluble surfactant	20.00
Diethylene glycol monoethyl ether (Transcutol)	Hydrophilic cosolvent	20.00
Caprylocaproyl Polyoxyl-8 glycerides (Labrasol)	Water soluble surfactant	up to 100 mL

Aspect: clear solution

C) The third prototype formulation is based on LFCS in order to obtain a type IIIB formulation (according to LFCS reported in Table 1.1).

Table 2.45 Quali-quantitative composition of the third 30 mg/mL formulation.

Component(s)	**Function**	**% w/v (g/100ml)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Active ingredient	3.00
Polyethylene glycol monostearate (Gelucire 48/16)	Water soluble surfactant	56.00
Diethylene glycol monoethyl ether (Transcutol)	Hydrophilic cosolvent	20.00
D, L-α-tocopherol	Antioxidant	1.50
Medium-chain triglycerides NF (Labrafac Lipophile WL1349)	Oil	up to 100 mL

Aspect: semisolid

Since, at this stage, the aim was to obtain a new liquid formulation containing 30 mg/mL of CB-03-10, this formulation was discarded because its semisolid consistency.

D) The fourth prototype formulation was developed based on formulation C using labrasol instead of Gelucire and adding the antioxidant.

Table 2.46 Quali-quantitative composition of the fourth 30 mg/mL formulation..

Component(s)	**Function**	**% w/v (g/100ml)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Active ingredient	3.00
Medium-chain triglycerides NF (Labrafac Lipophile WL1349)	Oil	18.00
Diethylene glycol monoethyl ether (Transcutol)	Hydrophilic cosolvent	20.00
D, L-α-tocopherol	Antioxidant	1.50
Caprylocaproyl Polyoxyl-8 glycerides (Labrasol)	Water soluble surfactant	up to 100 mL

Aspect: clear solution

2.4.3.3 DLS Characterization

Here below are reported the droplet size distribution by intensity of each liquid formulation tested:

Formulation A)

Results

			Size (d.nm...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	2998	Peak 1:	1681	41,6	343,5
PdI:	0,849	Peak 2:	788,7	26,3	128,9
Intercept:	0,825	Peak 3:	135,9	24,1	30,17

Result quality Refer to quality report

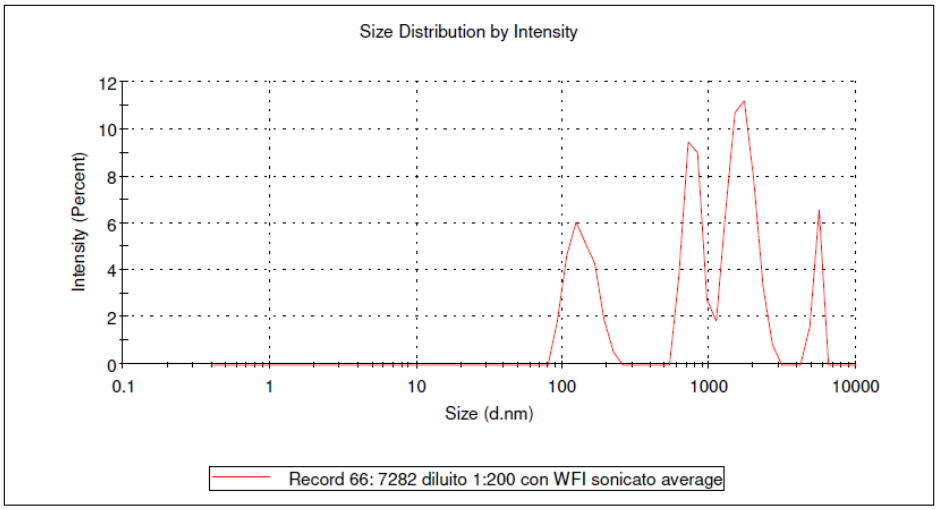


Figure 2.6 DLS results obtained for the Formulation A.

Formulation B)

Results

		Size (d.nm...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 177,7	**Peak 1:**	204,6	97,5	94,50
PdI: 0,213	**Peak 2:**	4711	2,5	777,9
Intercept: 0,941	**Peak 3:**	0,000	0,0	0,000

Result quality Good

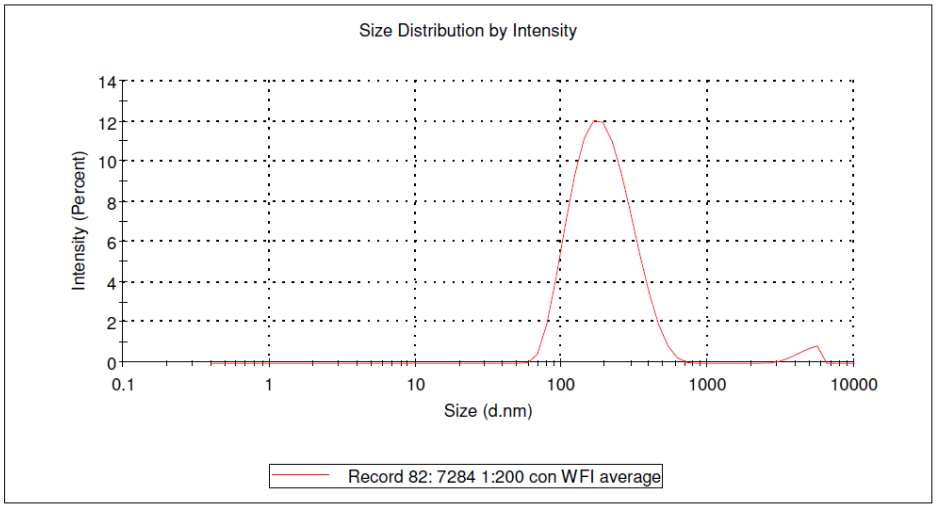


Figure 2.7 DLS results obtained for the Formulation B.

Formulation D)

Results

		Size (d.nm...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 208,7	**Peak 1:**	193,1	96,7	62,52
PdI: 0,304	**Peak 2:**	5065	3,3	607,1
Intercept: 0,953	**Peak 3:**	0,000	0,0	0,000

Result quality Refer to quality report

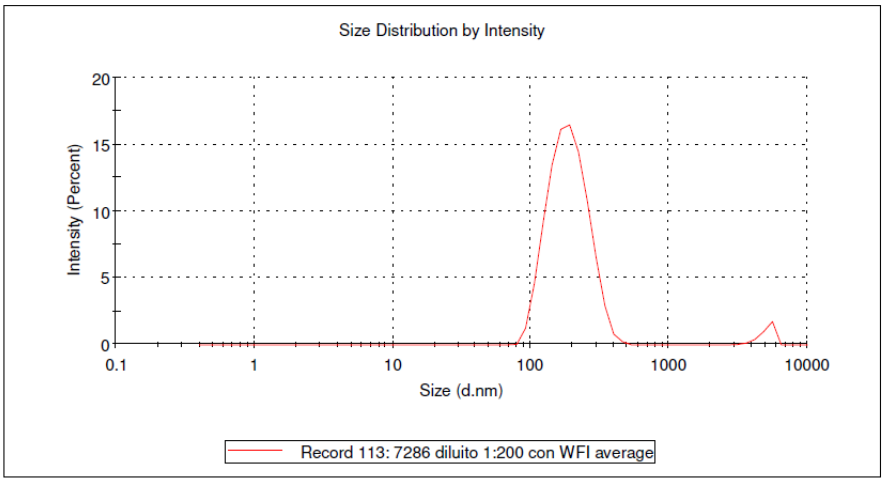


Figure 2.8 DLS results obtained for the Formulation D.

2.4.3.4 Discussion

Based on Size distribution by intensity results, the formulation B was chosen as self-emulsifying drug delivery system for CB-03-10.

In order to evaluate if it is needed to add an antioxidant to the selected formulation, two different formulations were prepared: one containing D, L-α-tocopherol (ratio API/ D, L-α-tocopherol 2:1) and the other one without antioxidant.

Purity profile of the API after oxidative stress test and temperature stress test at 50°C was performed.

- Formulation without antioxidant:

Table 2.47 Composition of the formulation without antioxidant.

Component(s)	**% w/v (g/100ml)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	3.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	20.00
Diethylene glycol monoethyl ether (Transcutol)	20.00
Caprylocaproyl Polyoxyl-8 glycerides (Labrasol)	up to 100 mL

- Formulation with antioxidant:

Table 2.48 Composition of the formulation with antioxidant.

Component(s)	**% w/v (g/100ml)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	3.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	20.00
Diethylene glycol monoethyl ether (Transcutol)	20.00
D, L-α-tocopherol	1.50
Caprylocaproyl Polyoxyl-8 glycerides (Labrasol)	up to 100 mL

2.4.3.4.1 Results

The results obtained after oxidative and temperature stress tests are reported in Table 2.49.

Table 2.49 Results obtained analyzing the samples with and without antioxidant agent after oxidative and temperature stress tests.

Batch	Condition	Cortexolone 21-propionate % (Average±sd; n=3)	Cortexolone 17α-valerate % (Average±sd; n=3)	Cortexolone 21-valerate % (Average±sd; n=3)	Any other related substances % (Average±sd; n=3)	Total impurities % (Average±sd; n=3)
with antioxidant	Room temperature	0.38±0.02	0.15±0.03	0.24±0.04	0.09±0.01	1.00±0.11
	T=50°C	0.38±0.03	0.16±0.04	0.25±0.03	0.10±0.02	1.04±0.13
	Oxidative stress	0.41±0.03	0.16±0.02	0.25±0.03	0.10±0.01	1.08±0.11
without antioxidant	Room temperature	0.38±0.01	0.08±0.02	0.19±0.03	0.07±0.02	0.77±0.10
	T=50°C	0.39±0.03	0.09±0.01	0.20±0.02	0.08±0.01	0.80±0.09
	Oxidative stress	0.40±0.02	0.09±0.04	0.20±0.03	0.07±0.01	0.84±0.12

The obtained results suggested that there is no need to add D, L-α-tocopherol as antioxidant agent.

2.4.3.5 Selected 30 mg/mL oral formulation

Based on both size distribution results and analytical tests, the formulation B, containing labrafil, labrasol and transcutol, was selected as final formulation for efficacy studies.

Table 2.50 Composition of the selected 30 mg/mL oral solution.

Component(s)	**Specification**	**Function**	**% w/V (g/100mL)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Internal monograph	Active ingredient	3.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	EP, USP	Surfactant	20.00
Diethylene glycol monoethyl ether (Transcutol HP)	EP, USP	Co-Solvent	20.00
Caprylocaproyl Polyoxyl-8 glycerides (Labrasol ALF)	EP, USP	Surfactant	up to 100 mL

2.4.3.6 Summary of the development batches

The summary of CB-03-10 30 mg/mL oral solution development batches is reported in Table 2.51.

Table 2.51 Summary of development batches.

Formulation Batch Number	**Batch Size (L of bulk preparation)**	**Batch use**
7294/1	1L	Efficacy study Ephor-11-18

2.4.3.7 CB-03-10 30 mg/mL oral solution Batch Analysis results

Table 2.52 Batch analysis results.

Test	**Provisional Specifications**	**Batch n° 7294**
Chemical Analysis		
Appearance	Colorless to light yellow clear solution	Light yellow clear solution
CB-03-10 (HPLC) Identification	Positive	Positive
CB-03-10 (HPLC) Assay	90.0 – 110.0% of labelled claim	2.996% g/100mL (9.99% of labelled claim)
Related Substances:		
Cortexolone	NMT 1%	<0.01%
Cortexolone-21-propionate	NMT 1%	0.39%
Cortexolone-17α-valerate	NMT 3%	0.15%
Cortexolone-21-valerate	NMT 3%	0.26%
Any other related substances	NMT 0.5%	0.09%
Total impurities	NMT 8%	1.00%
Density at 25°C	g/mL for information only	1.0198 g/mL
Viscosity at 25°C	cP for information only	52.1 cP
Microbiological Analysis		
Total Aerobic Microbial Count (TAMC)	NMT 1000 CFU/g	<10 CFU/g
Total Yeasts and Molds Count (TYMC)	NMT 100 CFU/g	<5 CFU/g
Escherichia Coli	Absent in 1 g	Absent in 1 g

2.4.3.8 Stability studies

The results of the stability study performed on CB-03-10 30mg/mL oral solution batch 7294 packaged in glass amber bottles are reported in Tables 2.53 and 2.54..

Table 2.53 Stability studies results obtained for CB-03-10 30 mg/mL oral solution batch 7294 stored at 25°C/60%RH.

Test	**Appearance**	**CB-03-10 identification**	**CB-03-10 Assay**		**Related substances**						**Viscosity**	**Density**	**Microbiological analysis**		
					Cortexolone	**Cortexolone 21-propionate**	**Cortexolone 17α-valerate**	**Cortexolone 21-valerate**	**Any other related substance**	**Total related substances**			**TAMC**	**TYMC**	**E. Coli**
Provisional Specification	Colorless to light yellow clear solution	Positive	27.0-33.0 mg/mL	90.0-110.0% vs labeled	% for information only						cP for information only	g/cm3 for information only	≤1000 CFU/g	≤100 CFU/g	Absent in 1 g
					NMT 1%	NMT 1%	NMT 3%	NMT 3%	NMT 0.5%	NMT 8%					
Initial values	Complies	Complies	30.0	100.0	<0.01	0.39	0.15	0.26	0.09	1.00	52.1	1.0198	<10	<5	Complies
25°C/60% RH															
03 Months	Complies	Complies	30.0	100.0	<0.01	0.38	0.16	0.26	0.13	1.35	--	1.0201	--	--	--
06 Months	Complies	Complies	29.7	99.0	<0.01	0.38	0.17	0.23	0.08	1.22	--	1.0189	--	--	--

--: not performed per protocol;

Table 2.54 Stability studies results obtained for CB-03-10 30 mg/mL oral solution batch 7294 stored at 40°C/75%RH.

Test	Appearance	CB-03-10 identification	CB-03-10 Assay		Related substances						Viscosity	Density	Microbiological analysis		
					Cortexolone	Cortexolone 21-propionate	Cortexolone 17α-valerate	Cortexolone 21-valerate	Any other related substance	Total related substances			TAMC	TYMC	E. Coli
Provisional Specification	Colorless to light yellow clear solution	Positive	27.0-33.0 mg/mL	90.0-110.0% vs labeled	% for information only						cP for information only	g/cm3 for information only	≤1000 CFU/g	≤100 CFU/g	Absent in 1 g
					NMT 1%	NMT 1%	NMT 3%	NMT 3%	NMT 0.5%	NMT 8%					
Initial values	Complies	Complies	30.0	100.0	<0.01	0.39	0.15	0.26	0.09	1.00	52.1	1.0198	<10	<5	Complies
40°C/75% RH															
03 Months	Complies	Complies	29.9	99.7	<0.01	0.38	0.19	0.29	0.20	1.49	--	1.0201	--	--	--
06 Months	Complies	Complies	29.7	99.0	<0.01	0.38	0.23	0.26	0.21	1.74	53.3	1.0202	<10	<5	Complies

--: not performed per protocol;

The results showed good stability of this formulation both in terms of chemical and microbiological results The results obtained in accelerated conditions show an increment of the total related substances, but always under 2%.

Both the oral formulations tested *in vivo* showed good safety profile. However, in both cases it was evidenced that the excipients selected to grant the drug substance solubility were also characterized by an awful taste that can not be masked with flavours and could jeopardizes the compliance of the oral administration in humans.
Therefore, it was decided to use capsules to administer the API solubilized in an appropriate formulation for human use.

At the end of the toxicological and efficacy studies in animal models, the aim was to optimize the oral formulation for the first time in man clinical trial because the concentration of the API into 10% w/w oral formulation corresponds to its solubility. Therefore, a restricted storage condition 20-25°C should be respected to avoid any API precipitation that can occur at a temperature below 20°C. While the concentration of the formulation containing 3% (30 mg/mL) of API is too low to obtain the desired dose in human clinical study because of the elevate number of capsules needed to reach the target dose.

Different formulations have been studied for the First Time in Man clinical trial.

2.4.4 New liquid formulation 80 mg/mL

The first new oral formulation is a liquid formulation developed based on the oral one containing 30mg/mL of API but the concentration of API is increased to 80 mg/mL in order to reduce the number of capsules administered to the patient.

Table 2.55 Quali-quantitative composition of the new oral formulation.

Component(s)	**Specification**	**Function**	**% w/V (g/100mL)**
CB-03-10 (cortexolone 17α-valerate-21- propionate)	Internal monograph	Active ingredient	8.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	EP, USP	Surfactant	20.00
Diethylene glycol monoethyl ether (Transcutol HP)	EP, USP	Co-Solvent	20.00
Caprylocaproyl Polyoxyl-8 glycerides (Labrasol ALF)	EP, USP	Surfactant	up to 100 mL

2.4.4.1 DLS Characterization

The prototype formulation has been characterized by Zetasizer Nano S upon dilution with WFI in order to test its ability to self-emulsify.

Here below are reported the droplet size distribution by intensity of the new liquid formulation tested:

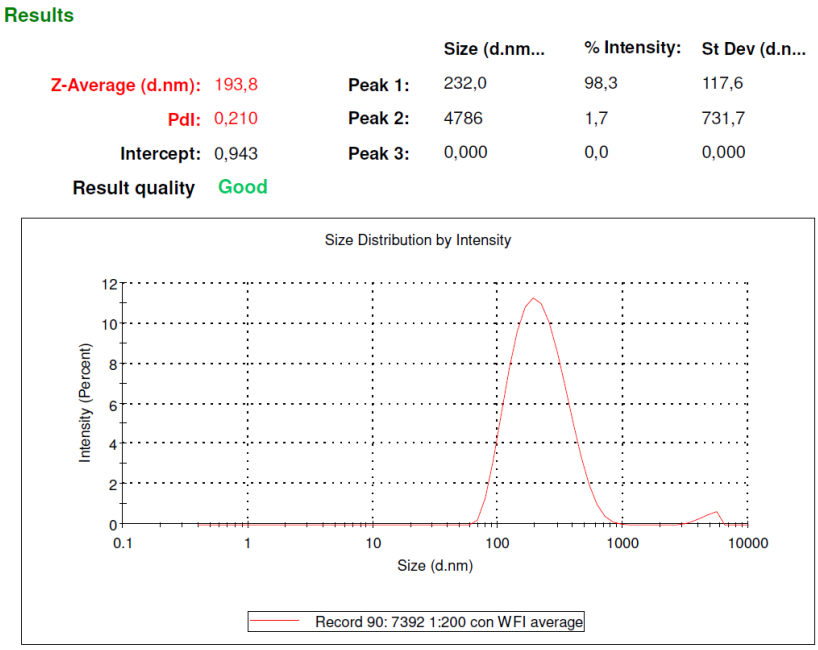


Figure 2.9 DLS results of the new liquid formulation prototype.

The DLS results upon dilution with WFI show a bimodal distribution. The 98.3% of the droplets have dimension of 232 nm and only 1.7% of the obtained emulsion presents dimensions over 4 µm. The quality of the result is good and the polydispersion index is low.

2.4.5 New semisolid formulation 8% w/w

In order to facilitate the filling procedures in hard gelatin capsules, a semisolid oral formulation was developed. The aim was to fill the formulation in hard gelatin capsules without the use of sealing or banding, that are required with liquid formulations.

Table 2.56 Quali-quantitative composition of the new semisolid formulation.

Component(s)	**Specification**	**Function**	**% w/w (g/100g)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Internal monograph	Active ingredient	8.00
Polyethylene glycol monostearate (Gelucire 48/16)	USP, JPE	Surfactant	50.00
Diethylene glycol monoethyl ether (Transcutol HP)	EP, USP	Co-Solvent	20.00
D,L-α-tocopherol	EP, USP	Antioxidant/solubilizer	1.50
Medium-chain triglycerides (Labrafac Lipophile WL1349)	EP, USP, JPE	Oil	20.50

2.4.5.1 DLS Characterization

The semisolid prototype formulation has been characterized by Zetasizer Nano S upon dilution with WFI in order to test its ability to self-emulsify.

Here below are reported the droplet size distribution by intensity of each the semisolid formulation tested:

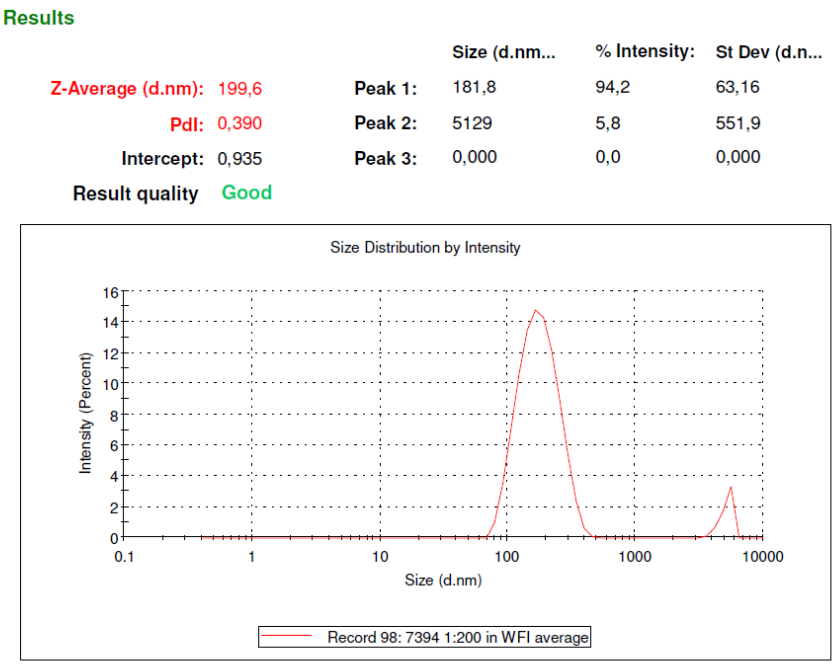


Figure 2.10 DLS results of the new semisolid prototype formulation.

The DLS results upon dilution with WFI show a bimodal distribution. The 94.2% of the droplets have dimension under 200 nm and 5.8% of the obtained emulsion presents dimensions over 5 µm. The quality of the result is good and the polydispersion index is 0.390.

2.4.5.2 Ternary diagram phase

In order to study the ratio between surfactants and oil in the semisolid formulation, a ternary diagram phase was created to evaluate the auto-emulsification properties.

The diagram was obtained using the titration method with water in order to simulate the physiological dilution with physiological fluids.

The results of the ternary diagram phase are reported in the Figure 2.11.

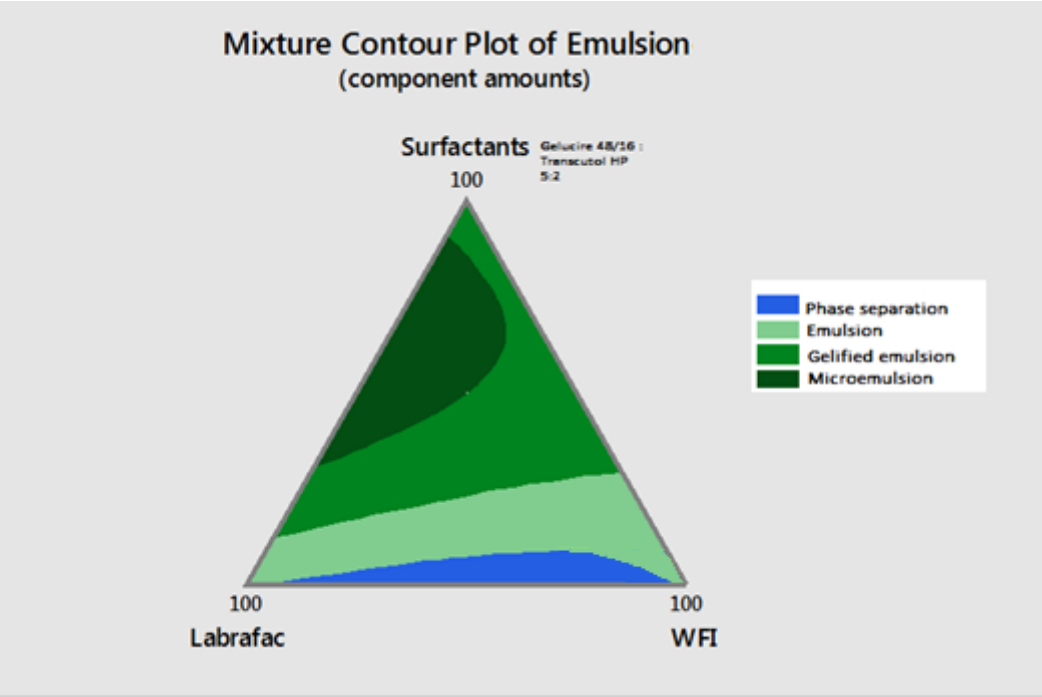


Figure 2.11 Schematic representation of the results.

The obtained results show that the self-emulsification properties depend on the ratio between surfactants and oil. Reducing the surfactants concentration below 30% the self-emulsification properties are reduced.

2.4.6 Alternative semisolid formulation

Based on the results obtained during ternary diagram phase trial a alternative semisolid formulation is developed, in order to facilitate the filling step of the capsules at lower temperature. This alternative formulation has the same qualitative composition of the first semisolid formulation but a different ratio between excipients. The aim was to obtain an alternative formulation sufficiently fluid to be machinable and with an adequate melting point compatible with the filling of the capsules.

Table 2.57 Quali-quantitative composition of the alternative semisolid formulation.

Component(s)	**Specification**	**Function**	**% w/w (g/100g)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Internal monograph	Active ingredient	8.00
Polyethylene glycol monostearate (Gelucire 48/16)	USP, JPE	Surfactant	48.50
Diethylene glycol monoethyl ether (Transcutol HP)	EP, USP	Co-Solvent	15.00
D,L-α-tocopherol	EP, USP	Antioxidant/solubilizer	1.50
Medium-chain triglycerides (Labrafac Lipophile WL1349)	EP, USP, JPE	Oil	27.00

The melting point of the alternative formulation is almost the same of the lead semisolid formulation even is its consistency is more fluid.

2.4.7 *In-vitro* dissolution test results and discussion

The dissolution test was performed on the three new prototype formulations (8% liquid formulation, 8% semisolid formulation (A) and 8% semisolid formulation (B)) compared with the liquid formulation containing 10% w/w of API used during pre-clinical studies.
The test was carried out on hard gelatin capsules containing 240 mg of formulated API each.
The quali-quantitative composition of the formulations tested are reported in Tables 2.58-2.61.

Table 2.58 Quali-quantitative composition of 10% liquid formulation

Component(s)	**% w/w (g/100g)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	10.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	56.00
D, L-α-tocopherol	5.00
Ethanol 96%	5.00
Polyethylene glycol 400 (PEG 400)	3.00
Medium-chain triglycerides NF (Labrafac Lipophile WL1349)	21.00

Table 2.59 Quali-quantitative composition of 8% liquid formulation

Component(s)	**% w/V (g/100mL)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	8.00
Oleoyl polyoxyl-6 glycerides NF (Labrafil M 1944 CS)	20.00
Diethylene glycol monoethyl ether (Transcutol HP)	20.00
Caprylocaproyl Polyoxyl-8 glycerides (Labrasol ALF)	up to 100 mL

Table 2.60 Quali-quantitative composition of 8% semisolid formulation (A).

Component(s)	**% w/w (g/100g)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	8.00
Polyethylene glycol monostearate (Gelucire 48/16)	50.00
Diethylene glycol monoethyl ether (Transcutol HP)	20.00
D,L-α-tocopherol	1.50
Medium-chain triglycerides (Labrafac Lipophile WL1349)	20.50

Table 2.61 Quali-quantitative composition of 8% semisolid formulation (B):

Component(s)	**% w/w (g/100g)**
CB-03-10 (cortexolone 17α-valerate-21-propionate)	8.00
Polyethylene glycol monostearate (Gelucire 48/16)	48.50
Diethylene glycol monoethyl ether (Transcutol HP)	15.00
D,L-α-tocopherol	1.50
Medium-chain triglycerides (Labrafac Lipophile WL1349)	27.00

The dissolution test results obtained after 60 minutes are reported in Figure 2.12.

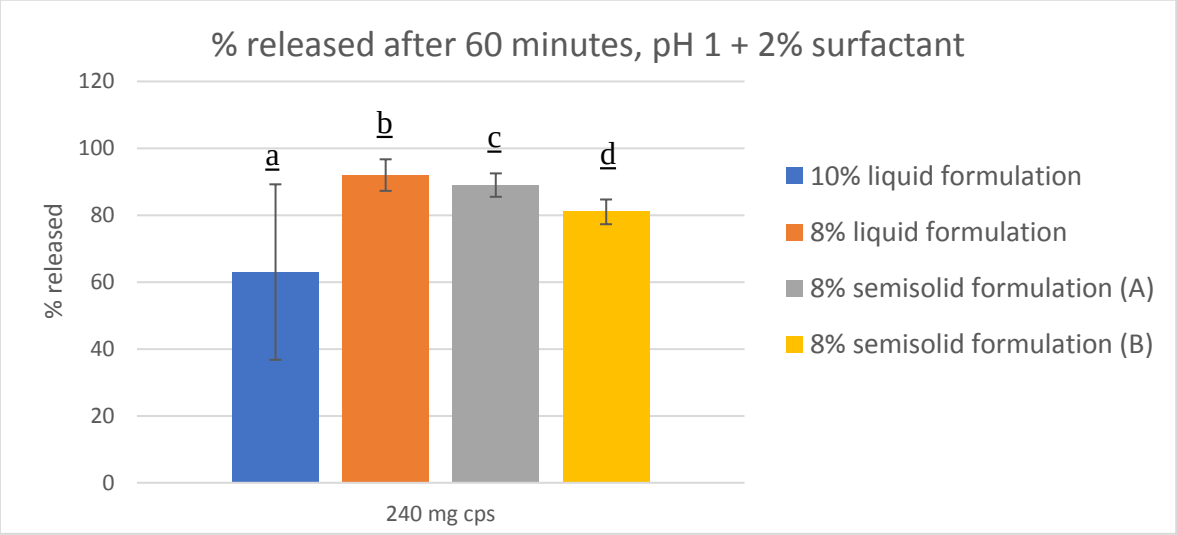


Figure 2.12 Dissolution test results obtained after 60 minutes (mean values ± sd; n=6). a vs b; b vs d; c vs d (t-test; $p<0.05$)

All the new formulations have a higher released value after 1 hour and a lower variability respect to the formulation used during pre-clinical studies (10% liquid formulation).
The Provisional Specification of release is fixed NLT 75%Q after 1 hour.
There is no statistically significant difference between the semisolid formulation (A) and the liquid formulation at 8% (80 mg/mL) of API (t-test; $p<0.05$). Therefore, the semisolid prototype formulation was chosen as lead formulation because it allows the filling of both hard gelatin capsules and soft gelatin capsules. The liquid formulation is selected as alternative formulation.

2.4.8 Lead Semisolid Prototype Formulation

2.4.8.1 Description and Composition of the Drug Product

Cortexolone-17α-valerate-21-propionate semisolid matrix filled in gelatin capsules is an off-white semisolid matrix. The qualitative- quantitative composition of CB-03-10, 8% w/w semisolid matrix filled in gelatin capsules is listed in Table 2.62.

Table 2.62 Qualitative - quantitative composition of CB-03-10 8% semisolid matrix.

Component(s)	Reference to standard	Function	% w/w (g/100g)
CB-03-10 (cortexolone 17α-valerate-21-propionate)	Internal monograph	Active ingredient	8.00
Polyethylene glycol monostearate	USP, JPE	Surfactant	50.00
D,L-α-tocopherol	EP, USP	Antioxidant/solubilizer	1.50
Diethylene glycol monoethyl ether	EP, USP	Co-solvent	20.00
Medium-chain triglycerides	EP, USP, JPE	Oil	20.50

All the components used in both the 8% semisolid matrix formulation and in the capsules are excipients of pharmaceutical grade and are compliant with the specifications of the relevant compendial tests.

2.4.8.2 Provisional specifications

The current provisional release specifications for CB-03-10 capsules to support the proposed clinical trial are reported in Table 2.63.

Table 2.63 CB-03-10 capsules provisional release and stability specifications.

Test	Method	Provisional Specification
Chemical Analysis		
Appearance	Visual inspection	Conform to the standard
Identification Cortexolone-17 α -valerate-21-propionate (HPLC)	In- house	Positive
Cortexolone-17 α -valerate-21-propionate Assay (HPLC)	In- house	90.0 – 110.0% of labeled claim
Related substances (HPLC) Cortexolone Cortexolone-21-propionate Cortexolone-17α-valerate Cortexolone-21-valerate Any other related substances Total impurities	In- house	 NMT 1% NMT 1% NMT 3% NMT 3% NMT 0.5% NMT 8%
Dissolution	USP <711>	pH1 after 1 hour NLT 75%Q
Uniformity of Dosage unit [1]	USP <905>	Meet the requirements
Microbiological Analysis		
Total Aerobic Microbial Count (TAMC)	USP <61> and <62>	NMT 1000 CFU/g
Total Yeasts and Molds Count (TYMC)		NMT 100 CFU/g
E. Coli		Absent in 1g

USP = United States Pharmacopoeia, current edition; CFU = colony forming unit; NMT = not more than.
[1] Test performed at release and on stability at T=0 only.

2.4.8.3 CB-03-10 capsules batch analysis

Table 2.64 Batch analysis results.

Test	**Provisional Specifications**	**Batch n° 7403**
Chemical Analysis		
Appearance	Conform to the standard	White gelatine capsules size 000
CB-03-10 (HPLC) Identification	Positive	Positive
CB-03-10 (HPLC) Assay	90.0 – 110.0% of labelled claim	101.9%
Related Substances:		
Cortexolone	NMT 1%	0.04%
Cortexolone-21-propionate	NMT 1%	0.29%
Cortexolone-17α-valerate	NMT 3%	0.53%
Cortexolone-21-valerate	NMT 3%	0.18%
Any other related substances	NMT 0.5%	0.15%
Total impurities	NMT 8%	1.47%
Dissolution	pH 1 after 1 hour NLT 75%Q	80%
Uniformity of dosage unit	Meet the requirements	Complies to USP <905>
Microbiological Analysis		
Total Aerobic Microbial Count (TAMC)	NMT 1000 CFU/g	50 CFU/g
Total Yeasts and Molds Count (TYMC)	NMT 100 CFU/g	<10 CFU/g
Escherichia Coli	Absent in 1 g	Absent in 1 g

NMT: not more than.

2.5 Conclusions

During one of the first preliminary pre-clinical trials, 4 weeks oral toxicity study in rats (ref. A2086), CB-03-10 was administered to the animals in suspension of aqueous solution of carboxymethylcellulose (0.5% CMC).

This study planned to administer to the animals growing doses of API in order to evaluate its toxic effects.

Even at highest dose administered to the animals, no side effects was evidenced and the analysis of plasma samples revealed that, in most sample, CB-03-10 concentrations were below quantification limit of the method (0.50 ng/mL). Therefore, no Toxicokinetic parameters was derived.

These results indicated that this API has either a very low absorption or a rapid biotransformation into the main metabolite (that is the most important circulating molecular species), before reaching the systemic circulation, indicating a very low bioavailability after oral administration, complicated by the poor water solubility of the drug molecule.

During the next step, the study was focused on the development of an oral formulation able to improve as much as possible the bioavailability after oral administration.

The data obtained during the bioavailability study in dogs (ref. 525/16) demonstrated an improved oral bioavailability of the oral formulation containing 10%w/w of API.

Plasma samples obtained during pharmacokinetic study of CB-03-10 in Beagle Dogs were analyzed using HPLC/MS/MS for CB-03-10 and its metabolite CB-03-05 content. The CB-03-10 and CB-03-05 plasma levels revealed similar PK profiles in male and female subjects. Plasma concentration-time profiles of CB-03-10 and CB-03-05 and derived non-compartmental pharmacokinetic parameters Cmax, tmax, and AUC0-48h were used as descriptors of systemic exposure of CB-03-10 and CB-03-05.

The PK profiles obtained following oral dosing with CB-03-10 10% oral solution versus the intravenous dosing were compared and expressed in term of absolute oral bioavailability.

CB-03-10 10% oral solution allowed to obtain an absolute median oral bioavailability equal to 6,35%. These results indicate an increased bioavailability in accordance to the aim of the formulation study.

The Results of pre-clinical studies demonstrated that CB-03-10 has shown to be able to provide a tumor growth suppression via inhibition of the Androgenic Receptor (AR) and Glucocorticoid Receptor (GR).

CB-03-10 induces cell death in various tumor cell lines grown in vitro and shows a marked inhibition of tumor growth in experimental animal models of cancer, mainly through apoptotic mechanisms, at well-tolerated dose levels.

Both CB-03-10 10% oral solution and CB-03-10 30 mg/mL oral solution showed good *in vivo* safety profile. However, in both cases it was evidenced that the excipients selected to grant the drug substance solubility were also characterized by an awful taste that can not be masked with flavours and could jeopardizes the compliance of the oral administration in humans.

Therefore, it was decided to use capsules to administer the API solubilized in an appropriate formulation for human use.

At the end of the toxicological and efficacy studies in animal models, the aim was to optimize the oral prototype formulations for the first time in man clinical trial because the concentration of the API into 10% w/w oral formulation corresponds to its solubility. Therefore, a restricted storage condition 20-25°C should be respected to avoid any API precipitation that can occur at a temperature below 20°C. While the concentration of the formulation containing 3% (30 mg/mL) of API is too low to obtain the desired dose in human clinical study because of the elevate number of capsules needed to reach the target dose.

Different prototype formulations, both liquid and semisolid, containing 8% of CB-03-10 have been studied for the First Time in Man clinical trial. Both liquid and semisolid prototype formulations allowed to obtain good results but the semisolid formulation was chosen as lead formulation for its versatility during the production step because it allows the filling of both hard gelatin capsules and soft gelatin capsules.

The lead semisolid formulation is a SEDDS filled in capsules and it was presented in IND application and it obtained the FDA allowance.

Based on the obtained results, the lead semisolid formulation submitted in the IND will be tested in phase I clinical trial in tumor bearing patients in order to evaluate safety, pharmacokinetics and pharmacodynamic activity.

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CHAPTER 2

Parenteral Formulations Pharmaceutical Development

3.1 Aim

Aim of the study was to develop a parenteral therapeutic system to be used during preclinical studies in comparison with the oral formulation and in order to have also an injectable formulation prototype for future clinical studies. The goal was to find a vehicle able to solubilize 4.0 mg/mL of CB-03-10.

3.2 Quality target product profile

Based on the clinical purpose, a Quality Target Product Profile for CB-03-10 solution for injection was defined in Table 3.1.

Table 3.1 Quality Target Product Profile for CB-03-10 solution for injection.

QTPP Elements		Target
Route of administration		Parenteral
Dosage form		Liquid
Characteristics		Good performance *in vitro* and *in vivo*
Product quality attributes	Physical attributes	Functional aim requirement: Must meet the same compendial or other applicable (quality) standards (i.e., identity, assay, purity, and quality).
	API Assay	
	API Purity	
	pH	
	Viscosity	
	Density	
	Injectability	
	Osmolality	
	Bacterial endotoxin limit	
	Sterility	
Stability		At least 36 month shelf-life at room temperature or in ICH conditions.
Container closure system		Container closure system qualified as suitable for this product.

3.3 Materials and methods

Materials and methods related to the preparation, characterization and analysis of the parenteral prototype formulation are reported here below.

3.3.1 Materials

3.3.1.1 Drug substance: CB-03-10

See paragraph 2.3.1.1.

3.3.1.2 Excipients

Several excipients/vehicles, including those usually used in toxicology studies, were considered in order to achieve the optimal solubilization of the API.
The main excipients used during the pharmaceutical development were reported below:

- Sodium carboxymethylcellulose
- Polysorbate 80 (Tween 80)
- Benzyl alcohol
- Polyethylene glycol 400 (PEG 400)
- Ethanol
- Dimethyl sulfoxide (DMSO)
- 2-Hydroxypropyl-β-cyclodextrin

In Table 3.2 are summarized the tolerated dose of the most common excipients used to improve the solubility of the insoluble drugs.

Table 3.2 Typical maximum used levels of excipients in various animal species and common adverse findings [1].

	Rodents (rat and mouse)		Dogs		Monkeys		Common adverse findings
	Oral % w/w (ml/kg)	IV % w/w (ml/kg)	Oral % w/w (ml/kg)	IV % w/w (ml/kg)	Oral % w/w (ml/kg)	IV % w/w (ml/kg)	
DMSO	50 (0.5)	100 (0.1)	50 (0.5)	100 (0.05)	50 (0.5)	Do not use	-
PEG-400	100 (2)	50 (1)	80 (2)	30 (0.5)	25 (2)	30 (0.5)	Loose stools, emesis
Propylene glycol	80 (2)	50 (0.5)	50 (2)	30 (0.5)	50 (0.5)	10 (0.5)	Loose stools, emesis
Ethanol	50 (0.5)	20 (0.5)	50 (2)	20 (0.5)	25 (2)	20 (0.5)	Peritoneal adhesions, liver and spleen peritonitis
Tween 80	50 (2.5)	2 (0.25)	25 (1)	Do not use	25 (1)	0.5 (0.005)	Anaphylaxis in dogs when dosed intravenously
Poloxamer	15 (1.5)	15 (0.5)	15 (1.5)	15 (0.5)	15 (1)	15 (0.3)	Loose stools
Cremophor EL	10 (0.5)	10 (0.5)*	10 (0.5)	10 (0.1)*	10 (0.5)	Do not use	-
Oils	100 (5)	15 (1.5)‡	100 (2)	15 (1.5)‡	100 (2)	15 (1.5)‡	Loose stools
Sulfobutyl ether cyclodextrin	40 (5)	20 (5)§	40 (2)	20 (2)§	40 (2)	20 (2)§	Renal tubule vacuolation and foamy macrophages at large doses

Levels are provided as % w/w concentration and maximum dose volume (ml/kg) to give the maximum dose. The concentrations and dose volume can be varied as long as the total dose is kept within these limits. In addition, these levels are typical for acute and subchronic dosing (up to 7 days).
*Only acute use is recommended (single dose).
‡Used in emulsion as a slow-push injection (30 min).
§12.5% w/w recommended to maintain isotonicity.
DMSO: Dimethly sulfoxide; IV: Intravenous.

Sodium carboxymethylcellulose

Carboxymethylcellulose sodium is widely used in oral and topical pharmaceutical formulations, primarily for its viscosity-increasing properties. Viscous aqueous solutions are used to suspend powders intended for either topical application or oral and parenteral administration.

Encapsulation with carboxymethylcellulose sodium can affect drug protection and delivery. In animal studies, subcutaneous administration of carboxymethylcellulose sodium has been found to cause inflammation, and in some cases of repeated injection fibrosarcomas have been found at the site of injection.

It is GRAS listed. Accepted as a food additive in Europe. Included in the FDA Inactive Ingredients Database (dental preparations; intraarticular, intrabursal, intradermal, intralesional, and intrasynovial injections; oral drops, solutions, suspensions, syrups and

tablets; topical preparations). Included in nonparenteral medicines licensed in the UK. Included in the Canadian Natural Health Products Ingredients Database [2].
Supplier: EIGENMANN & VERONELLI S.p.A.

Polysorbate 80

Polysorbate 80 is a hydrophilic nonionic surfactant. It is utilized as a surfactant in soaps and cosmetics and also as a lubricant in eye drops. In food or pharmaceutical products, it can act as an emulsifier. Polysorbate 80 is an excipient that is used to stabilize aqueous formulations of medications for parenteral administration or vaccinations. A solubilizing agent acts as a surfactant and increases the solubility of one agent in another. A substance that would not normally dissolve in a particular solution is able to dissolve with the use of a solubilizing agent. It is also known as an emulsifier, which helps ingredients mix together and prevent separation, and water-containing small amounts of salts, and is included in several vaccines licensed in the USA [3].
Polysorbate 80 is used as a solubilizing agent in IV infusions of the antiarrhythmic drug amiodarone. Rare case reports of liver toxicity have been published suggesting polysorbate 80 may contribute to liver toxicity with the IV formulation of amiodarone. The package labeling of amiodarone warns that polysorbate 80 is also known to leach DEHP (dioctyl phthalate) from PVC and dosing recommendations must be followed closely.
Moderately toxic by IV route [2, 3].
Polysorbate 80 is GRAS listed. It is accepted as food additives in Europe and included in the FDA Inactive Ingredients Database (IM, IV, oral, rectal, topical, and vaginal preparations). Polysorbate is included in parenteral and nonparenteral medicines licensed in the UK. Polysorbate 80 is included in the Canadian Natural Health Products Ingredients Database [2].
Supplier: Croda.

Benzyl alcohol

Benzyl alcohol is an antimicrobial preservative used in cosmetics, foods, and a wide range of pharmaceutical formulations, including oral and parenteral preparations.
It is included in the FDA Inactive Ingredients Database (dental injections, oral capsules, solutions and tablets, topical, and vaginal preparations) and in parenteral and nonparenteral

medicines licensed in the UK. Included in the Canadian Natural Health Products Ingredients Database [2].

Polyethylene glycol 400 (PEG 400)

Polyethylene glycols are widely used in a variety of pharmaceutical formulations. Generally, they are regarded as nontoxic and nonirritant materials. Adverse reactions to polyethylene glycols have been reported, the greatest toxicity being with glycols of low molecular weight. However, the toxicity of glycols is relatively low.

In parenteral formulation PEG 400 is used as solvent.

In concentration up to approximately 30% v/v has been used as vehicle for parenteral dosage forms.

Safety: LD50 (mouse, IV): 8.6 g/kg; LD50 (rat, IV): 7.3 g/kg

Included in the FDA Inactive Ingredients Database. Included in the Canadian Natural Health Products Ingredients Database [2].

Supplier: EIGENMANN & VERONELLI S.p.A.

Ethanol

Ethanol is primarily used as solvent also in parenteral solutions.

Parenteral products containing up to 50% of alcohol (ethanol 95 or 96% v/v) have been formulated. However, such concentrations can produce pain on intramuscular injection and lower concentrations such as 5–10% v/v are preferred. Subcutaneous injection of alcohol (ethanol 95% v/v) similarly causes considerable pain followed by anesthesia. If injections are made close to nerves, neuritis and nerve degeneration may occur.

Included in the FDA Inactive Ingredients Database (dental preparations; inhalations; IM, IV, and SC injections; nasal and ophthalmic preparations; oral capsules, solutions, suspensions, syrups, and tablets; rectal, topical, and transdermal preparations). Included in the Canadian Natural Health Products Ingredients Database. Included in nonparenteral and parenteral medicines licensed in the UK [2].

Supplier: Silcompa SpA

Dimethyl sulfoxide (DMSO)

Dimethyl sulfoxide is a highly polar substance that is aprotic, therefore lacking acidic and basic properties. It has exceptional solvent properties for both organic and inorganic components, which are derived from its capacity to associate with both ionic species and neutral molecules that are either polar or polarizable.

Dimethyl sulfoxide has low systemic toxicity but causes local toxic effects. It is readily absorbed after injection or after oral or percutaneous administration and is widely distributed throughout the body. Dimethyl sulfoxide acts as a primary irritant on skin,

causing redness, burning, itching, and scaling; it also causes urticaria. Systemic symptoms include nausea, vomiting, chills, cramps, and lethargy; dimethyl sulfoxide can also cause increases in intraocular pressure. Administration of dimethyl sulfoxide by any route is followed by a garlic-like odor on the breath.

Safety: LD50 (dog, IV): 2.5 g/kg; LD50 (rat, IV): 5.3 g/kg; LD50 (rat, SC): 12 g/kg; LD50 (mouse, IV): 3.8 g/kg.

Included in the FDA Inactive Ingredients Database (IV infusions, SC implants, and topical preparations). Included in the Canadian Natural Health Products Ingredients Database. Available in the USA as a 50% solution for irrigation in the treatment of interstitial cystitis. Also available in Canada as a 70% solution for use as a topical antifibrotic, and in Germany as a topical gel containing 10% dimethyl sulfoxide for the treatment of musculoskeletal and joint disorders. Included in topical formulations of idoxuridine and diclofenac licensed in the UK [2].

2-Hydroxypropyl-β-cyclodextrin (HP-β-CD)

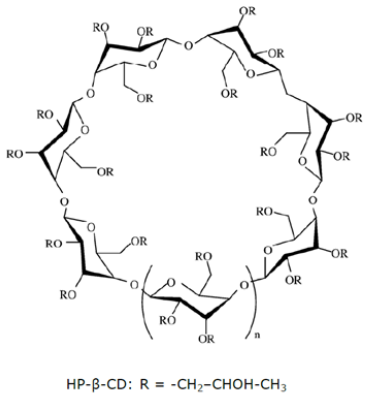


Figure 3.1 HP- β-CD structure.

The solubility of HP-β-CD is quite high, exceeding 600 mg/ml. Viscosity is not an issue in concentrations below 55%.

Cyclodextrins (CDs) are cyclic oligosaccharides used for the improvement of water-solubility and bioavailability of medicinal products.

CD derivatives such as amorphous HP-β- and SBE-β-CDs have been widely investigated for parenteral use on account of their high aqueous solubility and minimal toxicity. HP- β-CD with much higher aqueous solubility allows parenteral administration of various drugs with no significant toxicity problems and hence is more often used in parenteral formulations. An itraconazole parenteral injection containing HP-β-CD (40% w/v) has been commercialized in the United States and Europe. Applications of CDs in parenteral delivery are solubilization of drugs, reduction of drug irritation at the site of administration, and stabilization of drugs unstable in the aqueous environment [4].

It is generally accepted that in aqueous solutions cyclodextrins form what is called "inclusion complexes" where water molecules located within the lipophilic central cavity are replaced by a lipophilic guest molecule or a lipophilic moiety on, for example, a drug molecule. However, the hydroxy groups on the outer surface of the cyclodextrin molecule are able to form hydrogen bonds with other molecules and cyclodextrins can, like non-cyclic oligosaccharides and polysaccharides, form water-soluble complexes with lipophilic water-insoluble compounds [5].

The Pharmaceutical toxicology of hydroxypropyl betadex has been reviewed and the material was found to be of low toxicity. However, in pure form it may cause eye, skin or respiratory tract irritation.

It has been suggested that hydroxypropyl betadex may have a synergistic toxic effect with, for example, carcinogens, by increasing their solubility and thus bioavailability [2].

β-CD (Betadex) are listed in the European Pharmacopoeia (Ph.Eur.). A monograph for HP-β-CD (Hydroxypropyl-betadex) is available in the Ph.Eur. HP-β-CD is cited in the FDA's list of Inactive Pharmaceutical Ingredients [6].

Included in an injectable preparation licensed in UK for intramuscular or intravenous administration [2].

Supplier: Giusto Faravelli Spa

3.3.1.3 Analytical materials

Purified Water, HPLC grade; Acetonitrile, HPLC grade; Cortexolone 17α-valerate-21-propionate W.S.

3.3.2 Methods

3.3.2.1 Solubility studies

The solubility of CB-03-10 in several media, including those usually used in toxicology studies, was carried out; the goal was to find a vehicle able to solubilize 4.0 mg/mL of CB-03-10.

A description of the main vehicles used in the previous toxicology studies was reported in Table 3.3.

Table 3.3 Vehicles used in previous studies and their composition.

Vehicle	**Composition**
SV17874	0.4% (v/v) Tween 80; 0.5% sodium carboxymethylcellulose and 0.9% benzyl alcohol in 0.9% physiological saline
PEG400-Ethanol-WFI	50% Polyethylene glycol 400, 20% Ethanol, 30% sterile water
DMSO-HP β cyclodextrin-WFI	12.5% DMSO- 35% (w/v in H_2O) 2-hydroxypropyl-β-cyclodextrin

The solubility of saturated solution of CB-03-10 in several media was carried out.

3.3.2.1.1 HPLC Analytical Conditions

See paragraph 2.3.2.3.1.

3.3.2.2 Osmolality, pH and injectability evaluation

Osmolality (measured using a Micro-Osmometer Type 15), pH and injectability (through 26 G needle) were evaluated on CB-03-10 saturated solutions after filtration by 0.45 µm membrane filter.

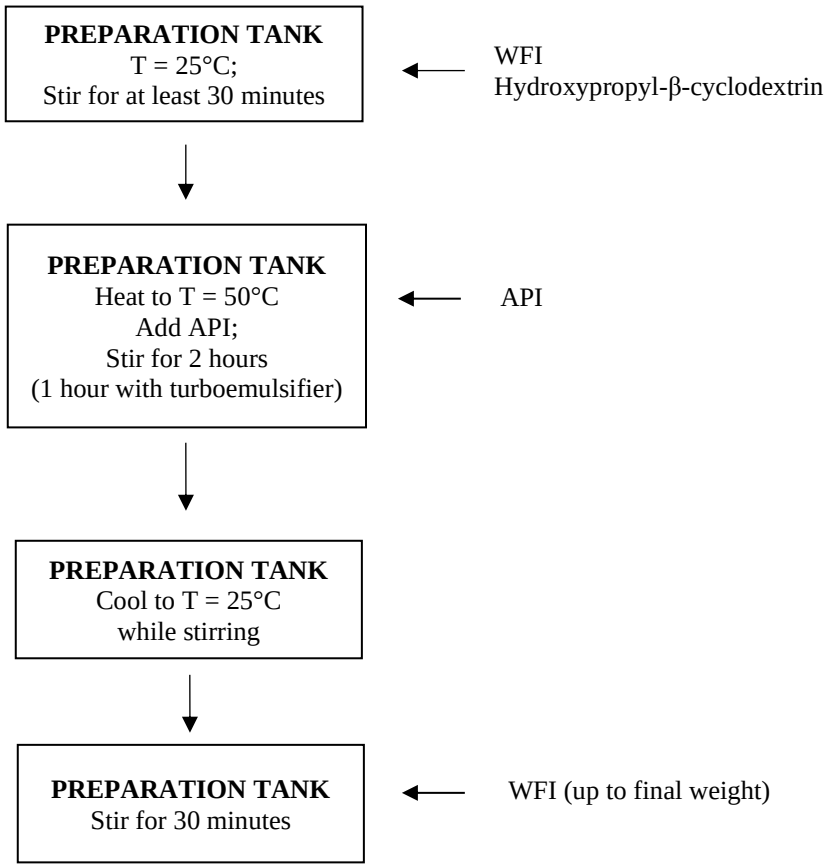
3.3.2.3 Manufacturing Process

3.3.2.3.1 Compounding

The API CB-03-10 needs the presence of Hydroxypropyl-β-cyclodextrin in solution before its addition. Hydroxypropyl-β-cyclodextrin was solubilized in the total amount of WFI and then CB-03-10 was added. In order to accelerate the solubilization of the API, the liquid mixture was heated at 50°C and then cooled at room temperature. The final step was the addition of WFI up to final weight.

The manufacturing process was optimized in order to reduce the manufacturing time and minimize product degradation.

The main studied parameter was the time required to solubilize the API at 50°C. Based on the analytical results, the minimum time required to solubilize the API is 2 hours. For this reason, the selected manufacturing process, related to the compounding phase, is summarized in the following flow-chart:



3.3.2.3.2 Brief description of the manufacturing process

PHASE I (Hydroxypropyl-β-cyclodextrin solubilization)

In the Preparation Tank pour Water for injection. Then add slowly under high speed stirring, Hydroxypropyl-β-cyclodextrin. Stir until complete dissolution is obtained maintaining the temperature around 25°C.

In Process Controls:

- Visual inspection: Clear Solution

PHASE II (Heating and CB-03-10 solubilization)

Heat up to 50°C and then add slowly under high speed stirring, CB-03-10. Stir for 2 hours (1 hour with turboemulsifier) maintaining the temperature around 50°C.

In Process Controls:

- Visual inspection: Clear Solution

PHASE III (cooling phase)

Cool to 25°C while stirring.

In Process Controls:

- Visual inspection: Clear Solution

FINAL WEIGHT

Add water for injection up to final weight.

In Process Controls:

- CB-03-10 assay and purity
- Visual inspection: Clear Solution
- pH: 5-8
- Density: for information only
- Viscosity: for information only

3.3.2.3.3 Primary packaging

Container: Type I Borosilicate neutral glass 10cc Vial, h 53.6 mm, Ø mouth: 20 mm, Ø bottom: 25.4 mm

Stopper: butyl rubber stopper Ø 20 mm + aluminium flip-off cap. Ø 20 mm

Filling volume: 10.5 mL

3.3.2.4 Sterilization

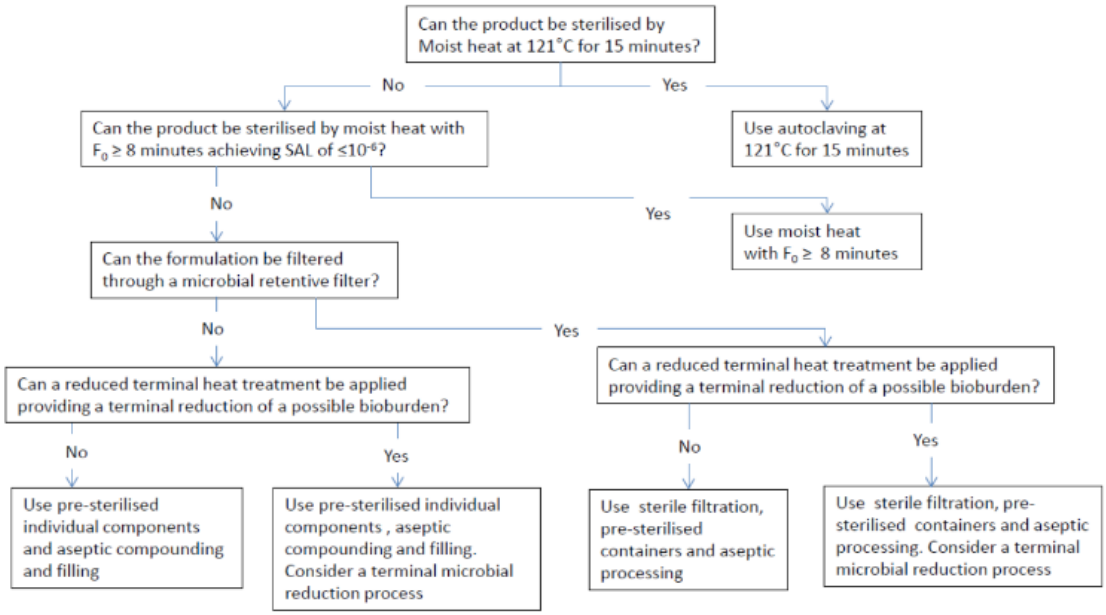


Figure 3.2 Decision tree for sterilization choice for aqueous product [7].

According to European Medicines Agency Guideline, Sterility is a critical quality attribute for all sterile products. Sterility of the medicinal product cannot be assured by testing, it needs to be assured by the use of a suitable and validated manufacturing process. Sterility is dependent on several factors such as the bioburden of the formulation components, the sterilization procedure, the integrity of the container closure system, and in the case of aseptic processing, the use of satisfactory aseptic technique. Terminal sterilization is preferred to sterilization by filtration and/or aseptic processing because it provides a sterility assurance level (SAL) that is possible to calculate, validate and control, and thus incorporates a safety margin.

3.3.2.4.1 Choice of the type of filter

The first step was to select the suitable filter for the parenteral formulation.
The tests have been carried out in collaboration with Sartorius. The aim of the study was to find a sterilizing grade filter able to treat a maximum batch size of 100 L.
The filterability trials were performed using a pressure tank in constant pressure mode. The pressure and the amount of filtrate have been recorded by a pressure transducer and a balance in relation to time. The corresponding data was transmitted to a computer.

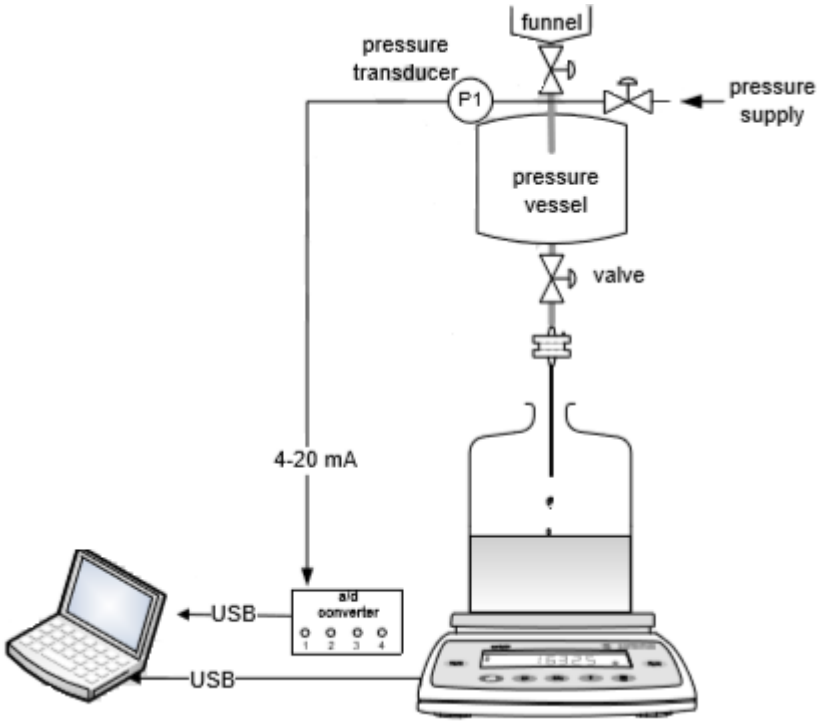


Figure 3.3 Schematic representation of the test**.**

Two different kinds of filter, previously selected based on the composition of our parenteral formulation, were tested:

- Sartopore® 2: sterilizing grade and Mycoplasma retentive pharmaceutical filter, with high total throughput and flow rate performance in a broad range of applications with broad chemical compatibility.
- Sartolon®: sterilizing grade pharmaceutical filter with nylon membrane with broad chemical compatibility.

The above filters were tested using SartoScale 25 device. SartoScale 25 single-use filters are the ideal tool for screening of filter materials in bio-pharmaceutical development and process optimization. Their unique design and small surface area permits to carry out trials with lowest volume.
Consistency of results is assured by using the identical filter material including supportive fleeces as process scale elements.

Test Condition: 20-25°C and 40°C, 1 bar.

The targeted filtration volume was taken into account for the selection of the filter type and filter area.

3.3.2.4.2 Autoclave treatment

Three different autoclave cycles were performed on the selected parenteral formulation:

- 121°C for 15 minutes
- 100°C for 30 minutes
- 100°C for 15 minutes

3.3.2.5 Analytical procedures for the selected parenteral formulation

3.3.2.5.1 CB-03-10 identification (HPLC)

Perform the test during the assay, using the same sample preparation.
The retention time of the main peak in the sample solution chromatogram corresponds to that of the standard solution.

3.3.2.5.2 CB-03-10 assay (HPLC)

Analytical Conditions

Reagents
Purified Water, HPLC grade;
Acetonitrile, HPLC grade;

Chromatographic conditions

Instrumentation:	High Performance Liquid Chromatograph equipped with autosampler, pump, UV detector and PC workstation for data acquisition and analysis.
Column:	Zorbax Extend C18 1.8 µm, 100 x 2.1 mm or equivalent
Detector wavelength:	240 nm
Flow rate:	0.6 mL/minute
Injection volume:	4 µl
Column temperature:	35° C
Mobile phase:	Phase A: water Phase B: acetonitrile Mixed according to gradient program reported in Table 3.4:

Table 3.4 Gradient program.

Time	Phase A	Phase B
0	60	40
0.5	60	40
7	30	70
8	30	70
8.1	60	40
10	60	40

Standard Solution and Control Standard Solution

In a 100 ml volumetric flask weigh accurately 20 mg of Cortexolone 17α-valerate-21-propionate W.S., dissolve in acetonitrile and bring to volume with the same solvent.

Filter through a 0.2 µm membrane filter before injection. (Standard Solution: Cortexolone 17α-valerate-21-propionate theoretical concentration: 0.2 mg/ml).

Sample preparation

Prepare a Sample Solution containing Cortexolone 17α-valerate-21-propionate 0.18 mg/mL: on the basis of the theoretical concentration of the formulation, in a 20 ml volumetric flask weigh accurately 1 g of Cortexolone 17α-valerate, 21propionate 0.36 % w/w solution for injection, dissolve in acetonitrile and bring to volume with the same solvent.

Sonicate for 10 minutes and stir on a magnetic stirrer for 4 hours.

Filter through a 0.2 µm cellulose membrane filter prior to injection.

System suitability test

Analyse the Standard solution and record the chromatograms.

Verify that the System Suitability Test criteria are fulfilled:

1) The USP tailing of Cortexolone 17α-valerate-21-propionate, calculated by the following formula, is not more than 1.5.

$$T = \frac{W_{0.05}}{2f}$$

where:

$W_{0.05}$ = width of the peak at 5% height;

f = distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

2) The Relative Standard deviation (RSD) of Cortexolone 17α-valerate-21-propionate peak areas, calculated on 5 replicate injections of the *Standard Solution,* is not more than 2.0%.

3) Analyse the *Control Standard Solution* at least after the injections of the Standard Solution (n=5) and at the end of HPLC sequence.
Cortexolone 17α-valerate-21-propionate recovery must range between 98.0% and 102.0%.

Calculation

Inject the Sample Solution. Record the chromatograms and calculate the peak areas.

Calculate the Cortexolone 17α-valerate,21propionate amount in the sample, as % w/w, using the following formula:

$$\text{Cortexolone 17α-valerate,21propionate (\% w/w)} = \frac{A_{SMP} \times C_S \times D_{SMP} \times 100}{A_S \times W_{SMP}}$$

where:

A_{SMP} = Cortexolone 17α-valerate-21-propionate peak area in Sample Solution chromatogram;

C_S = concentration (mg/mL) of Cortexolone 17α-valerate, 21propionate in Standard solution, corrected by its potency %;

D_{SMP} = sample dilution (mL);

A_S = mean peak area (n=5) of Cortexolone 17α-valerate, 21propionate in Standard Solution chromatograms;

W_{SMP} = sample weight (mg);

3.3.2.5.3 Related substances

Analytical conditions

Reagents

Purified Water, HPLC grade;

Acetonitrile, HPLC grade

Chromatographic conditions

Instrumentation: High Performance Liquid Chromatograph equipped with autosampler, pump, UV detector, and PC workstation for data acquisition and analysis

Column: Zorbax Extend C18 1.8 µm, 100 x 2.1 mm or equivalent

Detector wavelength: 240 nm

Flow rate: 0.6 mL/minute

Injection volume: 4 µl

Column temperature: 35° C

Mobile phase: Phase A: water

Phase B: acetonitrile

Mixed according to gradient program reported in Table 3.5:

Table 3.5 Gradient program.

Time	Phase A	Phase B
0	65	35
1	65	35
18	30	70
21	30	70
22	20	80
24	20	80
26	65	35
28	65	35

Resolution Solution

In a 50 ml volumetric flask weigh accurately 5 mg of Cortexolone, 5 mg of Cortexolone 17α-propionate W.S, 5 mg of Cortexolone 21-acetate, 5 mg of Cortexolone 21-propionate, 5 mg of Cortexolone17α-valerate, 5 mg of Cortexolone 21-valerate and 5 mg of Cortexolone 17α-valerate-21-propionate then dissolve and dilute to final volume with acetonitrile.

Transfer 1.0 ml of this solution into a 20 ml volumetric flask, dilute to volume with acetonitrile (Resolution Solution). Filter the solution by a 0.2 μm membrane filter.

Standard Solution and Control Standard Solution

In a 100 ml volumetric flask weigh accurately 20 mg of Cortexolone 17α-valerate-21-propionate W.S., then dissolve and bring to volume with acetonitrile.

Transfer 2.5 ml of this solution in a 100 ml volumetric flask, bring to volume with acetonitrile (Standard Solution, Cortexolone 17α- valerate,21-propionate theoretical concentration: 0.005 mg/ml).

Filter the solution by a 0.2 μm membrane filter.

Sample preparation

Prepare a Sample Solution containing Cortexolone 17α-valerate-21-propionate 0.36 mg/mL on the basis of the theoretical concentration of the formulation.

Stir on a magnetic stirrer and filter through a 0.2 μm cellulose membrane filter prior to injection.

System Suitability Test

Chromatograph the Resolution Solution and the Standard Solution (6 replicate injections) and record the chromatograms.

Verify that the System Suitability Test criteria are fulfilled:

1) The resolution (R) between Cortexolone 21-acetate and Cortexolone 17α-propionate peaks in the chromatogram of the *Resolution Solution* is not less than (NLT) 2.0. Peak resolution is calculated by the following formula:

$$R = \frac{1.18\,(t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

where:

t_R = retention time measured from time of injection to time of elution of peak maximum;

w_h = peak widths at half-height.

Identify the known impurities using the impurities retention time in chromatogram of the *Resolution Solution.*

2) The symmetry factor (A_s) of Cortexolone 17α-valerate-21-propionate peak in the chromatogram of *Standard Solution* is not more than (NMT) 1.5 and is calculated by the following equation:

$$A_s = \frac{w_{0.05}}{2f}$$

where:

$W_{0.05}$ = width of peak at one-twentieth of the peak height;

F = distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

3) System precision: the Relative Standard Deviation (R.S.D.) of Cortexolone 17α-valerate-21-propionate peak areas calculated on 6 replicate injections of the Standard Solution is not more than 5%.

4) Analyse the *Control Standard Solution* at least after the injections of the Standard Solution (n=6) and at the end of HPLC sequence.
Cortexolone 17α-valerate-21-propionate recovery must range between 90.0% and 110.0%.

Calculations

Inject the *Standard* and the *Sample Solution*. Record the chromatograms and measure the peak areas.

Calculate each related substance amount as % respect to Cortexolone 17α-valerate-21-propionate content by the following formula:

$$\text{Related substance amount (\%)} = \frac{A_I \times C_S \times D_{SMP} \times 10000}{A_S \times W_{SMP} \times C}$$

where:

A_I = peak area of each related substance in the *Sample Solution* chromatogram;

C_S = concentration of Cortexolone 17α-valerate-21-propionate W.S. in *Standard Solution*, corrected by its potency (mg/ml);

D_{SMP} = sample dilution (ml);

A_S = Cortexolone 17α-valerate-21-propionate mean peak area (n=6) in *Standard Solution* chromatograms;

W_{SMP} = sample weight (mg);

C = labelled concentration of Cortexolone 17α-valerate-21-propionate in the solution.

LOD = 0.01 %

LOQ = 0.03 %

3.3.2.5.4 Viscosity

Instrument:	Viscosimeter Brookfield LVDV III Ultra;
Spindle:	Enhanced UL Adapter (spindle n° 00)
Rotation rate:	60 rpm;
Sample temperature:	25° C.

Analyze about 30 ml of solution under exam and record three readings.
Express the result as mean of data collected.

3.3.2.5.5 Relative density

Perform the test at 25°C according to the current European Pharmacopoeia (2.2.5).

3.3.2.5.6 Sterility

The sterility test method was validated by Eurofins using batch 7248/1 (Beginning, Middle and End).

The other batches were analyzed using the validated method in order to test the sterility of the product.

Perform the test as directed under sterility tests, USP <71> by membrane filtration method.

3.3.2.5.7 Bacterial endotoxins

The evaluation of the bacterial endotoxin was performed and validated by Eurofins using batch 7248/1 (Beginning, Middle and End). The bacterial endotoxin was evaluated by LAL Kinetic Test.

The other batches were analyzed using the validated method in order to test the endotoxin of the product.

Perform the test as directed under Bacterial Endotoxins, USP <85>, by the kinetic turbidimetric method.

3.3.2.6 Stability study on CB-03-10 0.36% solution for injection

The storage conditions tested during stability for CB-03-10 solution for injection are reported in Table 3.6:

Table 3.6 Stability studies storage conditions.

Batch	Storage conditions
7142	25°C/60%RH; 40°C/75%RH; 5°C
7169	25°C/60%RH
7248	25°C/60%RH; 40°C/75%RH; 30°C/65%RH 5°C
7292	5°C
7328	5°C

3.4 Results and discussion

3.4.1 Vehicle selection

3.4.1.1 Solubility results

The solubility results are summarized in Table 3.7:

Table 3.7 Solubility results.

Vehicle/medium	**CB-03-10 solubility (mg/mL) Average ± sd (n=3)**
Physiological saline	$2.5 \cdot 10^{-04} \pm 0.1 \cdot 10^{-04}$
0.5% sodium carboxymethylcellulose high viscosity in WFI	$3.4 \cdot 10^{-04} \pm 0.2 \cdot 10^{-04}$
DMSO	263.18±0.53
43.75% 2-hydroxypropyl-β-cyclodextrin + 12.5% DMSO	1.57±0.01
50% w/v 2-hydroxypropyl-β-cyclodextrin in Physiological saline	3.96±0.01
40% w/v 2-hydroxypropyl-β-cyclodextrin in Physiological saline	3.72±0.01
Y 0.22 um 50% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	4.00±0.01
50% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	6.20±0.23
40% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	5.15±0.17
35% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	3.84±0.22
30% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	3.28±0.15
20% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	1.51±0.18
10% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	0.55±0.11

3.4.1.2 Osmolality, pH and injectability evaluation

The results of osmolality, pH and injectability evaluation are reported in Table 3.8.

Table 3.8 pH, osmolality and injectability results obtained on CB-03-10 saturated solutions after filtration by 0.45 µm membrane filter.

Vehicle/medium saturated	pH	Osmolality (mOsm/kg) Average ± sd (n=3)	Injectability (26 G)
50% w/v 2-hydroxypropyl-β-cyclodextrin in normal saline	6.25	**	Yes
40% w/v 2-hydroxypropyl-β-cyclodextrin in normal saline	6.14	+1155±6	Yes
50% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	7.29	+1440±35	Yes
40% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	7.61	+824±25	Yes
35% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	6.33	+597±10	Yes
30% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	5.84	+433±11	Yes
20% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	5.26	+213±3	Yes
10% w/v 2-hydroxypropyl-β-cyclodextrin in WFI	5.31	+87±1	Yes
50% Polyethylene glycol 400, 20% Ethanol, 30% WFI	/	**	/

*** impossible to determine because the solution is not frozen at -6.2°C*

The osmolality of SV17874 vehicle was evaluated as is, without CB-03-10.

Table 3.9 Osmolality results obtained for SV17874 vehicle as is (without CB-03-10).

Vehicle/medium	Osmolality (mOsm/kg) Average ± sd (n=3)
SV17874(with sodium carboxymethylcellulose medium viscosity)	+394±1
SV17874 (with sodium carboxymethylcellulose low viscosity)	+398±1

3.4.1.3 Discussion

- **SV17874 vehicle**

The solubility of CB-03-10 in SV17874 vehicle (independently of the sodium carboxymethylcellulose viscosity) is very low and for this reason the SV17874 vehicle is not optimal for solubilize CB-03-10.

- **50% Polyethylene glycol 400, 20% Ethanol, 30% WFI**

The solubility of CB-03-10 in these three excipients is ethanol>PEG400>WFI.

Based on toxicity data, it is clear that it is not possible to increase the concentration of Ethanol or PEG400 (in order to increase CB-03-10 solubility) because 20% (for ethanol) and 50% (for PEG400) is the maximum levels for IV administration. for this reason, it is impossible to modify this vehicle in order to increase the API solubility and 1.87 mg/mL is the maximum solubility obtained in the optimal ratio of these three excipients.

- **2-hydroxypropyl-β-cyclodextrin vehicles**

Based on solubility results it is possible to relate the solubility of CB-03-10 with 2-hydroxypropyl-β-cyclodextrin concentration in WFI (Figure 3.4).

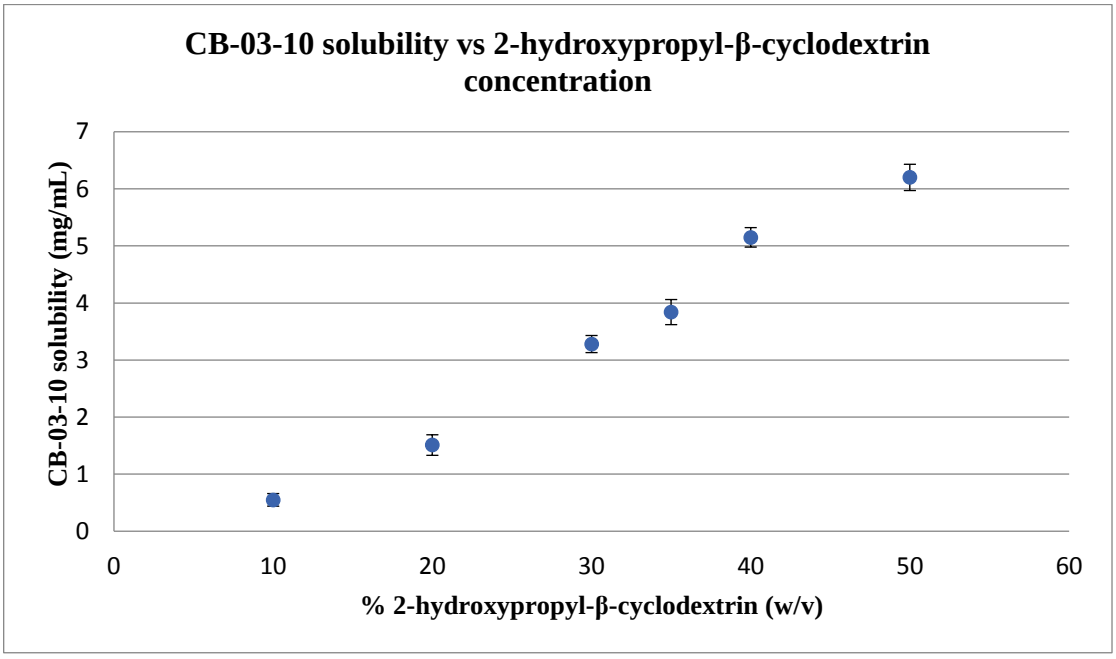


Figure 3.4 CB-03-10 solubility vs 2-hydroxypropyl-β-cyclodextrin concentration in WFI (mean value±sd; n=3).

CB-03-10 solubility increases with 2-hydroxypropyl-β-cyclodextrin concentration.

CB-03-10 solubility in 35% w/v 2-hydroxypropyl-β-cyclodextrin in WFI is about twice of solubility in 50% Polyethylene glycol 400, 20% Ethanol, 30% WFI (3.87 mg/mL vs 1.87 mg/mL).

The osmolality of CB-03-10 solution in 35% w/v 2-hydroxypropyl-β-cyclodextrin in WFI is +597 mOsm/kg (hypertonic solution). For drug products intended for intravenous or intravascular injection, the recommended upper limit should be generally controlled under 1000 mOsm/kg for small-volume injections (<100 mL) and 500 mOsm/kg for large-volume injections (>100 mL). Several options are available for minimization of hypertonicity-induced pain upon product administration [8].

For this stage an osmolality upper limit of +600 mOsm/kg was fixed as formulation selection criterion.

The vehicle containing 35% w/v 2-hydroxypropyl-β-cyclodextrin in WFI was selected.

3.4.2 Vehicle optimization

The concentration of cyclodextrin was then adjusted in order to shift from w/v to w/w. During this step the concentration of 2-hydroxypropyl-β-cyclodextrin was corrected for loss on drying.

A solution containing 35% w/w of 2-hydroxypropyl-β-cyclodextrin in WFI was prepared but it was excluded because of its high osmolality (+775 mOsm/kg).

Therefore, based on loss on drying, a solution of 32% w/w of 2-hydroxypropyl-β-cyclodextrin in WFI was tested and provided positive results in terms of osmolality, pH and solubility.

Table 3.10 Results obtained for 32% w/w 2-hydroxypropyl-β-cyclodextrin in WFI.

Vehicle/medium saturated	**CB-03-10 salubility (mg/ml) Average ± sd (n=3)**	**pH**	**Osmolality (mOsm/kg) Average ± sd (n=3)**	**Injectability (26 G)**
32% w/w 2-hydroxypropyl-β-cyclodextrin in WFI	4.84±0.36	6.7	+598±11	Yes

3.4.3 Selected parenteral formulation

Based on solubility data, pH value and injectability the optimal vehicle for parenteral administration is 32% w/w 2-hydroxypropyl-β-cyclodextrin in WFI.

The only disadvantage of this vehicle could be its hyperosmolality but according to literature[2] the recommended upper limit should be generally controlled under 1000 mOsm/kg for small-volume injections (<100 mL) and 500 mOsm/kg for large-volume injections (>100 mL) [1].

Therefore, the formulation (Table 3.11) containing 32% w/w of 2-hydroxypropyl-β-cyclodextrin and 0.36 % w/w of API (lower than solubility) was selected for its pH near to neutrality, osmolality under 600 mOsm/kg, and injectability through 26 G needle (Table 3.12).

Table 3.11 Qualitative - quantitative composition of CB-03-10 0.36% w/w solution for injection

Component(s)	**Reference to standard**	**Function**	**% w/w**
CB-03-10 (cortexolone 17α-valerate-21- propionate)	Internal monograph	Active ingredient	0.36
Kleptose HPB parenteral grade (Hydroxypropyl-β-cyclodextrin)	USP	Solubilizer	32.00
Water for injection	USP	Solvent	67.64

Table 3.12 Characteristics of the selected formulation.

CQA	**Target**	**Achievement**
pH	Physiological	6.7
Osmolality	<600 mOsm/kg[8]	+598 mOsm/kg
Injectability	Injectable through 26G needle	Injectable through 26G needle

3.4.4 Sterilization

3.4.4.1 Choice of the type of filter results

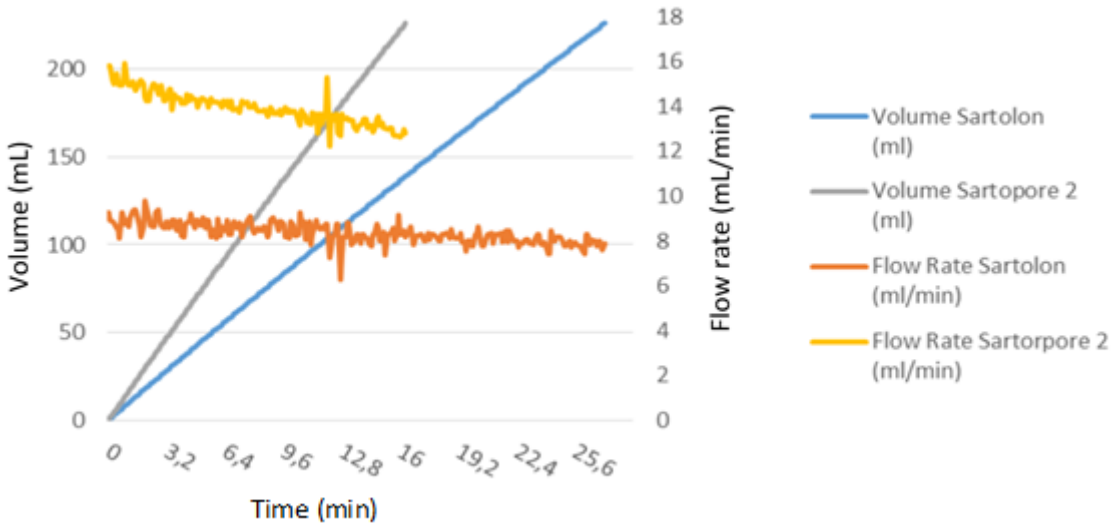


Figure 3.5 Sartopore 2 vs Sartolon

The results obtained for the tested filters were similar but Sartopore 2 was chosen because it allowed to obtain the same filtration performance in about 16 minutes instead of about 25 minutes, value obtained using Sartolon filter.

Taking into account the targeted filtration volume of 100 L per batch size, the required filtration area is ≈0.08 m^2 for Sartopore 2, therefore Sartopore 2 MidiCaps size 8 (5445307H8-SS, 0.1m^2) was selected.

3.4.4.2 Autoclave treatment

Terminal sterilization by autoclave was performed on CB-03-10 solution for injection, but the analysis of the product after a treatment at 121°C for 15 minutes allowed to a total impurities value higher than 2% (2.9%±0.2%; n=3) with an increment of 2.2% respect to the formulation before autoclave treatment (0.7%±0.2%; n=3). For this reason, the sample was tested with two further cycles: 100°C for 30 minutes and 100°C for 15 minutes.

The results obtained after these two autoclave treatments are reported in Table 3.13:

Table 3.13 Results obtained after two different autoclave treatment.

Autoclave treatment	**Total impurities value (%) Mean value ± ds (n=3)**
100°C; 15 minutes	1.02±0.05
100°C; 30 minutes	1.21±0.06

The best results in terms of purity have been obtained after the cycle at 100°C for 15 minutes. This kind of cycle is not a sterilizing treatment and, for this reason, the preclinical batches were sterilized using a sterilizing filtration followed by an autoclave treatment at 100°C for 15 minutes. This combination allowed to obtain a sterile product with an acceptable endotoxin limit.

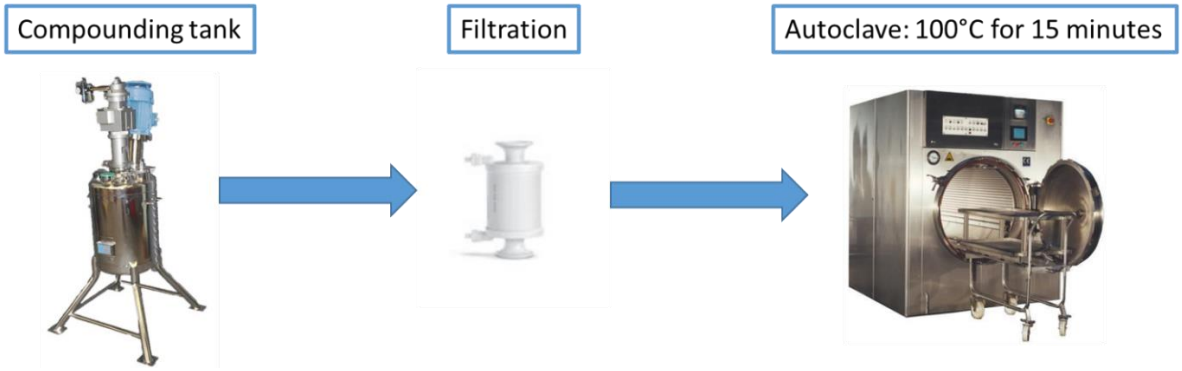


Figure 3.6 Schematic representation of the process.

During a future industrial scale, it will be evaluated if the treatment at 100°C for 15 minutes is necessary or if sterilizing filtration with aseptic filling is enough to obtain a sterile product.

3.4.5 Release test on the final product

Table 3.14 Release test on the final product and preliminary acceptance criteria

Test		**Preliminary Acceptance Criteria/Tentative specification**
Appearance		Colourless Clear Solution
Cortexolone 17α-valerate-21-propionate identification (HPLC)		Positive
Cortexolone 17α-valerate-21-propionate Assay (HPLC)		90.0-110.0%
Related substances (HPLC)	Cortexolone	NMT 1%
	Cortexolone 21-propionate	NMT 1%
	Cortexolone 17α-valerate	NMT 3%
	Cortexolone 21-valerate	NMT 3%
	Any other related substance	NMT 0.5%
	Total related substances	NMT 8%
Density at 25°C		For information only
Viscosity at 25°C		For information only
Osmolality		≤ 600 mOsm/kg
Sterility		Sterile
Lal Test		≤ 1.0 EU/mL

3.4.6 Summary of the development batches

The summary of the development batches is provided below in Table 3.15:

Table 3.15 Summary of the development batches.

Formulation Batch Number	API Batch number	Batch Size (Kg of bulk preparation)	Batch use
7142	TF10216 (2122V00 0021615)	4 kg	Efficacy study Ephor-11-17. MTD and 14-day DRF Study 527/16
7169	TF10216 (2122V00 0021615)	6 kg	MTD and 14-day DRF Study 527/16
7248	TF10216 (2122V00 0021615)	18 kg	Sterility and endotoxin methods validation Preliminary toxicity Study E0263
7292	TF10316 (2122V00 0031615)	3 kg	Efficacy Study Ephor-02-18 Toxicity Study in rats A3173
7328	TF10417 (2122V00 0031715)	21 kg	Toxicity Studies in dogs A3171 and A3172

3.4.7 Parenteral formulation batch analysis

Table 3.16 Batch analysis results.

Test	**Provisional Specifications**	**Batch n°**				
		7142	**7169**	**7248**	**7292**	**7328**
Chemical Analysis						
Appearance	Colourless Clear Solution	Colourless Clear Solution	Colourless Clear Solution	Colourless Clear Solution	Colourless Clear Solution	Colourless Clear Solution
CB-03-10 (HPLC) Identification	Positive	Positive	Positive	Positive	Positive	Positive
CB-03-10 (HPLC) Assay	90.0 – 110.0% of labelled claim	99.2%	99.7%	99.4%	99.7%	97.8%
Related Substances:						
Cortexolone	NMT 1 %	<0.025%	<0.025%	<0.01%	<0.01%	<0.03%
Cortexolone-21-propionate	NMT 1%	0.25%	0.22%	0.23%	0.34%	0.26%
Cortexolone-17α-valerate	NMT 3%	0.25%	0.21%	0.21%	0.21%	0.57%
Cortexolone-21-valerate	NMT 3%	0.07%	0.12%	0.08%	0.17%	0.12%
Any other related substances	NMT 0.5%	0.13%	0.11%	0.07%	0.08%	0.11%
Total impurities	NMT 8%	1.04%	1.01%	0.82%	0.93%	1.44%
Density at 25°C	g/mL for information only	1.1148 g/mL	1.1141 g/mL	1.1140 g/mL	1.1128 g/mL	1.1140 g/mL
Viscosity at 25°C	cP for information only	5.70 cP	5.72 cP	5.61 cP	5.51 cP	5.38 cP
Osmolality	≤ 600 mOsm/kg	598 mOsm/kg	599 mOsm/kg	593 mOsm/kg	598 mOsm/kg	595 mOsm/kg
Microbiological Analysis						
Sterility	Sterile	n.a.	n.a.	Sterile	Sterile	Sterile
LAL test	≤ 1.0 EU/mL	n.a.	n.a.	≤ 1.0 EU/mL	≤ 1.0 EU/mL	≤ 1.0 EU/mL

n.a.: data not available because the test was not in force at time of testing. NMT: not more than

3.4.8 Stability studies

The results of the stability study performed on CB-03-10 0.36% solution for injection batch 7428 are reported in Tables 3.17-3.20.

Table 3.17 Stability studies results obtained for CB-03-10 0.36% solution for injection batch 7248 stored at 25°C/60%RH.

Test	**Appearance**	**CB-03-10 identification**	**CB-03-10 Assay**		**Related substances**						**Viscosity**	**Density**	**Microbiological analysis**	
					Cortexolone	**Cortexolone 21-propionate**	**Cortexolone 17α-valerate**	**Cortexolone 21-valerate**	**Any other related substance**	**Total related substances**			**Sterility test**	**LAL test**
Provisional specification	Clear solution	Positive	0.324-0.396% w/w	90.0 - 110.0% vs labeled	% for information only						cP for information only	g/cm^3 for information only	Complies to sterility test	<1.0 EU/ml
					NMT 1%	NMT 1%	NMT 3%	NMT 3%	NMT 0.5%	NMT 8%				
Initial values	Complies	Complies	0.358	99.4	<0.01	0.23	0.21	0.08	0.07	0.82	5.61	1.1140	Complie	<0.5
25°C/60% RH														
01 Month	Complies	Complies	0.356	98.9	<0.01	0.27	0.25	0.18	0.07	0.86	--	1.1139	--	--
03 Months	Complies	Complies	0.358	99.4	<0.01	0.25	0.32	0.06	0.07	0.78	--	1.1131	--	--
06 Months	Complies	Complies	0.358	99.4	<0.01	0.25	0.46	0.11	0.07	1.01	5.53	1.1140	Complie	<0.5
09 Months	Complies	Complies	0.356	98.9	<0.01	0.30	0.62	0.10	0.07	1.21	--	1.1129	--	--
12 Months	Complies	Complies	0.356	98.9	<0.01	0.32	0.77	0.19	0.07	1.57	5.43	1.1137	Complie	<0.5
18 Months	Complies	Complies	0.358	99.4	<0.01	0.32	1.03	0.09	0.11	1.73	5.32	1.1136	--	--

--: not performed per protocol;

Table 3.18 Stability studies results obtained for CB-03-10 0.36% solution for injection batch 7248 stored at 40°C/75%RH.

Test	**Appearance**	**CB-03-10 identification**	**CB-03-10 Assay**		**Related substances**						**Viscosity**	**Density**	**Microbiological analysis**	
					Cortexolone	**Cortexolone 21-propionate**	**Cortexolone 17α-valerate**	**Cortexolone 21-valerate**	**Any other related substance**	**Total related substances**			**Sterility test**	**LAL test**
Provisional specification	Clear solution	Positive	0.324-0.396% w/w	90.0 - 110.0% vs labeled	% for information only						cP for information only	g/cm^3 for information only	Complies to sterility test	<1.0 EU/ml
					NMT 1%	NMT 1%	NMT 3%	NMT 3%	NMT 0.5%	NMT 8%				
Initial values	Complies	Complies	0.358	99.4	<0.01	0.23	0.21	0.08	0.07	0.82	5.61	1.1140	Complies	<0.5
40°C/75% RH														
01 Month	Complies	Complies	0.353	98.1	<0.01	0.30	0.44	0.17	0.08	1.15	--	1.1132	--	--
03 Months	Complies	Complies	0.351	97.5	<0.01	0.32	1.01	0.09	0.07	1.59	--	1.1140	--	--

--: not performed per protocol;

Table 3.19 Stability studies results obtained for CB-03-10 0.36% solution for injection batch 7248 stored at 30°C/65%RH.

Test	**Appearance**	**CB-03-10 identification**	**CB-03-10 Assay**		**Related substances**						**Viscosity**	**Density**	**Microbiological analysis**	
					Cortexolone	**Cortexolone 21-propionate**	**Cortexolone 17α-valerate**	**Cortexolone 21-valerate**	**Any other related substance**	**Total related substances**			**Sterility test**	**LAL test**
Provisional specification	Clear solution	Positive	0.324-0.396% w/w	90.0 - 110.0% vs labeled	% for information only						cP for information only	g/cm^3 for information only	Complies to sterility test	<1.0 EU/ml
					NMT 1%	NMT 1%	NMT 3%	NMT 3%	NMT 0.5%	NMT 8%				
Initial values	Complies	Complies	0.358	99.4	<0.01	0.23	0.21	0.08	0.07	0.82	5.61	1.1140	Complie	<0.5
30°C/65% RH														
03 Months	Complies	Complies	0.356	98.9	<0.01	0.28	0.42	0.06	0.07	0.91	--	1.1139	--	--
06 Months	Complies	Complies	0.357	99.2	<0.01	0.28	0.67	0.12	0.07	1.30	5.53	1.1141	Complie	<0.5
09 Months	Complies	Complies	0.353	98.1	<0.01	0.33	1.02	0.11	0.07	1.67	--	1.1135	--	--
12 Months	Complies	Complies	0.351	97.5	<0.01	0.34	1.22	0.21	0.07	2.04	5.46	1.1141	Complie	0.8

--: not performed per protocol;

Table 3.20 Stability studies results obtained for CB-03-10 0.36% solution for injection batch 7248 stored at 5°C.

Test	**Appearance**	**CB-03-10 identification**	**CB-03-10 Assay**		**Related substances**						**Viscosity**	**Density**	**Microbiological analysis**	
					Cortexolone	**Cortexolone 21-propionate**	**Cortexolone 17*α*-valerate**	**Cortexolone 21-valerate**	**Any other related substance**	**Total related substances**			**Sterility test**	**LAL test**
Provisional specification	Clear solution	Positive	0.324-0.396% w/w	90.0 - 110.0% vs labeled	% for information only						cP for information only	g/cm^3 for information only	Complies to sterility test	<1.0 EU/ml
					NMT 1%	NMT 1%	NMT 3%	NMT 3%	NMT 0.5%	NMT 8%				
Initial values	Complies	Complies	0.358	99.4	<0.01	0.23	0.21	0.08	0.07	0.82	5.61	1.1140	Complie	<0.5
5°C														
01 Month	Complies	Complies	0.354	98.3	<0.01	0.25	0.21	0.17	0.08	0.91	--	1.1130	--	--
03 Months	Complies	Complies	0.357	99.2	<0.01	0.24	0.20	0.06	0.06	0.65	--	1.1137	--	--
06 Months	Complies	Complies	0.360	100.0	<0.01	0.22	0.21	0.11	0.07	0.70	5.56	1.1136	Complie	<0.5
09 Months	Complies	Complies	0.357	99.2	<0.01	0.25	0.24	0.09	0.07	0.72	--	1.1137	--	--
12 Months	Complies	Complies	0.358	99.4	<0.01	0.23	0.23	0.15	0.06	0.83	5.46	1.1139	Complie	<0.5
18 Months	Complies	Complies	0.364	101.1	<0.01	0.24	0.25	0.07	0.06	0.75	5.28	1.1137	--	--

--: not performed per protocol;

The stability results show an increment of the impurities, especially the hydrolysis impurities, but always within the preliminary specifications. The assay value is always within the limits as well as the microbiological results. No substantial variations have been observed for both density and viscosity parameters as function of time.

The results suggest that the best storage condition is +2/+8°C.

3.5 Conclusions

The aim of the formulation study was reached since it was obtained a liquid solution of 2-hydroxypropyl-β-cyclodextrin able to solubilize the target dose of CB-03-10. The selected formulation meets the target requirements: physiological pH, osmolality lower than 600 mOsm/kg and injectability through 26G needle.

The stability results suggest that the formulation is stable and that the best storage conditions are +2/+8°C.

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