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CICLO XXXIII

Buccal route for fast multimodal analgesia for the treatment of moderate-to-severe acute pain: feasibility and enhancement strategies

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1 INTRODUCTION

1.1 DEFINITION OF PAIN

During the last years, the concept of pain has evolved from a mono-dimensional to a multi-dimensional phenomenon involving sensorial, cognitive and affective characteristics. The International Association for the Study of Pain (IASP) described pain as an unpleasant sensation associated with tissue damages. Pain not only involves the anatomical pathways of painful stimulus but it also has an emotional impact evoked by this experience. Thus, pain is a subjective phenomenon because the emotional and sensory components may evoke different degrees of suffering[1].

Pain is classified depending on the anatomic location, etiology, frequency and source of origin while the duration of painful stimulus is the traditional categorization. Acute pain is relatively brief phenomenon, last less than 30 days and typically caused by trauma or specific disease. It is a defense system but it can turn into a pathological condition if it is maintained for long time. Pain that lasts more than six month usually is defined chronic. Both acute and chronic pain can be debilitating and may be associated with psychological and physical impairment[2]. The main reason to classify type of pain is to facilitate the communication with patients in order to ensure the safest and most efficacious treatment.

1.2 GENERAL PAIN PATHWAY

The subjective experience of pain can be summarised in four step process: transduction, transmission, modulation and perception (**Figure 1**).

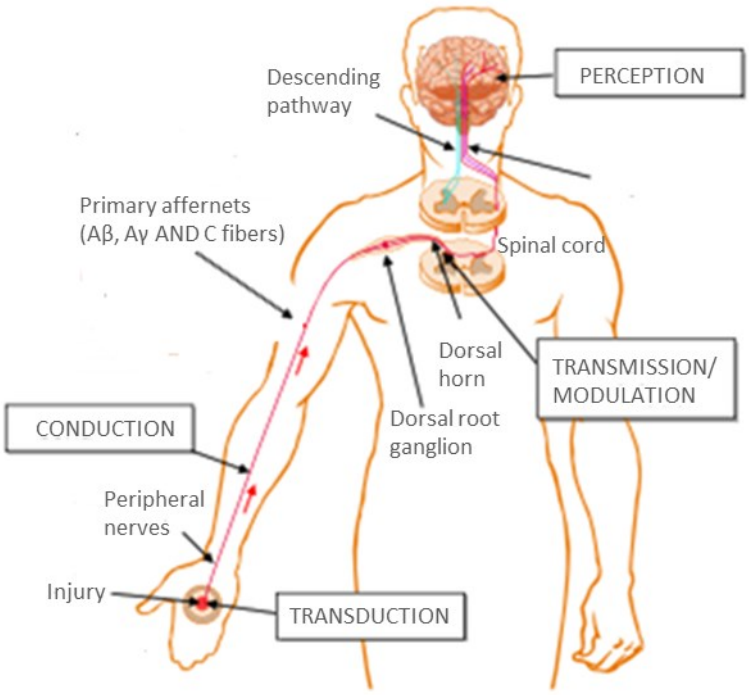


Figure 1: Schematic phases of painful transmission. (Adapted from[3])

Damaging stimuli from periphery of the tissues are converted by sensory cells, called nociceptors, in action potentials which are conducted along the primary afferent neurons to the dorsal horn of spinal cord. Here modulation take place and can be excitatory or inhibitory, increasing or decreasing pain. The last step of the ascending pathway involves generation of affective and cognitive responses to the painful stimulus, leading to pain perception.

1.2.1 Nociceptive Pain

Nociceptors are located at the free nerve endings of the primary afferent neuron. They can be found in the periphery of the body and can be activated from different stimuli. Three types of nociceptors exist:

- **Mechanical** nociceptors, detects pricking pain
- **Thermal** nociceptors
- **Polymodal** nociceptors, detects mechanical, thermal and chemical stimuli.

Nociceptive information is transmitted to the spinal cord by at least two type of primary afferent neurons called Aδ and C fibers. Signals from mechanical and thermal

nociceptors are transmitted to the dorsal horn of the spinal cord predominantly by **Aδ fibres.** These myelinated fibres have a low threshold and a fast conduction speed. Polymodal nociceptors transmit their signals into the dorsal horn through **C fibres**. C fibres are unmyelinated and lead to poor localisation of pain with a slow conduction speed. The peripheral activation of first order neurons release glutamate in the synapse activating glutamate receptors on the second order neuron, called spino-thalamic neuron. At this level, the presence of opiate pre-synaptic receptors can modulate the ability to transmit nociceptive information. The stimulation of μ-opioids receptors causes the inhibition of the synapses and the lacking of neurotransmitters provides a partial refractoriness to damaging stimuli. Spinothalamic tract transmits sensorial information up to the thalamus where second order neurons make synapsis with the third order fibers that directly innervates the somatosensory cortex[4].

1.2.2 Modulation of pain and gate control theory

Two main mechanisms modulate the pain transmission at the spinal cord level. The *"gate control theory"* was proposed by Melzack and Wall in 1960s. The theory suggested that the stimulation of non-noxious **Aβ fibers** activate the inhibitory interneurons in the dorsal horn. The "gate is closed and pain transmission is blocked". On the other hand the activation of **C** and **Aδ neurons** inhibit interneurons and painful stimulus take place. This physiological modulation allow the second order neuron to discriminate painful or non-painful stimulus. The concept of the gate control theory is that non-painful input closes the gates to painful signalling, which results in prevention of the pain sensation from traveling to the CNS. Pain is also modulated by descending pathways that project nerves from the midbrain to the dorsal horn and inhibit pain transmission. These pathways are monoaminergic, utilising noradrenaline and serotonin. These neurotransmitters act directly on their receptor located on pre- and post-synaptic first order neurons and inhibits synaptic transmission. The persistence of monoamines in the synapse explains the analgesic effect, while their activity is stopped by the re-uptake. The same pathway also activate spinal interneurons that release enkephalin. This endogenous opioid inhibits synaptic pain transmission at the spinal level, through the activation of μ-receptors of the spinothalamic neuron. Together, these mechanisms

(**Figure 2**) reduce the firing of action potentials in the second order neuron, blocking the transmission of pain signals.

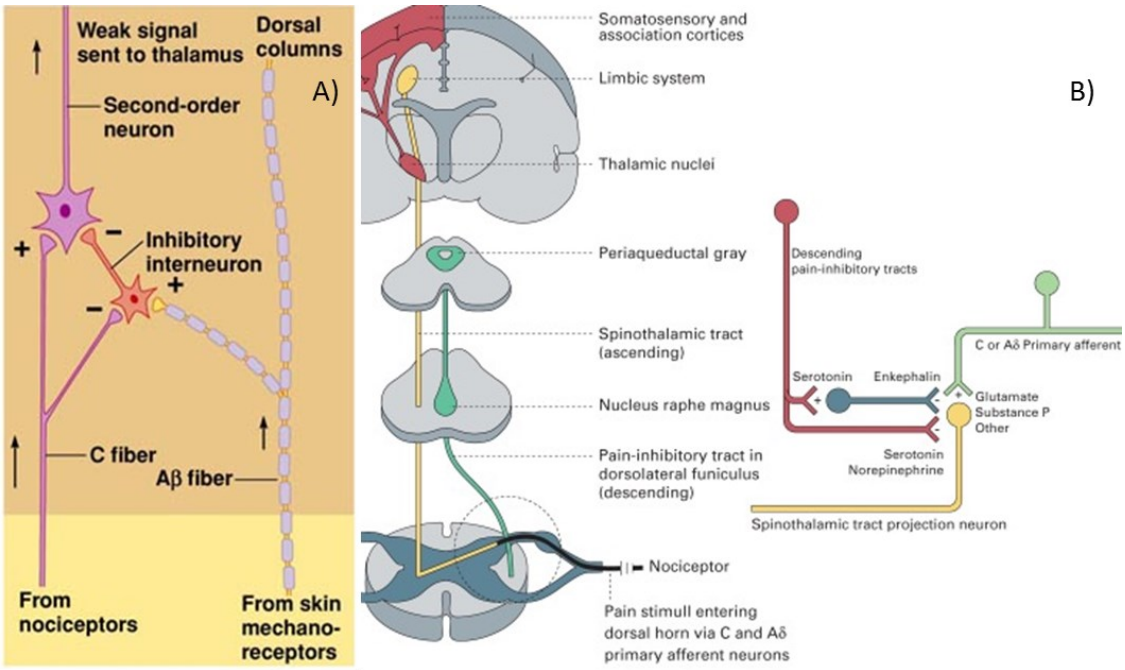


Figure 2: Schematic representation of the gate control theory (panel A) and descending pathway releasing serotonine and noradrenaline (panel B). (adapted from[5])

1.2.3 Neuropathic pain

Neuropathic pain is caused by damage to nerves in the central or peripheral nervous system. Peripheral neuropathy alters the electrical properties of sensory nerves, which unbalance pain modulation systems such as inhibitory interneurons and descending control pathways of the midbrain level[6]. The transmission of sensory signals and disinhibition or facilitation mechanisms of pain are altered at the level of the dorsal horn neurons in the spinal cord. These changes shift the sensory pathways to a state of hyperexcitability and this result in the perception of pain even in the presence of non-noxious stimuli.

1.2.4 Inflammatory pain

Inflammation is a natural biological response produced by the tissues as a reaction to the harmful stimuli in order to initiate the tissue repairing process. Tissue injuries results

in the release of inflammatory mediators including histamine, substance P, bradykinin, prostaglandins (**Figure 3**).

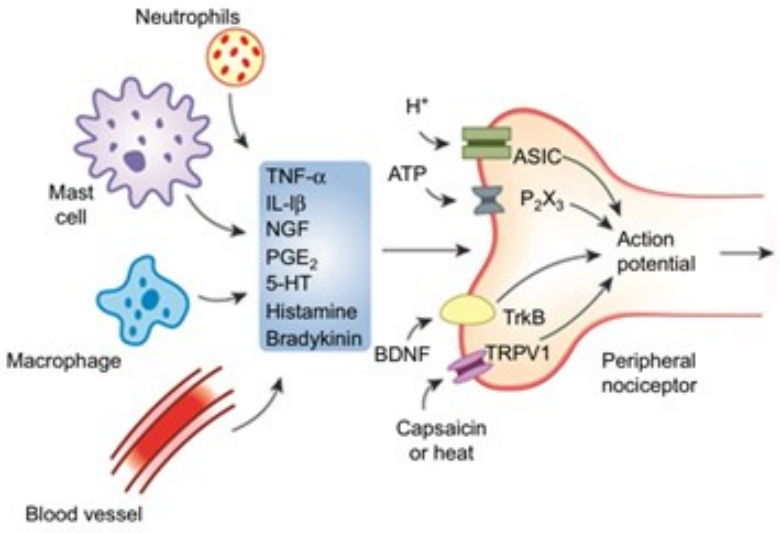


Figure 3: Schematic representation of peripheral sensitization (adapted from[7])

All these inflammatory mediators bind their respective receptors that are located on the postsynaptic primary neurons. For instance, the involvement of Bradykinin in inflammation and pain was demonstrated in in vivo models. It was found that Selective Bradykinin B2 receptors antagonist blocks inflammatory hyperalgesia in animal models[8]. The same effect was shown for several cytokines like IL-6 and IL-1 because intradermal injections of these substances causes peripheral sensitization[9].

Thus, they trigger intercellular signalling pathways in the peripheral terminals making those afferents to become more responsive to painful stimuli.

1.3 GUIDELINES FOR PAIN MANAGEMENT

The World Health Organization (WHO) in 1986 proposed a three-stage analgesic ladder as standard for pain treatment. It was a stepwise strategy to treat pain based on the use of drugs with growing pharmacological activity, from non-opioid to opioid drugs, depending on the intensity of pain. Although at first it is referred to the treatment of cancer pain, it was also used in the past to treat acute and chronic non cancer pain[10].

The real efficacy of this application was considered as a controversial issue in the past. WHO suggested a non-opioid medication as the first step, which include acetaminophen or NSAIDs, and move up step by step to weak or strong opioid drugs if pain has not solved. Codeine, dihydrocodeine, tramadol, dextropropoxyphene of the second step were prescribed to control mild pain while morphine, methadone, oxycodone, buprenorphine, fentanyl to control moderate to severe pain. Moreover, additional substances called "adjuvants" should be used in order to calm fears and anxiety[5]. About this approach, the use of assessment scales play a key role in order to establish different degrees of pain. Pain measurements help determine the severity, type, and duration of the pain, and are used to determine the most appropriate analgesic regimen and its effectiveness. Pain intensity is commonly assessed using different scales like Numerical Rating Scale (NRS), Verbal Rating Scale (VRS) or Faces Pain Scale-Revised (FPS-R) for children with poor language skills. NRS is the most common type of scale used in the hospital and the most accepted by patients[11].

During the last two decade WHO ladder has been revised and criticized[12]. The knowledge acquired on the multifactorial nature of pain represented the driving force for a modern multitarget approach that combine drugs belonging to different steps of the traditional WHO ladder.

1.3.1 Multimodal analgesia

Multimodal analgesia involves the administration of a combination of drugs with different mechanism of action on pain pathway. Recently the multimodal approach has been studied for its potential to provide additive or synergic analgesic effects. In this sense the simultaneous administration of two drugs acting on different level of pain pathway could require lower dosages of each drug minimizing side effect, often dose-related. This aspect provides a better pain relief and efficacy/safety ratio resulting in a better compliance for patients[3]. Nausea, dizziness and somnolence were the most common related side effect in pain treatment. For these reasons nowadays several organizations like WHO and the American Pain Society (APS) recommend analgesic combinations to treat pain. In clinical practice drugs combination is becoming more common, especially in fixed and ad hoc dose ratio. The use of acetaminophen with weak

opioid is a well established approach in fixed analgesic combination. Acetaminophen plus codeine is a traditional analgesics combination that allows improvements to treat pain compared to acetaminophen alone[13].

The success of multimodal analgesia depends on the proper selection of both drugs as well as the most appropriate ratio for their combination. Fixed dose ratio can produce reproducible clinical effect compared to personalized therapy. However, the fixed dose ratio must be designed in order to find the appropriate dose ratio which provide the best analgesic effect[14].

Dex-ketoprofen/tramadol provide different analgesic efficacy in different dose ratio, however the 1:3 ratio (25 mg : 75 mg) provided the best pain relief in a dental pain model.

The therapeutic mixture of two enantiomers of tramadol has a dual mechanism of action, opioid and non-opioid, that gives tramadol (**Figure 4**) an important role in the management of pain: plus enantiomer showing higher activity for μ-receptors while minus enantiomer for noradrenaline re-uptake.

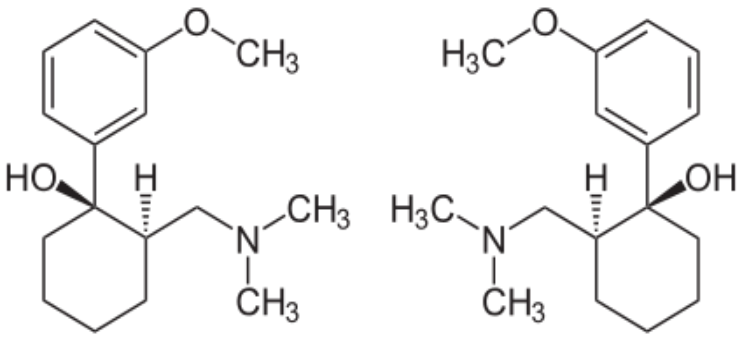


Figure 4: Structure of tramadol

The double mechanism of tramadol acts synergistically on the descending inhibitory pathway, resulting in the modulation of second order neuron. The main metabolite of tramadol, O-Desmetyl-tramadol (M1), acts on μ-receptors located in the posterior horns of spinal cord where there is the synapsis between the nociceptive neurons and the spinothalamic ones. The stimulation of μ-opioids receptors causes the pre-synaptic

inhibition of glutamate release and hyperpolarization of the spinothalamic neurons. The second mechanism of tramadol inhibits the re-uptake of monoamines released by the descending tracts at the dorsal horn level: noradrenaline and serotonin are accumulated in synapsis and their analgesic effect persists.

Ketoprofen (**Figure 5**) stops prostanoid synthesis by the inhibition of Cyclooxygenase 1 and 2 isoforms (COX-1 ; COX-2) and has anti-inflammatory effects in the peripheral tissues. COX-1 occurs constantly in the body and it has a cytoprotective effects for the gastric mucosa facilitating the production of mucus and bicarbonates while COX-2 is manly induced by inflammation.

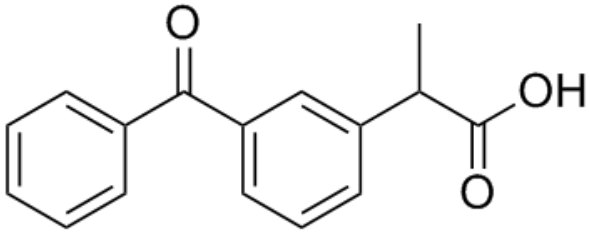


Figure 5: Structure of ketoprofen

The inhibition of prostanoid synthesis mediated by COX-2 isotype has an important role in the pain management, because sometimes peripheral receptors become extremely sensitive to noxious stimuli in presence of inflammation mediators, like prostaglandins[5].

1.4 ADMINISTRATION ROUTES IN PAIN MANAGEMENT

The administration routes influence the absorption rate as well as the analgesic onset of action. The preferential administration routes for the treatment of pain are the oral and intravenous route. The oral route appears to be the most suitable to prescribe a therapy which is simple to be administered and easy to be managed. Nevertheless the slow onset of action and the side effect typical of opioid make this route less attractive for patients. Analgesics given by the intravenous route bypass the absorption phase and provide a fast analgesic effect. However injections is limited to high soluble drugs and it

needs trained personnel. The intramuscular route is not recommended for pain management, because intramuscular injections are often painful and drug absorption is variable and unpredictable. On the contrary subcutaneous administration is associated with a higher and predictable bioavailability and it is commonly used in palliative care. Transdermal drug delivery system provide administration without pumps or needles. Fentanyl is available in a transdermal drug delivery system that provides continuous opioid administration for patients with chronic pain but it is not indicated for the treatment of breakthrough pain. For transdermal delivery, the adherence of the patch on the skin for long time may be problematic. Among non-invasive administration route pulmonary, nasal and oral transmucosal are considered to be the ones leading to a fast onset of action.

1.4.1 Buccal administration

Buccal drug delivery is becoming an attractive alternative to conventional administration routes as it offers many advantages in terms of bioavailability of drugs and fast onset of action. Buccal tissues is strongly supplied by a dense capillary vessels network and drugs can reach systemic circulation directly, bypassing gastric degradation and hepatic first pass metabolism. These aspects play a key role in pain management because buccal administration allow lower drug doses and induces less related side effect during pain treatment. Drugs having high first pass metabolism, low aqueous solubility, pH gastric instability and low molecular weight can be delivered via buccal route.

The oral mucosa has a complex stratified structure and differences in thickness, function, and vascularity are observed according to the region of the entire oral cavity. The oral cavity consists of masticatory, lining and specialized mucosae that comprise 25%, 60% and 15% respectively of the total surface area of the oral mucosa. The first is characterized by a keratinized epithelium and located in regions particularly sensitive to mastication stress (hard palate and gum). The mucus membrane lining, on the other hand, is subject to less masticatory shears and consequently has a non-keratinized epithelium. The mucosa of the dorsum of the tongue is a specialized mucosa and the surface presents papillae and receptor. Thus three different approaches characterize

drug delivery via the oral mucosa including sublingual delivery across mucosa lining the floor of the mouth, buccal delivery via mucosa lining the cheeks, both for systemic delivery, and not site specific into the oral cavity for the treatment of oral local delivery of drugs. The selection of the optimal approach depends on the site of action (local or systemic) or on the anatomical differences which exists in different sites of the oral mucosa. The surface of the oral mucosa consists in a differentiated stratified squamous epithelium and its thickness changes depending on the site in the oral cavity from 600 µm for buccal mucosa to 200 µm for gingival and palatal tissue. The epithelium is separated from the underlying connective vascularized layer, called lamina propria, by the basal membrane that provides adherence between them. The connective tissues is not an effective barrier because its structure is insufficiently dense to prevent penetration of drugs[15].

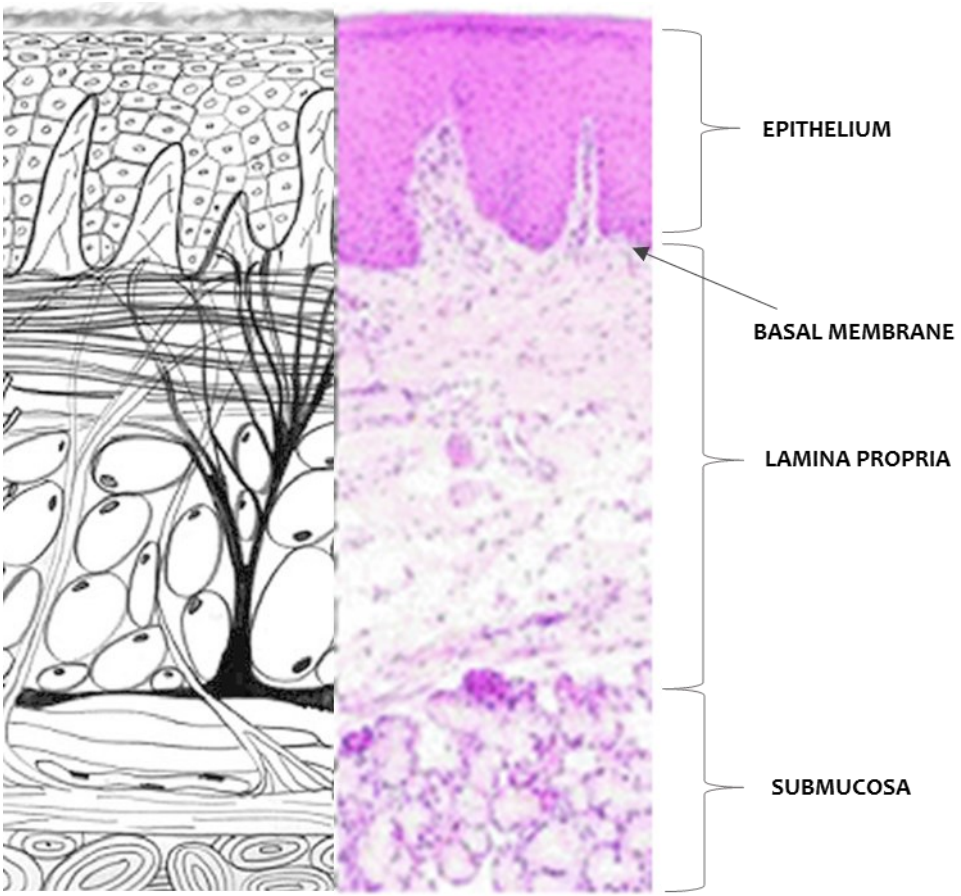


Figure 6: Structure of the oral mucosa. (adapted from[15])

Scientific literature suggests that the permeability of the oral mucosa depends on the thickness of the epithelium, the presence of keratin and the nature of intercellular lipidic material, which is extruded by membrane-coating granules (MCGs) in the epithelium. Squire and colleagues demonstrated the involvement of the nature of extruded intercellular lipid as barrier through their extraction. The treatment of oral mucosa with chloroform/methanol negatively affects the barrier properties and enhance the water permeability of the tissue[16].

MCGs are small cytoplasmic granules probably derived from the Golgi region of the prickle cells layer of the mucosa. During differentiation of epithelial cells, membrane-coating granules become evident when cells reach the upper third of the epithelium. Organelles migrate to the superficial regions, fuse with cell membrane and discharge their lipid contents into the intercellular space of the upper epithelium. The chemical nature of these lipids was verified in various regions of porcine oral cavity after extraction, separation and identification by thin-layer chromatography. Keratinized palatal and gingival mucosae contained high quantities of ceramides and cholesterol, and a low proportion of cholesterol esters and glychosylceramides. In contrast, the non-keratinized buccal and sublingual mucosae, contained higher quantities of the more polar phospholipids, and glycosylceramides. Evidences supporting the location of the epithelial barrier was carried out by using Horseradish peroxidase as a tracer[17].

1.4.2 Absorption pathway

Substances permeates across the oral mucosa by endocytosis, carrier mediated diffusion or passive diffusion. The first two mechanisms are poorly represented for buccal absorption and permeation occurs mainly by passive diffusion. About passive diffusion, trans-cellular and paracellular route seems to be involved for permeation across the mucosa.

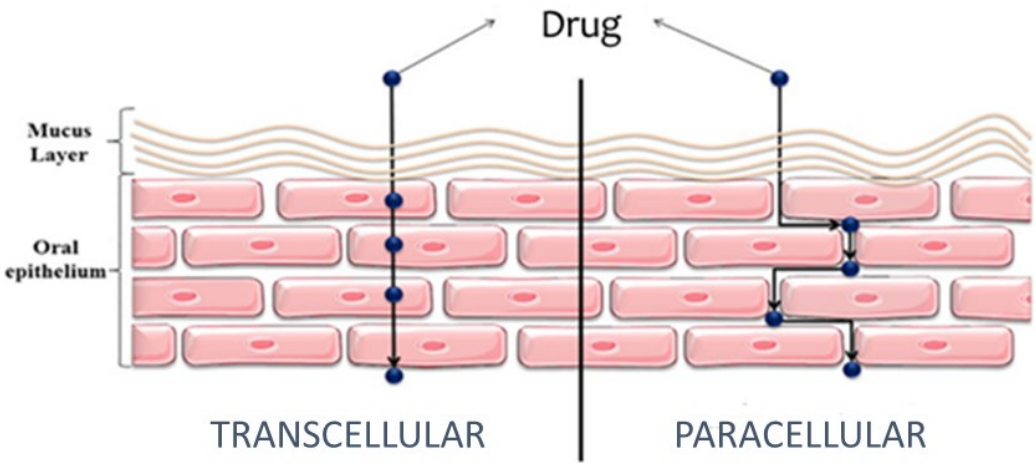


Figure 7: Schematic representation of penetration pathway through oral mucosa

Drugs can permeate using both routes simultaneously however permeation through the mucosa depends on physical and chemical properties of drugs such as the partition coefficient, lipophilicity and molecular weight. Hydrophilic permeants mainly permeate via paracellular route because the lipophilic nature of epithelial cells membrane represents a barrier for permeation. While intercellular spaces and cytoplasm are hydrophilic and provide a permeability barrier to lipophilic drugs.

Within the paracellular spaces, there are two other pathways. The hydrophobic route involves the intercellular lipid domains, while the hydrophilic polar pathway is related to the aqueous channels, which are associated with the polar head groups of the cells membrane lipids.

1.5 PERMEATION ENHANCEMENT

Drug delivery through the buccal mucosa is limited by the barrier nature of the epithelium, the small area available for absorption and swallowing. However different strategies were explored in order to enhance the absorption of drugs including the use of chemical penetration enhancers, mucoadhesive substances, prodrugs, and physical methods, such for example iontophoresis. The incorporation of chemical compounds in a buccal formulation is a typical approach to overcame the barrier properties of mucosa. A chemical penetration enhancer is a substance that increase temporarily the

membrane permeability or the absorption rate of drugs, without damaging tissues and causing toxicity. Tissues should revert into their normal activity and structure after the removal of the chemicals. In addition to their enhancing activity, chemicals can exhibits very important aesthetic effects for buccal application improving odour and texture.

Since permeation is mainly related to a passive diffusion process, according to Fick's first law of diffusion, the permeation of compounds can be enhanced by increasing the diffusivity of the permeant or its partition through the tissue. Chemical enhancers are classified according to their structures or mechanism of action. Thus the main penetration pathway is the intercellular route, chemical enhancers should modify intercellular lipid of mucosa. Two main categories were described depending on their interaction with the intercellular lipid: the partitioning of chemicals into the hydrophobic tails or the lipid extraction cause a fluidization of ceramide acyl chain and facilitate permeation of drugs.

- Surfactants and Bile salts

Surfactants are amphiphilic molecules that have a hydrophobic chain and a hydrophilic polar head group. The major drawback is their toxicity and irritancy, which define their limited use in vivo as chemical enhancer. They can be classified according to the nature of their polar group into: anionic, cationic, zwitterionic and non-ionic. Above the critical micellar concentration they can aggregate into micelles that influence their effectiveness as chemical penetration enhancers. In vitro permeation of caffeine through porcine buccal mucosa was enhanced at concentrations only above the CMC at of Sodium dodecyl sulphate (SDS). The major enhancement mechanisms consist in lipid extraction and perturbation of lipid packing mediated by the formation of micelles. However, it seems that SDS doesn't increase the permeation of more lipophilic molecule, thus demonstrating that surfactants only enhance the permeability of compounds which traverse the buccal mucosa via the paracellular route[18].

Bile salts are endogenous steroidal molecules that belong to the ionic surfactants class of chemical enhancers. The enhancing mechanism includes the extraction of lipids with a consequent membrane fluidization or the production of reverse micelles in the membrane creating aqueous channels in the phospholipid bilayer. It was demonstrated

that sodium glycocholate enhance the buccal transport of compounds in ionized form and not the more lipophilic bases which permeate through different pathways[19]. In this sense surfactants, enhance the buccal permeability of permeants depending on their lipophilicity and preferential permeation pathway.

- Terpenes

Terpenes and terpenoids are components of plant essential oils, with a pleasant taste and odour. They are widely used as flavouring agent or fragrance in oral and topical dosage formulations respectively. Terpenes are classified depending on the number of isoprene units (C_5H_8) joined together from head to tail, and the functional groups presente in the chemical structure: alcohol, ethers, esters and ketones. “Food and Drug Administration” (FDA) and “Flavour and extract Manufactures Association” (FEMA) classify terpenes as Generally Recognized as Safe (GRAS) substances[20]. Although few studies suggested terpenes as chemical enhancers for transbuccal application, they were greatly studied for transdermal delivery. These molecules were reported to have high percutaneous enhancement abilities for both hydrophilic and lipophilic model drugs[20]. In skin application different the efficacy as enhancer is influenced by different factors like concentration, size and lipophilicity. At low doses, a direct relationship exists between concentration and enhancement activity[21] while amphiphilic structures, such as nerolidol, are optimal to modify the organized lipid packing in stratum corneum facilitating permeation across the tissues[22].

- Fatty acids

Fatty acids are endogenous molecules and primary lipidic components cell membranes. For long time fatty acid was studied as penetration enhancers for transdermal and topical application. It was found that their effectiveness depends on the structure, chain length and degree of saturation. About the structure, among unsaturated fatty acids, cis conformation destabilize lipid packing more than trans fatty acids and a chain length of C10 to C12 was optimal to obtain a good enhancing effect. While saturated fatty acids need a longer chain length to be effective enhancers for skin application[23]. More recently the use of fatty acids as possible promoters of macromolecules have received

more attention for buccal application. In this case the better efficacy of less lipophilic fatty acids was explained by the different nature of the buccal mucosa compared to the skin[24].

- Chitosan

Chitosan is a natural positively charged polymer of N-acetyl-glucosamine and d-glucosamine produced by partial deacetylation of chitin. It was used for different applications such as tissue engineering but its natural mucoadhesive property makes it a great candidate to enhance permeation of drugs via transmucosal route. Different theories explain bio-adhesion including electrostatic, diffusion or wetting mechanisms. However the muco-adhesion process generally consists in a contact phase followed by the consolidation stage that involves molecular interactions between mucus components and materials. In the case of chitosan, being positively charged, muco-adhesion depends on the electrostatic forces with the negatively charged residues of glycoproteins called mucins on the mucosal surface. This feature increases the retention time on the mucosal surface thus facilitating the permeation of drugs through the tissue. Chitosan also can interacts with cell membranes and modifies the tight-junction between cells, thus facilitating permeation of hydrophilic drugs via paracellular route[25]. However this mechanism is poorly represented the in buccal mucosa because of the absence of tight-junctions and the enhancing activity of chitosan is mainly related to its muco-adhesive property[26].

2 AIM OF THE WORK

The general aim of this thesis was to evaluate the feasibility of buccal route, for the simultaneous administration of analgesics used for multimodal pain therapy in order to obtain a faster onset of the analgesic effect. For this work, the combination of tramadol and ketoprofen was selected. In particular the objectives of the work were:

1. Set up and validation of an analytical method for the simultaneous quantification of the drugs;
2. Passive permeation studies in order to evaluate the permeation parameters of the two drugs across pig esophageal epithelium as model for buccal mucosa;
3. Evaluation of different chemical enhancers (fatty acids, terpenes, bile salts) and their associations on the permeation of tramadol and ketoprofen, considering co-administration conditions.
4. Development and evaluation in vitro of a buccal hydrogel formulation containing both drugs and eventually a permeation enhancer.

3 EXPERIMENTAL

3.1 MATERIALS

Acetic acid (VWR Chemicals, Radnor, USA)

Acetonitrile HPLC analytical grade (Sigma-Aldrich, St. Luis, MO, USA)

Capric acid (Sigma-Aldrich, St. Louis, USA)

Chitosan 80-200 KDa, 96% deacetilation (Chitochemicals)

Eucalyptol (ThermoFischer , Kandel, Germany).

Hydroxy-propil-beta-cyclodextrin (Sigma-Aldrich, Steinheim, Germany)

Ketoprofen (SIMS-Società Italiana Medicinali Scandicci, Italia)

Limonene (ThermoFischer , Kandel, Germany).

Menthol (ACEF, Fiorenzuola D'Arda, Italy)

Nerolidol (ThermoFischer , Kandel, Germany).

Octanol (Alfa Aesar, Karlsruhe, Germany)

Phosphoric acid 85% (Acef S.p.A, Fiorenzuola d'Arda, Piacenza, Italia)

Poloxamer 407 - Lutrol F127 (BASF, Ludwigshafen, Germany)

Potassium dihydrogen phosphate (H_2KPO_4) (Fluka Chemika, Buchs, Switzerland)

Sodium chloride (Acef S.p.A., Fiorenzuola, d'Arda, Piacenza, Italia)

Sodium Hydrogen Phosphate (Na_2HPO_4) (Alfa Aesar, Karlsruhe, Germany)

Sodium Hydroxide anhydrous pellets (Carlo Erba, Chaussée du Vexin, France)

Tramadol hydrochloride (Contramal® solution for injection, Grunenthal GmbH, Stolberg, Germany)

Transcutol (Gattefossè, Saint-Priest, France)

Ultrapure water (ELGA LabWater, PURELAB ® Pulse system)

3.2 DETERMINATION OF KETOPROFEN APPARENT PARTITION COEFFICIENT

PBS buffer at different pH values and octanol (1.1, v/v) were mutually saturated overnight and then separated. 5 ml of pre-saturated buffer solution, containing known concentration of drug (100 µg/ml of ketoprofen) were equilibrated with an equal volume of pre-saturated octanol and shaken for 1 h at room temperature. After separating the two phases, the concentration of ketoprofen in the aqueous phase, in the absence or presence of tramadol, was determined by HPLC.

3.3 PERMEATION EXPERIMENTS

3.3.1 Tissue preparation

Pig esophageal epithelium was prepared according to the protocol performed by Diaz Del Consuelo and collaborators [27]. Fresh esophagi were obtained from a local abattoir and transported to the laboratory in isotonic buffer (PBS, pH: 7.4). The esophageal mucosa was separated from the outside muscular layer with a scalpel. The mucosa was cut longitudinally and then excised in pieces of adequate dimensions. The epithelium was peeled off from the connective tissue after immersion in ultrapure water for 120 seconds at 60-65 ˚C. The isolated samples were frozen at -20°C until use.

3.3.2 Apparatus and conditions

Permeation experiments were performed in vertical Franz type cells (DISA, Milan, Italy) with a diffusion area of 0.6 cm^2 (**Figure 8)**.

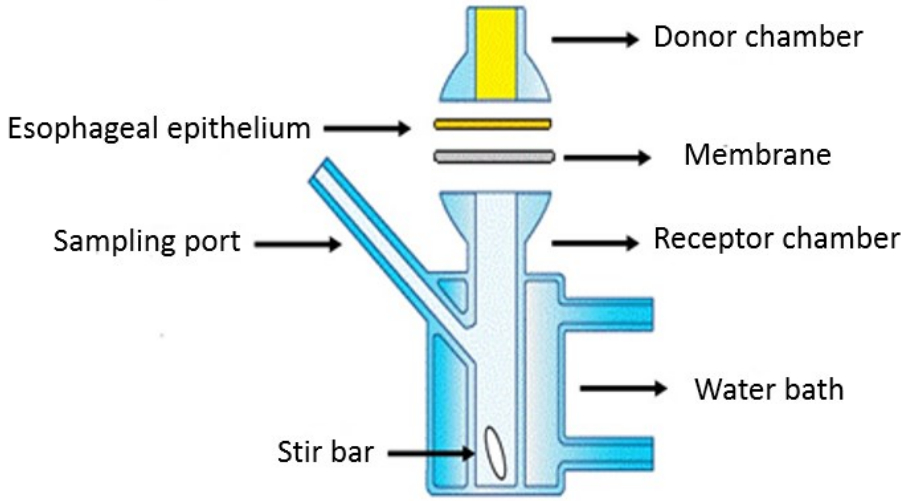


Figure 8: Franz type diffusion cell

Franz type diffusion cell consist in two primary chambers called donor and receptor compartment. Epithelial tissue supported by a regenerated cellulose filter membrane (0.45 µm, pore size) was clamped between the chambers of the diffusion cell. The receptor compartment was filled with about 4 ml of PBS (pH: 7.4),maintained at 37 ± 1˚C and stirred at 300 rpm. Experiments lasted 6 hours and 0.3 ml of receptor solution were sampled hourly, restoring the initial volume with fresh buffer. The amount of drugs permeated was determined by HPLC.

The following conditions were tested:

1. Passive diffusion

The donor compartment was filled with 0.5 ml of tramadol hydrochloride (5 mg/ml) and/or ketoprofen (3 mg/ml) solution in SSF pH 6.8.

2. Pre-treatment with permeation enhancers solution

Pre-treatment of the esophageal epithelium was performed by applying 20 µl ethanol enhancer solution (10% w/v) in correspondence of the available diffusion area. After 1 h pre-treatment in non-occluded condition, 0.5 ml of tramadol hydrochloride (5 mg/ml) and/or ketoprofen (3 mg/ml) solution in SSF pH 6.8 was applied in the donor.

3. Permeation enhancer co-administration

These experiments were performed using either sodium taurocholate (NaTC) or menthol. In the first case, the solution contained NaTC 20 or 100 mM and tramadol hydrochloride (5 mg/ml) and ketoprofen (3 mg/ml) in PBS pH 6.8. In the case of menthol, 0.4% (w/v) of the terpene, tramadol hydrochloride (5 mg/ml) and ketoprofen (3 mg/ml) were dissolved in PBS pH 6.8: Transcutol (3:1, v/v). In both cases, 0.5 ml of donor solution were applied in the donor chamber.

3.3.3 Data analysis

The cumulative amount of drugs permeated was plotted as a function of the time. The transmucosal flux of tramadol and ketoprofen (J, μg/cm²h) was calculated as the slope of the regression line in the steady state interval 2-6 h while the permeability coefficient was calculated in steady state condition as P= J/Cd where Cd is the drug concentration in the donor chamber. The enhancement factor (EF) was calculated as the ratio of flux values obtained in enhanced and passive condition. All data are reported as mean value ± sd. Statistical differences were evaluated by t-test (significance level $p < 0.05$).

3.4 PREPARATION OF POLOXAMER HYDROGEL

Poloxamer hydrogel was prepared by the cold method. Polymer (final concentration 15.5%, w/w) was slowly added to cold PBS pH 6.8 solution at 4°C, maintaining constant stirring until a clear solution was obtained. The obtained solution was stored at 4°C. Hydrogels containing tramadol hydrochloride (5 mg/ml) and ketoprofen (3 mg/ml) were obtained by adding drugs solutions to the preformed gel.

In the case of hydrogel containing xanthan gum 0.25% (w/w), the gum was hydrated in SSF (pH:6.8) under gentle stirring at 4°C. Poloxamer 407 was then added to the cold solution at a final concentration of 15.5% (w/w). Hydrogel was then loaded with drugs as described before.

3.4.1 T sol-gel transition temperature evaluation

Tsol-gel transition temperature was determined by the “magnetic stirrer method”. The poloxamer solution was heated progressively under magnetic stirring. When the

magnetic bar stopped moving due to gelation, the temperature displayed on the external thermo-sensor was registered as the transition temperature.

3.4.2 Viscosity evaluation

Apparent viscosity was measured using a rotational viscometer (FUNGILAB-PRO SERIES) using a TR11 spindle at a fixed speed of 30 rpm corresponding to 7.5 (s^{-1}) shear stress. Viscosity was evaluated as a function of temperature in the range 25-34°C. The transition temperature was extrapolated as the intercept on x-axis of the regression line in the linear portion of the sigmoidal curve obtained[28].

3.4.3 In vitro release studies

In vitro release studies were carried out using the same apparatus and conditions described for the permeation experiments across regenerated cellulose membrane (0.45 µm pore size).

3.5 INSTRUMENTS AND CHROMATOGRAPHIC CONDITIONS

Both drugs were quantified simultaneously by HPLC (FLEXAR ™ PerkinElmer Waltam, MA, USA) using Zorbax C18 column (3.5 µm, 4.6x150 mm, Agilent, Santa Clara, CS,USA). The mobile phase was a mixture of acetonitrile/acetic acid water solution 0.25% (45:55, v/v) pumped at 1 ml/min. The injection volume was 100 µl and UV detector wavelength was set at 280 nm. In these conditions, the retention times of tramadol and ketoprofen were 2 and 3.4 min respectively. Stock solution of ketoprofen (5 mg/ml) was prepared in ethanol 95%. Standard solutions were prepared by appropriate dilutions of tramadol hydrochloride (50 mg/ml stock) and ketoprofen stock solution with PBS at pH 7.4.

The analytical method was validated in terms of selectivity, linearity, precision, accuracy, lower limit of quantification (LLOQ). Calibration curve was performed with 5 concentration levels.

- Selectivity

The selectivity of the method was evaluated by the analysis in the optimized chromatographic conditions, of the receptor solution of a blank experiments (PBS pH 7.4 left in contact in a Franz's cell with the esophageal epithelium for 6 h in absence of

drugs in the donor compartment). The absence of interfering peaks with the same retention time of the two analytes proved the selectivity of the method.

- Linearity

The linearity of the method was verified through the correlation coefficient between analytical peaks areas and the corresponding nominal concentration standards obtained by the regression line analysis.

- Accuracy and precision

Accuracy was expressed as percentage of the relative error (RE%) between the nominal standard concentrations and the corresponding calculated values. Precision was expressed as (CV%) and calculated as the ratio between the standard deviation of each calculated concentrations and its mean.

- Calibration curve

Calibration curve was developed at 7 concentration levels on the basis of concentrations expected in permeation studies in the range 0.44 – 44 µg/ml and 0.5 – 50 µg/ml for tramadol and ketoprofen respectively. Each calibration standard was repeated 5 times.

- LLOQ

LLOQ was calculated as the lowest concentration of the calibration standard that could be determined with acceptable accuracy and precision.

4 RESULTS

Tramadol is a synthetic opioid structurally related to morphine and codeine. It has 2 chiral centres in the cyclohexane ring and consequently 4 stereoisomers are possible (1R,2R; 1S,2S; 1R,2S; 1S,2R;). However it is mostly available in hydrochloride salt form as racemic mixture of RR and SS also designed as (+) and (–) enantiomers. The salt form is actively soluble in water and ethanol. It has a pKa value of 9.41 and the Log partition coefficient is 1.35 at pH:7.

Ketoprofen is a non-selective non-steroidal anti-inflammatory drug (NSAID) of propionic acid family. The asymmetric carbon of the drug generate two enantiomers, S-(+)-enantiomer known as dex-ketoprofen that is reported to reduce inflammation and pain (cit) and R-(-)- enantiomer is used as toothpaste additive to prevent periodontal disease[14]. Ketoprofen is a week acid molecule (pKa: 4.39)[29] that belongs to the class II of Biopharmaceutics Classification System (BCS) with a low water solubility and good permeability. Ketoprofen solubility in pure water at ambient temperature is reported as 0.010 mg/ml[30] and the logarithm of partition coefficient (Log P n-octanol/water) near to 3.0 is an optimal value to facilitate the absorption phase. Among NSAID's commonly used, ketoprofen has a low molecular M.W. (260 Dalton) compared to diclofenac (325 dalton) and piroxicam (330 dalton)[31]. Buccal administration of tramadol or ketoprofen have been poorly investigated. For tramadol, with exception of the paper of Zhang[32] that reported the tramadol bioavailability after administration via different routes in rats, few works concerning the transbuccal administration are available. In a paper of 2012, tramadol permeation across chicken pouch mucosa was studied from hydrogels containing microspheres loaded with the drug[33]. In 2017, Li and collaborators studied in vivo the analgesic effect produced in rats by the administration of buccal films loaded with tramadol[34]. Buccal administration of ketoprofen has been studied for the local treatment of different inflammatory conditions of the oral cavity such periodontitis or mucositis with nanofibers[35] or mucoadhesive film[36]. Miranda and collaborators provided evidences of analgesic synergism between tramadol and dexketoprofen and supported the advantages of using drugs with different mechanism for multimodal analgesia[37–39]. The combination allows to have analgesia at doses lower than those necessary for each

drug to produce comparable effect[40]. Different studies demonstrated that using tramadol with NSAIDs such as dexketoprofen[38], ibuprofen or acetaminophen[41] reduces opioid-induced constipation. Moreover clinical studies showed no drug to drug pharmacokinetic interactions. This complementary pharmacokinetic and pharmacodynamic activity makes Tramadol/ Dexketoprofen fixed dose combination an attractive option to manage different painful conditions. For instance, osteoarthritis is a dual pain phenomenon involving neuropathic and nociceptive mechanism. It is characterized by continuous pain with acute flare and tramadol / dexketoprofen combination represents a smart medication for acute exacerbation of osteoarticular pain. Their combination, targeting different sites of action is suitable for this mixed pain[42,43].

4.1 DEVELOPMENT AND VALIDATION OF THE ANALYTICAL METHOD

Routine laboratory analysis must be selective and accurate but they also need to be fast. For this reason, the first aim of this work was the setup and validation of an adequate HPLC analytical method for the simultaneous quantification of tramadol and ketoprofen in in vitro permeation experiments.

Initially, the quantification of tramadol was performed by modifying the method described by Zhang for the quantification in rat's plasma samples[44]. In our case, UV detection at 270 nm instead of fluorescence detection was used. The chromatographic separation was performed on a C18 reverse phase column using a mixture of water: acetonitrile: phosphoric acid (60:40:0.01, v/v/v) as mobile phase. A tentative for the quantification of ketoprofen was made but in these conditions it was no possible to detect any peak.

Different methods for the simultaneous determination of tramadol and other actives are reported in the literature. For the continuation of the work, the methods described for the quantification of tramadol and paracetamol[45] and of tramadol and ibuprofen[46] in solid pharmaceutical formulations, were considered. Both methods used, for the chromatographic separation, a C18 column and a mobile phase composed of acetonitrile and acetic acid mixture in different ratio. Similar condition were also described by

Allegrini and collaborators for the quantification of ketoprofen in human plasma[47]. In this case potassium dihydrogen phosphate 0.01 M at pH 3.5 and acetonitrile mixture was used as mobile phase and the separation was performed on a C18 column.

The first step was the selection of the detection wavelength. UV spectrum of tramadol hydrochloride and ketoprofen solutions was recorded in the range of 190-500 nm using (Jasco, V-570). The maximum absorption for both drugs occurs at 200 nm and in the range between 260 and 280 nm. Considering that biological interferences have their highest absorption in range from 190 to 200 nm, the wavelength of 280 nm was selected.

Using a C18 Zorbax and a mobile phase composed of a mixture of acetic acid 0.25% and acetonitrile (55:45, v/v) pumped at 1 ml/min at room temperature, the simultaneous separation of the two drugs was achieved with retention time of 1.8 and 3.4 min respectively for ketoprofen and tramadol.

Table 1 and **2** summarizes the calibration data of the analytical method respectively for tramadol and ketoprofen.

Table 1: Calibration data of the analytical method (tramadol).

Nominal concentration (µg/ml)	Fitted concentration (µg/ml)	RE %	CV%	Regression equation
0.44	0.42 ± 0.10	3.46	2.67	y=19012x + 34190 (R^2=0.9997)
8.78	8.69 ± 0.19	1.99	1.93	
43.89	44.08 ± 0.42	0.87	0.95	

Table 2: Calibration data of the analytical method (ketoprofen).

Nominal concentration (µg/ml)	Fitted concentration (µg/ml)	RE %	CV%	Regression equation
0.25	0.25 ± 0.01	3.83	5.07	y=69989x + 22659 (R^2=0.9996)
10	10.12 ± 0.12	1.26	1.01	
50	49.80 ± 0.76	1.29	1.52	

Linearity of the method was evaluated in the interval of concentration expected in the in vitro permeation experiment and in both cases the correlation coefficients (R^2) for was higher than 0.999. Moreover, as required by the EMA guideline[48], at each concentration level, accuracy (RE%) is lower than 15%.

The LLOQ, (the lowest concentration of the calibration curve that can be quantified with accuracy (RE%<20%) and precision (RSD%<20%)) resulted to be 0.44 and 0.25 µg/ml for tramadol and ketoprofen respectively.

4.2 DETERMINATION OF KETOPROFEN APPARENT PARTITION COEFFICIENT

Because of the different characteristics of ketoprofen (weak acid) and tramadol (weak base) is reasonable to hypothesize the formation of a ion pair that can promote the transport across a membrane[49,50]. The evaluation of the apparent partition coefficient of ketoprofen as a function of pH, in the presence or absence of tramadol is summarized in **Table 3**.

Table 3: Apparent partition coefficient of ketoprofen as a function of pH in absence or presence of tramadol.

pH	*KTP*	*KTP (+TMD)*
4.5	250.85 ± 13.74	250.51 ± 9.41
5.5	41.87 ± 1.61	42.92 ± 2.87
6.8	2.47 ± 0.25	2.67 ± 0.18
7.4	0.89 ± 0.13	1.07 ± 0.09

From the data obtained, the formation of an ion pair between ketoprofen and tramadol can be excluded.

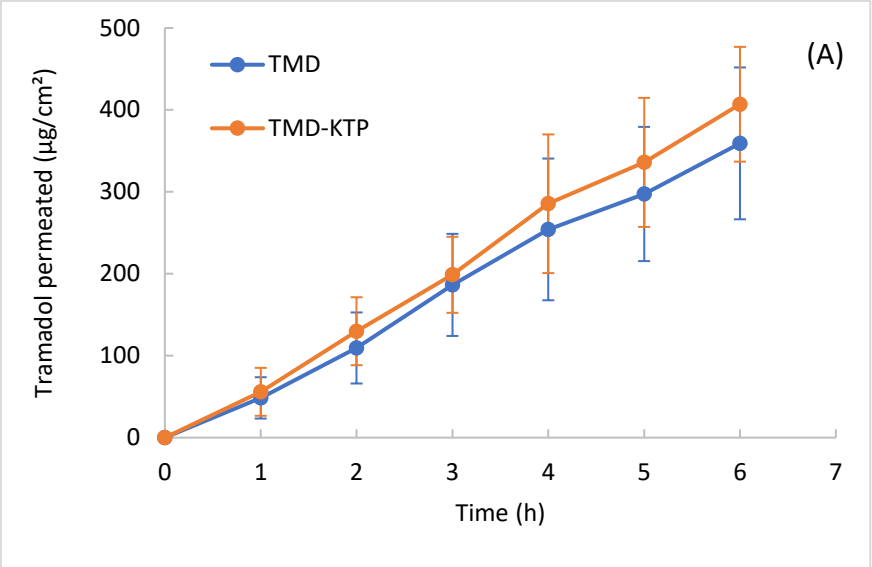
4.3 PERMEATION EXPERIMENTS

4.3.1 Passive permeation

Initially the passive permeation of tramadol and ketoprofen across pig esophageal epithelium was studied in infinite dose condition (0.5 ml/cm^2) from aqueous solutions at pH 6.8 containing one or both drugs, in order to evaluate the permeation parameters. As suggested by the pH partition theory, the transport of ionisable molecules depends on the pH of the medium in which they are dissolved. In fact, depending on the pka of the drug, at any give pH, the unionized and ionized species ratio changes. pH variation has an opposite effect on the two molecules object of this work being tramadol a weak base (pKa 9.41) and ketoprofen a weak acid (pKa 4.39). For this reason it has been decided to maintain constant the pH of the donor solution at 6.8, corresponding to the pH of simulated salivary fluid[51]. At this pH value, the percentage of the unionized form (calculated with the Henderson-Hasselbach equation) is 0.24 and 0.36% respectively for tramadol and ketoprofen. As barrier, pig esophageal epithelium, that has been shown to be a reasonable model for human buccal mucosa was used[24,52].

The cumulative amount of tramadol (panel A) and ketoprofen (panel B) across porcine esophageal epithelium are presented in **Figure 9** while the relevant permeation parameters are reported in **Table 4**.

The permeation profile obtained from the solution containing the single drug was compared to that obtained from the solution containing the combination of the drugs.



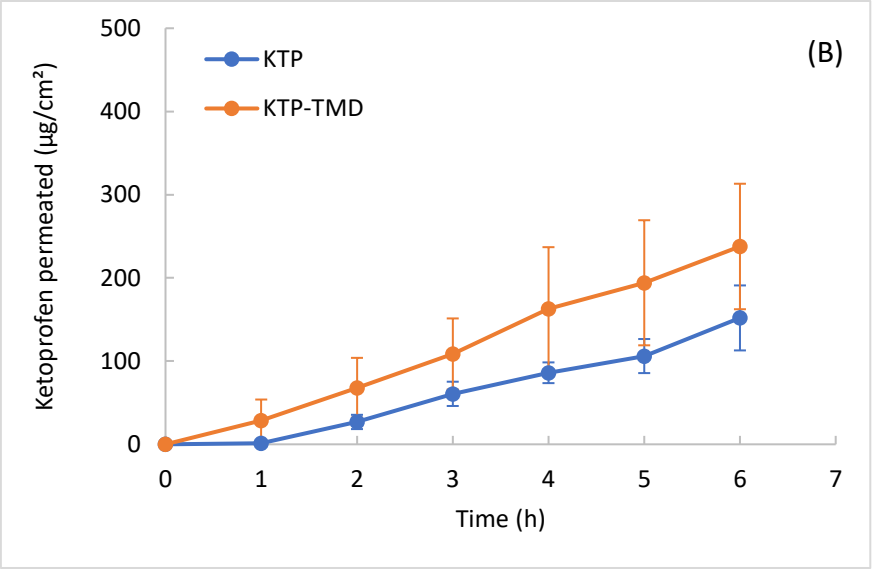


Figure 9: Permeation profiles of tramadol (A) and ketoprofen (B) across porcine esophageal epithelium. (mean value ± sd)

Table 4: Permeation parameters of tramadol and ketoprofen applied alone or in combination (mean ± sd)

Parameters	*TMD*	*TMD-KTP*	*KTP*	*KTP-TMD*
J (µg/cm²h)	61.04 ± 14.64	69.12 ± 8.98	29.21 ± 7.93	42.51 ± 11.77
T Lag (h)	0.27 ± 0.22	n.d.	1.25 ± 0.64	n.d.
Px10 ² (cm/h)	1.41± 0.34	1.67 ± 0.27	0.98 ± 0.26	1.51 ± 0.37

Despite at the pH considered the unionized fraction of both drugs is very low, tramadol and ketoprofen transport across esophageal epithelium is significant. Permeation

coefficient obtained in a parallel work by testing the same solutions across porcine full thickness skin (data not shown), resulted to be one order lower compared to that obtained in this work, confirming that the permeability of the buccal mucosa is higher than that of the skin[53]. The relative high permeability of the non-keratinized mucosa to ionizable molecules is in line with literature data reporting the buccal absorption of other highly ionized molecules such fentanyl citrate or sodium[27]. It is well known that the main permeability barrier of the buccal epithelium is located in the superior third of the tissue and it is due to the presence of intercellular lipidic material derived from the membrane coating granules (MCG). Two main routes are involved in the transport across the buccal mucosa: the transcellular (across the cells) and the paracellular (through the lipids present in the intercellular spaces) the route. Compared to the intercellular lipidic matrix of the stratum corneum, the lipid composition of the non keratinized mucosa is quite different being phospholipid's content abundant and ceramides' very low[52] . The presence of the polar groups of these lipids provokes the entrapment of many molecules of water that form an aqueous pathway that can explain the passage of polar molecules[54].

The co-administration of the two drugs does not modify the fluxes significantly and this results confirm the absence of differences in the partitioning of ketoprofen in presence and absence of tramadol.

To evaluate the clinical relevance of the flux obtained, the minimum flux required was calculated, as follows. Considering that at steady-state the rate of administration must be equal to the rate of elimination, it is possible to calculate the required flux to maintain the plasma therapeutic concentration from the following equation:

$$J = \frac{Ct\ x\ Cl}{A}$$

where J the flux at the steady-state, A is the area of the therapeutic system, C_t the plasma therapeutic concentration and Cl the clearance[55]. Considering an application area of 5 cm^2, the flux required for tramadol and ketoprofen is approximately 2160 and

544 µg/cm^{2}h respectively. This means that an enhancement of 31 and 13 times is required for tramadol and ketoprofen, respectively.

4.3.2 CHEMICAL ENHANCEMENT pre-treatment

4.3.2.1 Fatty acid

With the aim to increase the fluxes across the membrane, the effect of chemical enhancers on the transport of tramadol and ketoprofen was studied.

The first class of permeation enhancer considered was that of fatty acids, because most of them are considered as save by FDA and are approved as inactive ingredients in many formulations. Among them, capric acid, a C10 saturated fatty acid, was selected. In a previous work[24] the chain length of 10C has been identified as the "ideal" chain length for the optimal enhancement activity across the mucosa.

Capric acid 10% ethanolic solution was applied as pre-treatment on the mucosa. Despite the clinical applicability of this procedure is questionable, it offers the possibility to investigate a wider range of concentrations of the enhancer and allows a direct evaluation of the enhancer effect, avoiding drug-enhancer interactions. All other conditions remain unchanged compared to the passive permeation experiments, that is 0.5 ml of solution of tramadol hydrochloride (5 mg/ml) and ketoprofen (3 mg/ml) in SSF pH 6.8 in the donor and a duration of 6 h.

Figure 10 reports the permeation profiles obtained with 1-h pre-treatment of the tissue with 20 µl of capric acid 10% ethanol solution. The effect of pre-treatment with neat ethanol was also tested since it demonstrated to increase the buccal permeability in co-administration and as pre-treatment[56–58].

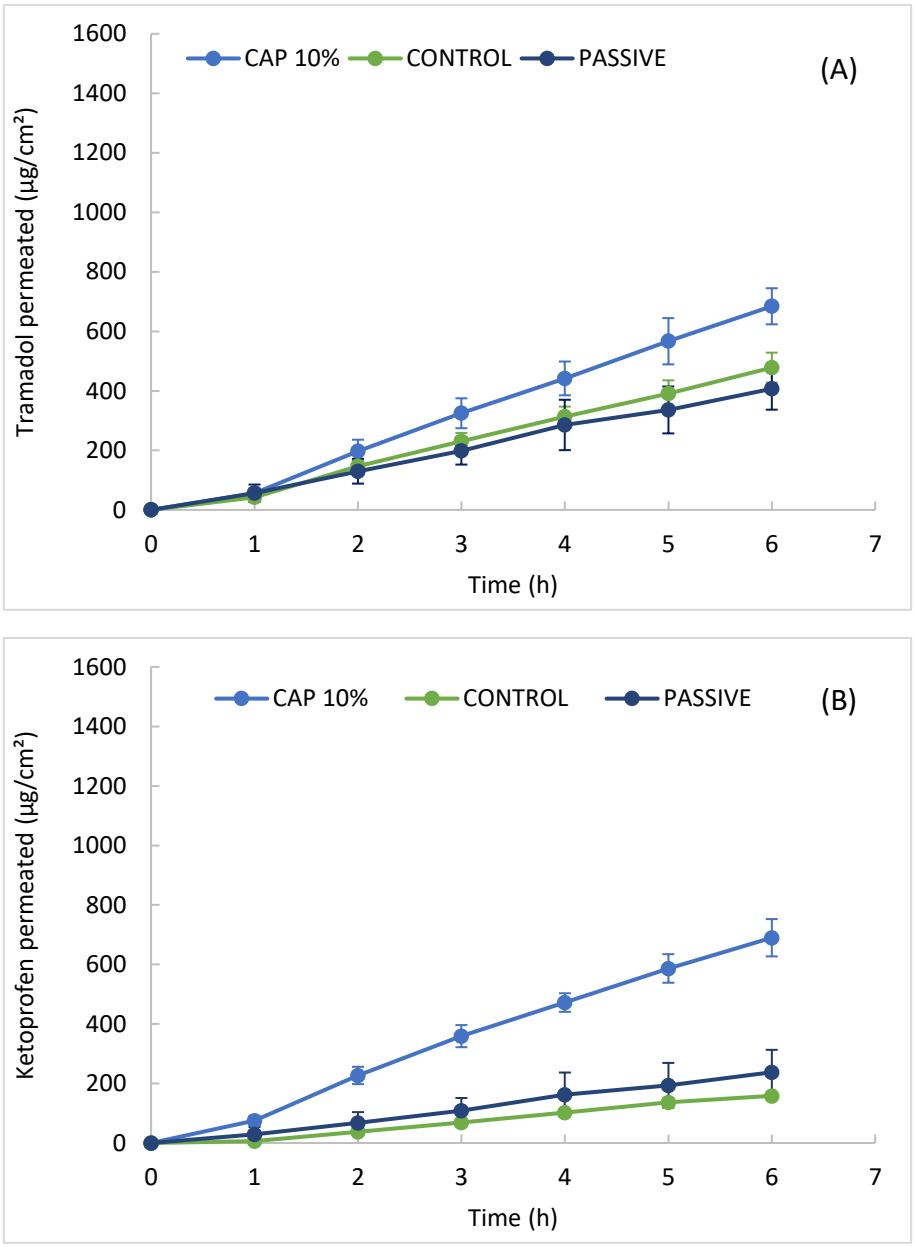


Figure 10: Effect of pre-treatment with capric acid 10% solution on the permeation of tramadol (panel A) and ketoprofen (panel B) across pig esophageal epithelium (mean ± sd).

Table 5 summarizes the permeation parameters calculated.

Table 5: Effect of pre-treatment with capric acid on the permeation parameters of tramadol and ketoprofen across pig esophageal epithelium (mean ± sd).

Drug	*Condition*	*J (µg/cm²h)*	*Px10 ² (cm/h)*	*EF*
Tramadol	Passive	69.12 ± 8.98	1.67 ± 0.27	
	Control	82.32 ± 8.07	1.89 ± 0.19	
	Capric acid	121.56 ± 7.90	2.92 ± 0.19	1.47 ± 0.09
Ketoprofen	Passive	42.51 ± 11.77	1.51 ± 0.37	
	Control	30.92 ± 0.91	1.25 ± 0.04	
	Capric acid	115.32 ± 10.93	3.52 ± 0.3	3.73 ± 0.35

The 1 h pre-treatment with ethanol in non occlusive conditions, does not modify the passive fluxes of both tramadol and ketoprofen. These findings agree with a previous study on buccal permeation of fluorescein isothiocyanate dextran of 4 kDa molecular weight[24], showing that in the conditions used, ethanol does not modify the epithelium permeability.

The effect of capric acid pre-treatment is different for the two drugs: almost negligible in the case of tramadol, with an EF of about 1.5, but significative ($p<0.001$, t-test) for ketoprofen with and EF of about 4. Although the exact mechanism by which fatty acids is not fully elucidate, it is reported that they act by increasing the fluidity of phospholipid domain, thus facilitating the transport through the hydrophobic pathway of the paracellular route. Despite the unionized fraction of both drugs in the condition used is very low, it is supposed it contributes partly to the total flow across the tissue. Being logP of the two drugs different (3 and 1.35 respectively for tramadol and ketoprofen), the higher lipophilicity of ketoprofen could be the reason for the higher EF observed probably because the transport through the hydrophobic pathway is more important for this drug.

4.3.2.2 Terpenes

The main reason that could severely limit the use of capric acid in a formulation for the buccal delivery of drugs is its represented by its organoleptic characteristics. As alternative, terpenes where considered because with their pleasant taste are often used as flavor in oromucosal formulations. In particular the permeation enhancer ability of menthol has been evaluated for nucleoside analogs[59], ropinirole[60], doxepin[61] and more recentely for and ketoprofen co-administered with lidocaine[62].

For the present work, in addition to menthol, limonene, eucalyptol and nerolidol were selected and their characteristics are summarized in **Table 6**.

Table 6: Characteristics of terpenes. [63]

Terpene	*Type*	*MW*	*LogP*
Limonene	Monoterpene	136.23	4.45
Eucalyptol	Monoterpene	154.25	2.82
Nerolidol	Sesquiterpene	222.37	5.32
Menthol	Monoterpene	156.26	3.20

As in the case of capric acid, the effect of terpenes was evaluated by pre-treatment of the tissue with enhancer's solution in ethanol. In all the cases, the concentration of the terpenes was 10%.

Figure 11 illustrates the permeation profiles obtained, while permeation parameters calculated are reported in **Table 7.**

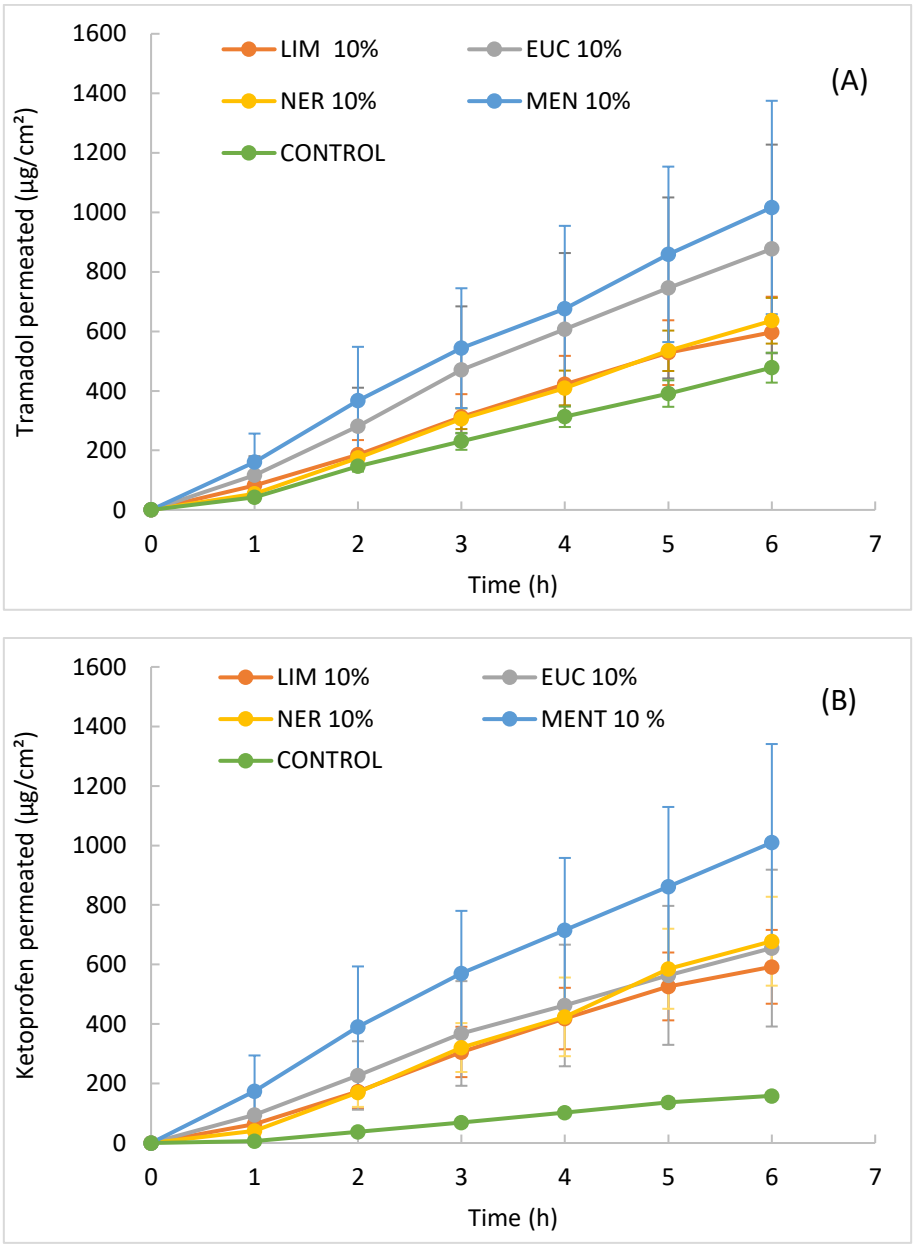


Figure 11: Effect of pre-treatment with terpenes 10% solution on the permeation of tramadol (panel A) and ketoprofen (panel B) across pig esophageal epithelium (mean ± sd).

Table 7: Effect of pre-treatment with terpenes on the permeation parameters of tramadol and ketoprofen across pig esophageal epithelium (mean ± sd).

Drug	*Condition*	*J (µg/cm²h)*	*Px10 ² (cm/h)*	*EF*
Tramadol	Control	82.32 ± 8.07	1.89 ± 0.19	
	Limonene	110.46 ± 19.99	2.57 ± 0.48	1.34 ± 0.24
	Limonene+Capric acid	129.00 ± 29.39	3.48 ± 0.79	1.57 ± 0.36
	Eucalyptol	146.69 ± 54.82	3.41 ± 1.17	1.78 ± 0.67
	Eucalyptol+Capric acid	139.92 ± 30.14	3.76 ± 0.81	1.70 ± 0.37
	Nerolidol	114.22 ± 14.35	2.67 ± 0.28	1.39 ± 0.17
	Nerolidol+Capric acid	151. 62 ± 25.57	3.74 ± 0.63	1.84 ± 0.31
	Menthol	161.32 ± 46.04	3.75 ± 1.01	1.96 ± 0.56
	Menthol+Capric acid	148.18 ± 41.67	3.37 ± 0,90	1.80 ± 0.51
Ketoprofen	Control	30.92 ± 0.91	1.25 ± 0.04	
	Limonene	112.69 ± 19.76	3.55 ± 0.64	3.64 ± 0.63 [a]
	Limonene+Capric acid	90.05 ± 3.43	3.64 ± 0.14	2.91 ± 0.11
	Eucalyptol	105.13 ± 37.20	3.12 ± 0.97	3.40 ± 1.20 [a]
	Eucalyptol+Capric acid	90.05 ± 3.43	3.64 ± 0.14	2.91 ± 0.11
	Nerolidol	125.52 ± 24.97	3.59 ± 0.64	4.05 ± 0.81 [b]
	Nerolidol+Capric acid	105.86 ± 12.24	4.03 ± 0.46	3.42 ± 0.39
	Menthol	153.02 ± 34.21	4.23 ± 0.83	4.23 ± 0.83 [c]
	Menthol+Capric acid	137.26 ± 21.09	4.03 ± 0.61	4.44 ± 0.68

Significantly different from the control [a]($p<0.05$), [b]($p<0.01$), [c]($p<0.001$)

From a general point of view, the results obtained agree with literature data confirming that the efficacy of a permeation enhancer is strictly dependent on the characteristics of the drug. All terpenes resulted to be more effective in promoting the ketoprofen flux (EF in the range 3.40-4.95) compared to tramadol (EF in the range 1.34-1.96).

Concerning tramadol, the flux observed after the pre-treatment increases in the order limonene<nerolidol<eucalyptol<menthol. However, no significant differences compared to the control were observed.

For ketoprofen, the EF obtained after the pre-treatment increases in the order eucalyptol<limonene<nerolidol<menthol. For all the terpenes, the increase resulted to be significantly different from the control.

Although also in the case of terpenes the real mechanism by which they increase the transbuccal permeation in not clear, it is supposed that they act by fluidizing the lipid membrane, similarly to fatty acids. As observed for fatty acid, ketoprofen flux significantly increases in presence of terpenes probably because a higher transport through the hydrophobic pathway of the paracellular route.

Since fatty acids and terpenes share the same hypothetical mechanism of action, pre-treatment experiments were performed also using a binary mixture of capric acid and the terpenes selected. In no case a synergic action was observed, being the EFs comparable to that obtained with the single enhancer (**Table 7**).

4.3.3 CHEMICAL ENHANCEMENT co-administration

From the clinical practice point of view, the pre-treatment of the tissue with a permeation enhancer solution before the application of the formulation, is not easy to perform, especially in the case of buccal administration. For this reason the study continued by evaluating the co-administration of drugs and permeation enhancer.

4.3.3.1 BILE SALTS – Sodium taurocholate (NaTC)

Bile salts are probably the most known class of buccal permeation enhancer. They have been extensively used to promote the buccal transport of both small molecules[64,65] and macromolecules[66].

The dependence of the effect from the concentration of bile salts is reported in the literature[67]. Nielsen and collaborators[68] reported that whereas the permeation enhancer effect of bile salts is correlated to their ability to form micelles, for NaTC, the bile salt selected for this work, the concentration had to be 2-3 higher than the critical micellar concentration (CMC) to increase tissue permeability. The CMC reported for

NaTC in the same work is 5 mM. For this reason the initial concentration of NaTC tested was 20 mM. **Figure 12** shows the in vitro permeation profiles of tramadol (panel A) and ketoprofen (panel B) obtained from solution of the drugs containing sodium taurocholate (NaTC) at different concentrations. **Table 8** reports the permeation parameters calculated.

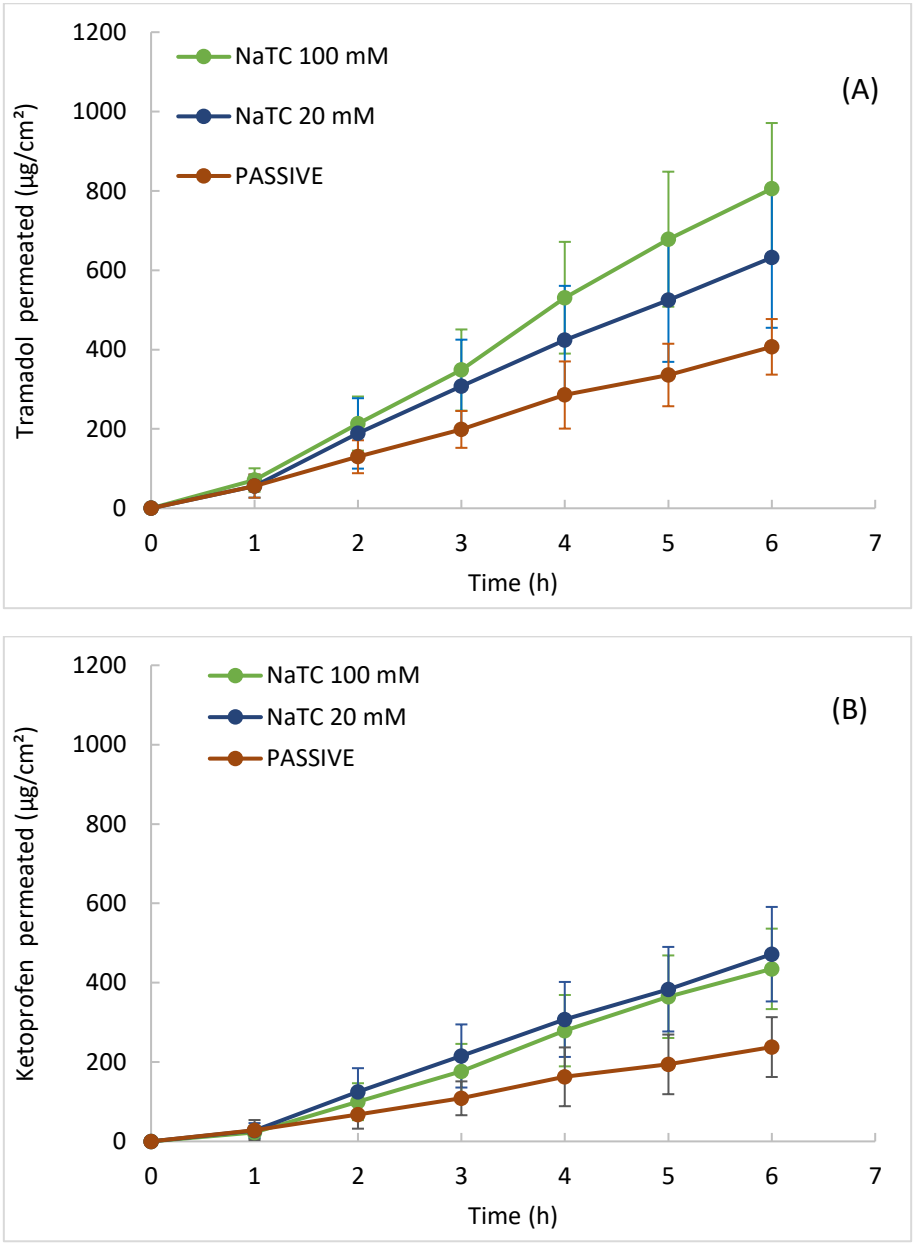


Figure 12: Effect of NaCT concentration on the permeation of tramadol (panel A) and ketoprofen (panel B) across pig esophageal epithelium (mean ± sd).

Table 8: Effect of NaCT concentration on the permeation parameters of tramadol and ketoprofen across pig esophageal epithelium (mean ± sd).

Drug	Condition	J (µg/cm²h)	Px10 ² (cm/h)	EF
Tramadol	Passive	69.12 ± 8.98	1.67 ± 0.27	
	NaTC 20 mM	110.33 ± 22.39	2.68 ± 0.54	1.60 ± 0.32
	NaTC 100 mM	151.37 ± 26.79	3.76 ± 0.66	2.19 ± 0.39
Ketoprofen	Passive	42.51 ± 11.77	1.51 ± 0.37	
	NaTC 20 mM	81.95 ± 15.51	2.81 ± 0.53	1.93 ± 0.36
	NaTC 100 mM	85.38 ± 14.87	3.18 ± 0.55	2.01 ± 0.35

For both drugs, an EF of about 2 was obtained in the presence of the bile salt, confirming the ability of NaTC to serve as buccal permeation enhancer. Considering the variability of tramadol data, a 5-fold increase of NaTC concentration, did not produce a significant analogue increase in drugs' transport showing that the relationship between EF and enhancer concentration is not linear.

4.3.3.2 Menthol

Menthol and capric acid showed to have comparable enhancement effect for both tramadol and ketoprofen transport in the pre-treatment experiments. Because of the definitely better taste, menthol was selected for the continuation of the work.

Menthol has a solubility in water of about 450 mg/ml[69]. Initially a saturated solution of menthol in SSF pH 6.8 was prepared. The addition of the two drugs provoked the formation of a precipitate, then this approach was abandoned and the addition of a co-solvent, namely Transcutol, was considered. Transcutol, diethylene glycol monoehtyl ether, is a solubilizer largely used in oral, topical, transdermal and injectable pharmaceutical product[70]. Besides the ability to solubilize substances otherwise insoluble in common solvents, Transcutol has proven to be an effective permeation enhancer across the skin, the cornea[71] and recently also across the buccal mucosa[72].

In order to maintain constant the amount of menthol applied in comparison to the pre-treatment experiments, a solution of 0.4% (w/v) of the terpene, tramadol hydrochloride

(5 mg/ml) and ketoprofen (3 mg/ml) were dissolved in PBS pH 6.8: Transcutol (1:3, v/v). As control, a solution with the same composition but without menthol was used. Flux curves of tramadol and ketoprofen in absence and in presence of Transcutol and menthol are reported in **Figure 13** while the relative permeation parameters are summarized in **Table 9**.

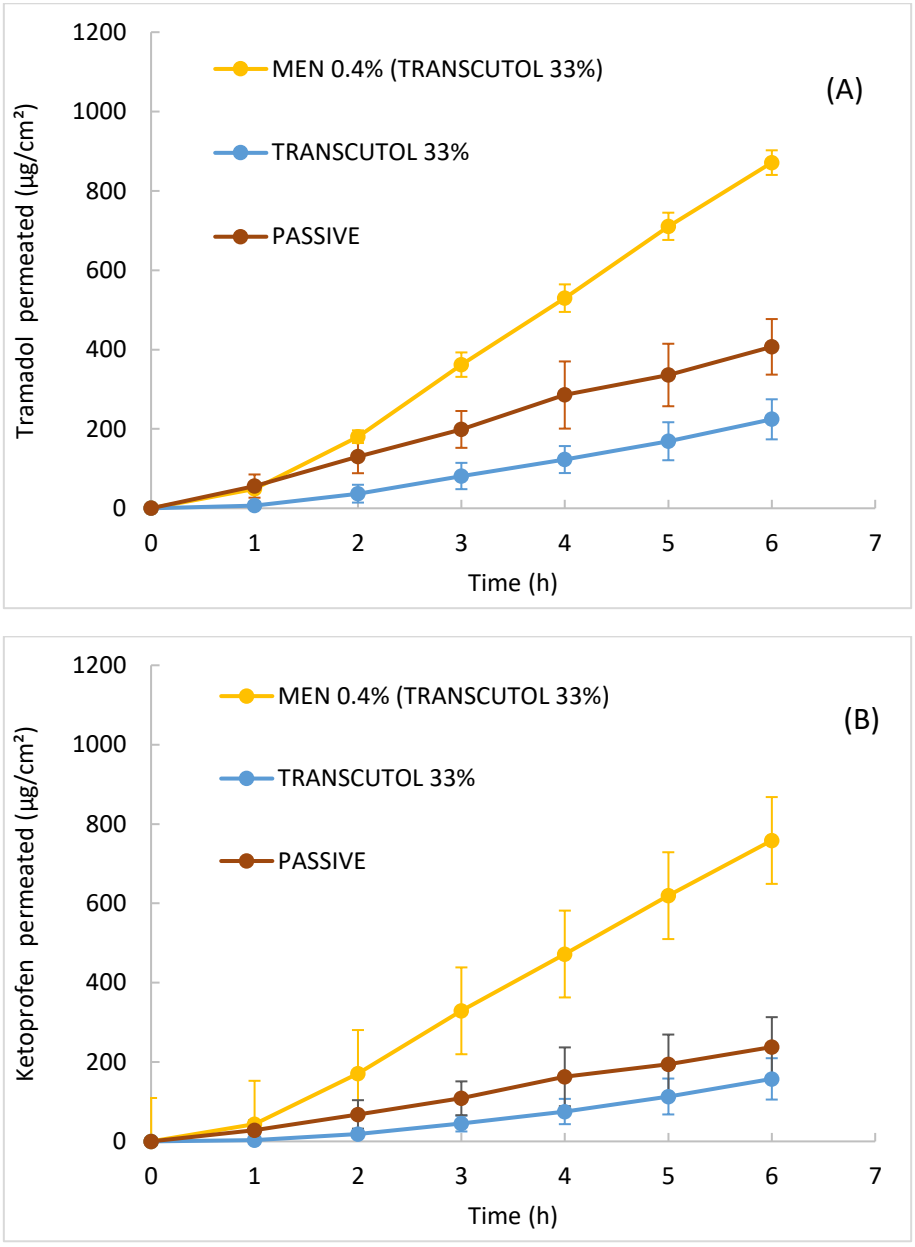


Figure 13: Effect of co-administration with menthol 0.4% on the permeation of tramadol (panel A) and ketoprofen (panel B) across pig esophageal epithelium (mean ± sd).

Table 9: Effect of co-administration with menthol 0.4% and transcutol 33% on the permeation parameters of tramadol and ketoprofen across pig esophageal epithelium (mean ± sd).

Drug	*Condition*	*J (µg/cm²h)*	*Px10 ² (cm/h)*	*EF*
Tramadol	Passive	69.12 ± 8.98	1.67 ± 0.27	
	Transcutol 33%	46.42 ± 6.83	1.30 ± 0.19	0.67 ± 0.10
	Men 0.4%-Tran 33%	173.01 ± 4.06	4.2 ± 0.1	2.50 ± 0.05
Ketoprofen	Passive	42.51 ± 11.77	1.51 ± 0.37	
	Transcutol 33%	34.68 ± 11.52	0.90 ± 0.30	0.81 ± 0.27
	Men 0.4%-Tran 33%	146.47± 2.27	4.09 ± 0.17	3.44 ± 0.15

In the presence of Transcutol the flux across the membrane for both tramadol and ketoprofen is reduced. Apparently Transcutol seems to acts as a retarder rather than as an enhancer. However, it must be considered that the presence of Transcutol produces an increase in the solubility of the drugs in the solvent and therefore a decrease of the thermodynamic driving force. Similar behavior was observed by Bialik and collaborators that found that the permeation of ibuprofen across human skin from neat Transcutol solution was practically zero and that it increased dramatically following dilution with water[73]. As observed for other permeation enhancers, it probably exists an optimal concentration for the enhancement effect. On the other hand, the combination of menthol and Trascutol has a synergist effect producing an increase in the fluxes of both drugs with an EF respectively of 2.5 and 3.44 for tramadol and ketoprofen. The permeation curves of both drugs in the presence of menthol and Transcutol are similar to the respective passive profiles (SSF pH 6.8) up to 1 h. Afterwards, both profiles rise significantly. This could mean that it takes a certain period of time before the menthol reaches the tissue and exerts its action. The enhancement produced in the case of the co-administration is analogous to the pre-treatment

4.4 POLOXAMER HYDROGELS

The results obtained in the first part of the work showed the feasibility of the buccal administration for the combination of tramadol and ketoprofen. The direct entry of the drugs in the systemic circulation, thanks to the rich bloody supply of the oral mucosa, can make faster the onset of the analgesic effect, that is the main limit of the oral route for the treatment of pain. Nevertheless, the use of conventional pharmaceutical forms, which can be easily removed from the buccal cavity due to salivation and tongue movements, could reduce the bioavailability of the drugs administered. Several innovative formulations, able to prolong the residence time in the oral cavity and thus the buccal absorption, have been developed and include, among others, patches, films, adhesive tablets and hydrogels. For this work the attention was focused on hydrogels and in particular on poloxamer based hydrogels.

Among poloxamers, poloxamer 407 (PL 407), with a molecular weight of 12600 Da, was selected because it has low toxicity, high solubilizing properties and high compatibility with other chemicals. Moreover, it has been approved as “inactive ingredient” by FDA for many drug products for oral, intravenous, ophthalmic and topical application and is listed in US and European Pharmacopoeia. Poloxamers are a family of non-ionic triblock water-soluble copolymers composed of a basic structure of hydrophobic polyoxypropylene (PPO) between two hydrophilic polyethylene (PEO) oxides units. The arrangement and the different number of these basic units allow the formation of micelles with a hydrophobic core and an hydrophilic corona. Their hydrophobic core architecture can acts as drug loading site by encapsulating poorly water soluble drugs. Concentrated solutions of Poloxamer 407 undergo in-situ gelation process caused by the formation of ordered packing micelles. With the increase of the temperature, PPO hydrophobic units dehydrate forming the micellar core and define the initial phase of gelation[74]. The thermal gelling phenomenon is reversible and it is related to a sol-gel transition temperature. Below this value, the poloxamer solution is liquid, while above it, turn into a semi-solid material.

Hydrogel for the buccal delivery of drugs have been proposed for other drugs. For example, Shin and collaborators used PL407-Carbopol gel for the vehiculation of

triamcinolone acetonide across buccal mucosa[75] while Choi e al. developed a delivery system of PL407 and polyethylene oxide for the buccal delivery of plactitaxel[76].

Thermosensitive properties of PL407 solution, in particular its transition sol gel temperature, depends on the concentration of the polymer and on the presence in solution of drugs, co-solvent or other additives[74].

As discussed before, it has been decided to maintain the pH value of the formulation at 6.8, thus the first part of the work consisted in the selection of the PL407 concentration to obtain an hydrogel with a transition temperature near to the body temperature. The concentration tested were in the range 15-20% (w/w). For PL407 concentration between 16 and 20%, the transition temperature was lower than the ambient temperature, while for 15% was higher than 37°C. The target was reached with a concentration of 15.5% that exhibit a transition temperature of 28.5°C.

Drugs were added to the pre-formed hydrogel solubilized in water (tramadol) or ethanol (ketoprofen) and the effect on the transition temperature was evaluated with the magnetic bar stirring method and by viscosity measurements. Results are reported in **Table 10**.

Table 10: Effect of ethanol ,drugs and chemical enhancers addition on Tsol-gel transition

Preparation	*Stir / Mag Tsol-gel*	*Calculated Tsol-gel*
PL407	28.5 °C	27.4°C
PL407+EtOH 5%	28.5 °C	27.8°C
PL407+EtOH 5%+Drugs	28.5 °C	28.0°C

Although Fakhari and coworkers demonstrated an inverse correlation between the ethanol concentration and the gelation temperature[77], the presence of ethanol at 5% and/or drugs doesn't modify this parameter. Similar results were obtained also by Giuliano with poloxamer 407 20% hydrogel loaded with rutin dissolved in ethanol[78].

Figure 14 reports the permeation profiles of tramadol and ketoprofen across pig esophageal tissue obtained from the hydrogel, while the relative permeation parameters are reported in **Table 11**.

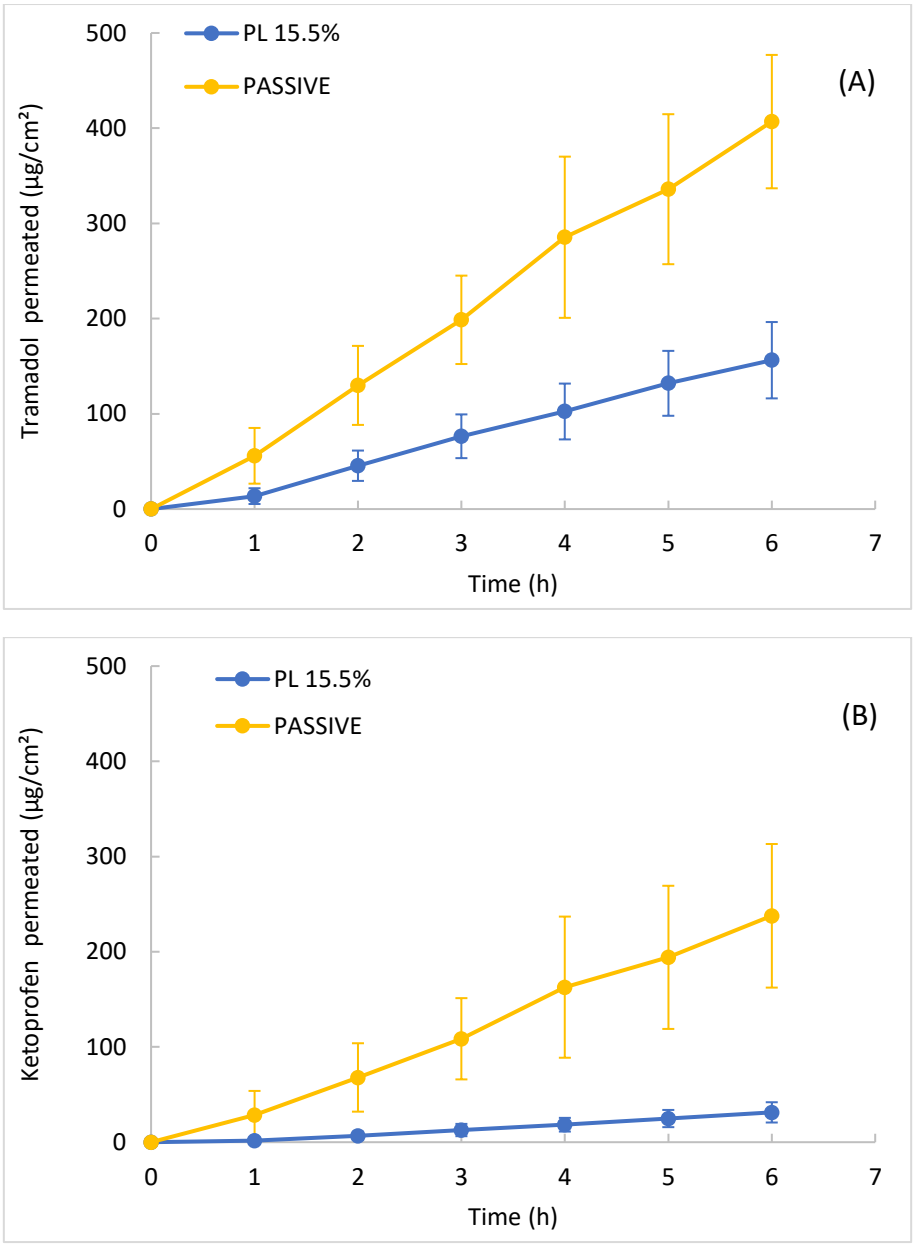


Figure 14: Effect of poloxamer 407 15.5% on permeation profile of tramadol (panel A) and Ketoprofen (panel B) across pig esophageal epithelium (mean ± sd).

Table 11: Effect of poloxamer 407 15.5% on permeation parameters of tramadol and ketoprofen across pig esophageal epithelium (mean ± sd).

Drug	*Condition*	*J (µg/cm²h)*	*Px10 ² (cm/h)*
Tramadol	Passive	69.12 ± 8.98	1.67 ± 0.27
	PL 15.5%	27.72 ± 6.52	0.58 ± 0.15
Ketoprofen	Passive	42.51 ± 11.77	1.51 ± 0.37
	PL 15.5%	6.01 ± 1.55	0.20 ± 0.04

As reference, the permeation in passive condition (from pH 6.8 solution in SSF) was considered. As expected, the increased viscosity of the formulation, decreased the fluxes of tramadol and ketoprofen, more evident for the last.

The mucoadhesiveness of non-ionic polymer is generally lower than ionic polymers[79]. For this reason xanthan gum, an anionic polymer that exhibits mucoadhesive properties[80] was added to the PL407 hydrogel to a final concentration of 0.25%. At the same time, in order to improve tramadol and ketoprofen permeation, menthol was added as permeation enhancer. The addition of these chemicals determined a decrease in the transition temperature of the hydrogel from 28.5°C to 27 and lower than 24°C respectively for the hydrogel with xanthan gum and with xanthan gum and menthol.

These results agree with those of Rhee et al, that demonstrated that monoterpenes and other chemicals, decrease the gelling point of poloxamer 407 aqueous solution following a concentration dependent trend[28]. The same work reports that terpenes facilitate the dehydration of PPO residues chain of poloxamer resulting in more viscous system at increasing temperature. And preliminary data on the characterization of the hydrogels prepared, confirms the increase of the viscosity as a result of the addition of the gum and the menthol.

The effect of xanthan gum and menthol on the profiles of tramadol and ketoprofen is illustrate in **Figure 15** while the permeation parameters are summarized in **Table 12**.

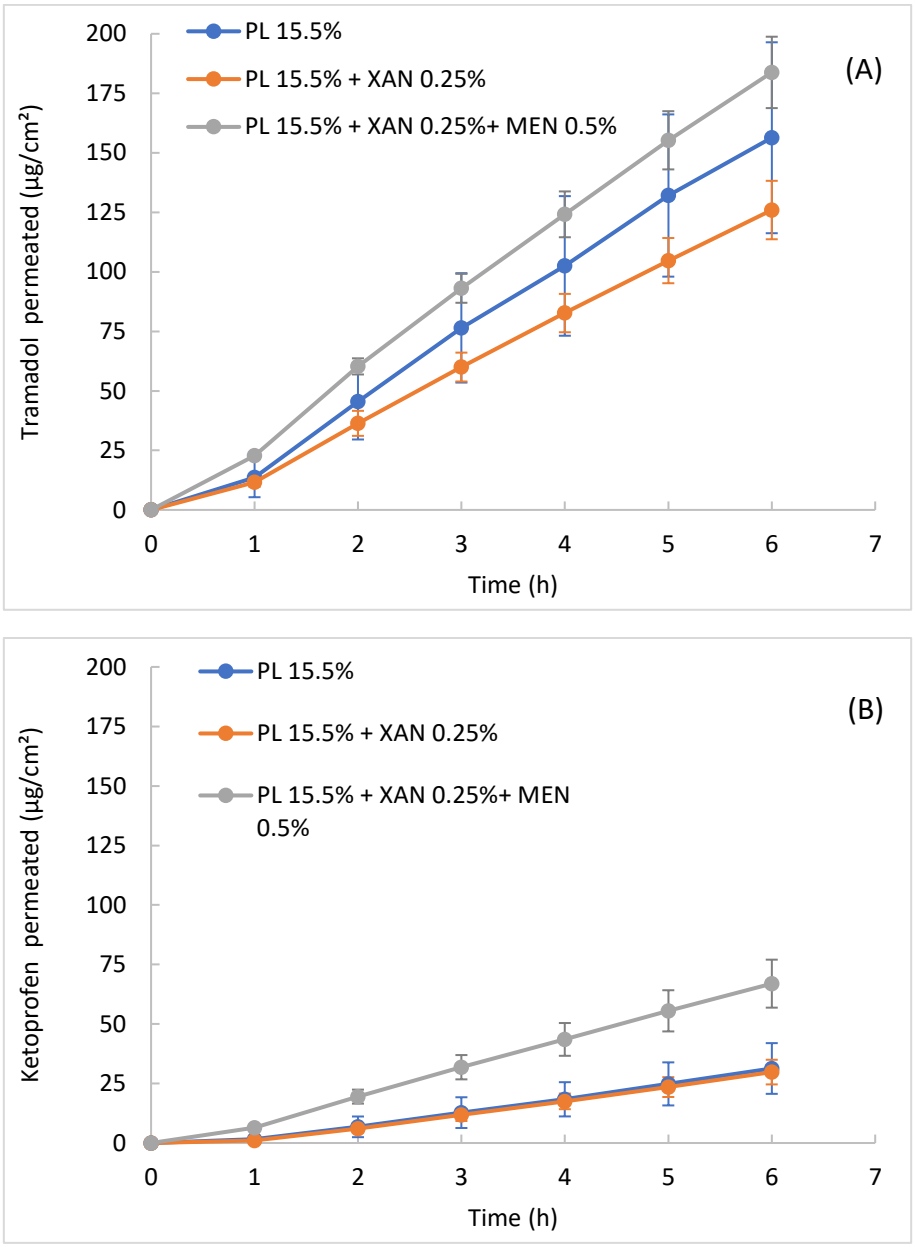


Figure 15: Effect of the xanthan gum 0.25% and menthol 0.5% on permeation profile of tramadol (panel A) and ketoprofen (panel B) from poloxamer 407 hydrogel (mean ± sd).

Table 12: Effect of the xanthan gum 0.25% and menthol 0.5% on permeation parameters of tramadol (panel A) and ketoprofen (panel B) from poloxamer 407 hydrogel (mean ± sd).

Drug	*Condition*	*J (µg/cm²h)*	*Px10 ² (cm/h)*	*EF*
Tramadol	Poloxamer 15.5%	27.72 ± 6.52	0.58 ± 0.15	
	Poloxamer15.5%+Xanthan 0.25%	22.39 ± 2.05	0.51 ± 0.07	0.81 ± 0.07
	Poloxamer15.5%+Xanthan 0.25%+Menthol 0.5%	30.92 ± 2.93	0.69 ± 0.06	1.11 ± 0.10
Ketoprofen	Poloxamer 15.5%	6.01 ± 1.55	0.20 ± 0.04	
	Poloxamer15.5%+Xanthan 0.25%	5.91 ± 0.92	0.22 ± 0.06	1.12 ± 0.17
	Poloxamer15.5%+Xanthan 0.25%+Menthol 0.5%	11.86 ± 1.80	0.51 ± 0.08	2.26 ± 0.34

The presence of 0.25% xanthan gum in the considered poloxamer formulations doesn't increase permeation parameters of drugs despite the increase of the viscosity of the system. On the other hand, menthol is able to increase the permeation of both drugs even if the effect is more evident in the case of ketoprofen, confirming the data obtained in the previous part of the work.

4.4.1.1 Poloxamer gels - release study

The release of both tramadol and ketoprofen from poloxamer 407 hydrogels was performed using Franz-Type diffusion cells using a regenerated cellulose membrane to separate donor and receptor compartment. The drugs release was monitored for 6 hours at 37 °C under continuously stirring in a water bath. **Figure 16** illustrates release of tramadol (panel A) and Ketoprofen (panel B) from poloxamer 407 hydrogels in presence of xanthan gum 0.25% incorporated alone or with menthol at 0.5 % (w/w).

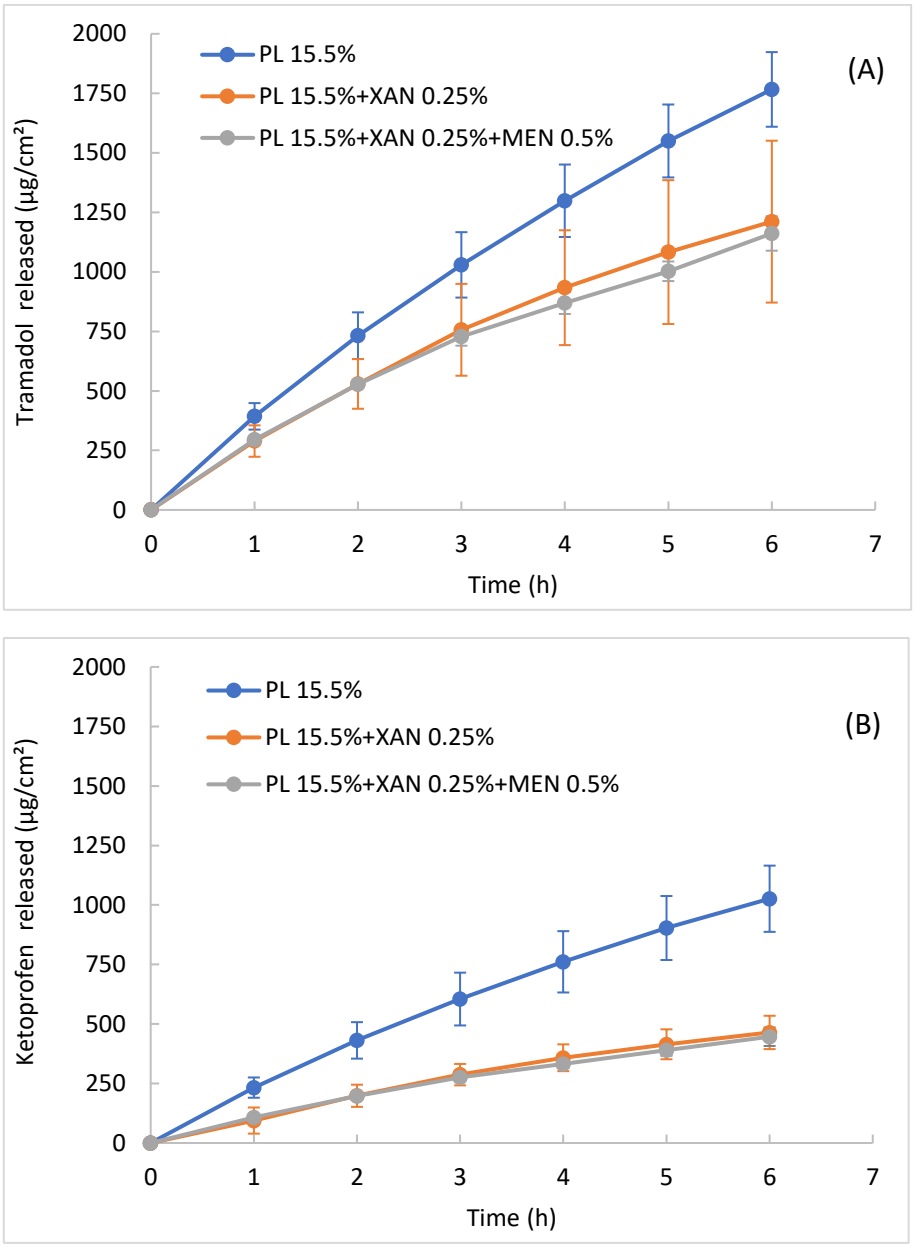


Figure 16: Effect of xanthan gum 0.25% and menthol 0.5% on release profile of tramadol (panel A) and ketoprofen (panel B) from poloxamer 407 hydrogel (mean±).

The cumulative amount of drugs released per unit area resulted to be linear as function of the square root of time. This means that the release of both drugs from poloxamer hydrogels follow the Higuchi kinetic and the system act as a matrix (Table 13).

Table 13: Release parameters for poloxamer 407 hydrogel formulations according to Higichi mathematic model (mean ± sd)

Drug	*Condition*	*% Kh*[81]	*R²*
Tramadol	Poloxamer 15.5%	24.22 ± 1.96	0.996 ± 0.003
	Poloxamer15.5%+Xanthan 0.25%	18.43 ± 7.89	0.998 ± 0.002
	Poloxamer15.5%+Xanthan 0.25%+Menthol 0.5%	13.93 ± 1.04	0.998 ± 0.001
Ketoprofen	Poloxamer 15.5%	21.00 ±1.86	0.996 ± 0.003
	Poloxamer15.5%+Xanthan 0.25%	10.12 ± 1.70	0.997 ±0.003
	Poloxamer15.5%+Xanthan 0.25%+Menthol 0.5%	11.06 ±1.19	0.998 ± 0.002

Generally, drug release from poloxamer formulation into the receptor medium depends on the drug diffusion in the gel matrix and the poloxamer dissolution rate. In membrane release models the diffusion is the predominant mechanism in releasing drugs, and the increased viscosity observed explain the reduction of drugs' release rate in the presence of xanthan gum 0.25% and menthol 0.5%. The presence of xanthan gum reduces from 40% to 20% the release of both drugs after 6 hours compared to the PL407 hydrogel. The same trend was observed in the case of permeation studies. The further addition of menthol produced no effect on the release of both drugs, contrary to what was previously observed across the esophageal epithelium to underline that the permeation enhancer acts on the tissue and not on the formulation.

5 CONCLUSIONS

On the basis of the results obtained, the buccal co-administration of tramadol and ketoprofen is feasible. Both drugs are able to cross in significant amount the pig esophageal epithelium used as model for buccal mucosa although the evaluation of the clinical relevance of the fluxes obtained showed that an enhancement strategy is needed.

Different classes of permeation enhancers were tested under both pretreatment and co-administration conditions. All of them resulted to be able to increase the fluxes pf tramadol and ketoprofen, although to a different extent. The best results were obtained with menthol, a terpene usually added as flavour in oromucosal or oral pharmaceutical products.

A thermosensitive hydrogel formulation based on poloxamer 407 and xanthan gum and containing menthol was successfully developed and proved to be a potentially effective system for the buccal administration of tramadol and ketoprofen.

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