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CASE REPORT

Quantification of Anti-Inflammatory Drugs Retained in Different Layers of Skin after in Vitro Permeation Studies by Liquid Chromatography Assay

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ABSTRACT

Over the past decades, in vitro and in vivo permeation studies have been widely used in the pharmaceutical area due to their ability to provide important pharmaceutical data for the rational development of topical and transdermal delivery systems. In order to achieve success on topical or transdermal therapy, the drug must be able to penetrate into the skin - a semi permeable barrier which controls the entrance of molecules into the body. Furthermore, monitoring drug penetration across the skin and quantifying its levels in different skin layers have been constant challenges due to the low detection limitations of the current bioanalytical methods, as well as the inherent interference in this tissue. Advances in bioanalytical methods, indispensable tools to study drugs across the skin, have been reported in the last years and were explored and discussed in this review, emphasizing anti-inflammatory drugs. The review was structured highlighting the skin as an important route of drug administration and its structure, in vitro assay most used in the study of drug delivery to the skin were also reported. Noteworthy, it was provided a solid discussion of the main skin samples processing techniques in order to recovery the drug retained into the skin by solid-liquid extraction process and subsequent chromatographic analysis. Finally, new perspectives were highlighted regarding liquid chromatography method findings achieved with several anti-inflammatory drugs in the field of topical or transdermal therapy. This review has a comprehensive approach in order to help researchers at the design of their experiments and update the applicability and advances in this area of expertise.

INTRODUCTION

Topical delivery systems are placed on the skin to deliver drugs to the local tissue directly under the application site or within tissues under and around the site of application; transdermal delivery systems goal is the drug penetration across the skin reaching the bloodstream. The transdermal route for anti-inflammatory drugs may be an interesting strategy considering the convenience to the patient, pain-free self-administration, patient compliance (especially when long-term treatment is required, as in chronic pain treatment), absence of hepatic metabolism and the reduction of possible side effects that can occur with systemic administration. Even with the low market share that those systems possess, the patients' acceptability for topical and transdermal medicines is very high, considering that the transdermal drug delivery market worth $12.7 billion dollars in 2005 was reached $32 billion in 2015 [1].

Approximately 15 drugs constitute the whole segment of the transdermal drug market [1]. Advances in biotechnologies field have been resulting in an increase of studied drugs delivered transdermally, including those with low skin permeability due their excessive hydrophilicity or lipophilicity and high-molecular-weight [1]. Much effort is spent in formulating drug delivery systems for anti-inflammatory topical or transdermal use, with the ultimate goal to control the drug permeation to deeper skin layers. Studies using different transdermal and topical delivery systems containing anti-inflammatory drugs, such as celecoxib, dexamethasone, ketoprofen and others, have been demonstrated for increased the penetration into skin [2-4].

According to the literature, the epidermis and dermis are directly related to the flux and percutaneous absorption of anti-inflammatory transdermal drugs, as these skin layers could be a reservoir to the posterior drug passage to the systemic circulation [4]. In that way, monitoring the anti-inflammatory drug amount retained into the skin after their topical application is a crucial step to assure the therapy efficacy. In this work, a review of the most relevant reports in the field of skin samples processing techniques in order to recovery the drug retained into the skin by solid-liquid extraction process and subsequent chromatographic analysis considering anti-inflammatory drugs is provided. We searched the Web of Science database using keywords, such as "anti-inflammatory drugs", "chromatographic assay", "drug skin recovery", "solid-liquid extraction process", and we have limited our search for the period from 2000 to 2017. This effort resulted in more than 20.000 citations and the most relevant studies were selected for the review.

SKIN MEMBRANE AND THEIR APPLICABILITY

The primary function of the skin is to protect the body from unwanted influences from the environment. The skin is the largest human body organ and performs important functions such as thermoregulation, absorption, excretion, immune protection and protection against loss of ions, water and proteins from the organism [5]. The skin protection action is provided primarily by the Stratum Corneum (SC). The SC (usually ranging from 10 to 20 μm) is the outmost layer of skin and consists of corneocytes (anuclear, flattened, keratin-rich dead cells) surrounded by lipid regions, and represents the main barrier of skin [6-8]. Underlying the SC is the viable epidermis (50–100 μm thick), which is a hydrophilic layer composed by stratified layers of keratinocytes at different stages of differentiation, melanocytes (responsible for melanin production), Merkel cells (important on sensory perception) and Langerhans cells (involved on immunological function) and responsible for generation of the stratum corneum [8]. In general, more lipophilic anti-inflammatory drugs are absorbed faster, while hydrophilic compounds penetrate slowly. That happens due to the formidable barrier provided by the stratum corneum [2]. The pathways of drug penetration into the skin is still under investigation it is not completely understood. However, as a rule, three passive routes (intracellular, extracellular and appendageal routes) are known (Figure 1).

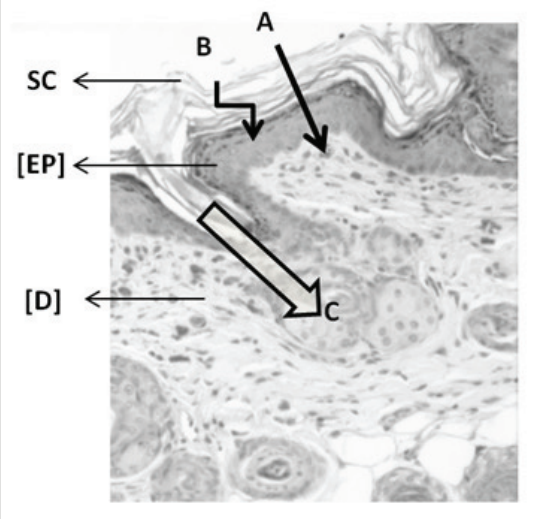

Figure 1: Structure of the skin showing routes of penetration: (A) intracellular pathways, across the intact horny layer, (B) intercellular pathways, between the intact horny layers or (C) through the hair follicles with the associated sebaceous glands. Skin section stained with haematoxilin and Eosin. (SC) Stratum Corneum, [EP] viable epidermis and [D] dermis.

In the intracellular route, (Figure 1-A) the compounds pass through the corneocytes lipid membrane (lipophilic pathway) and cytoplasm (hydrophilic center of the cell) and thus, the compounds must have amphiphilic characteristic to be able to be taken by this route. Penetration between corneocytes is the route that most compounds penetrate the skin and it is known as intercellular pathway [9]. Considering that corneocytes are not stacked parallel to one another in the SC layers, the compound has a sinuous pathway to pass when penetrating between them (Figure 1-B). The appendageal pathway (Figure 1-C) is believed not to be a significant penetration pathway for most molecules since the number of sebaceous glands on the total skin surface represents not more than 0.1% [9]. However, it is an important site of action for acne treatment since it targets the hair follicles.

In order to overcome these strong barrier exerted by the SC, the skin permeability status can be actively modified by different external and internal factors such as climate, physical stressors, and a number of skin disorders [10]. The literature also reports the association of strategies to improve anti-inflammatory drug penetration through the skin [11]. Anti-inflammatory drugs can be loaded in conventional pharmaceutical forms as cream or ointments and systems in nanoscale such as nanodispersions, nanoemulsions, liposomes and others [2,12]. However, for the evaluation of all these and other systems, it's necessary a reliable bioanalytical method for quantifying real drug concentration within and across the skin after in vitro and in vivo permeation studies [6]. Percutaneous penetration of different drugs can be studied in vivo using healthy volunteers. However, due to regulatory restrictions, in vitro studies usually employ animal skin membranes (snake, porcine, mouse) and more recently, new technologies of reconstituted human skin models are being envisioned as important sources, even considering the human skin as the 'golden standard' [3,13-18].

Ideally, the use of human skin to evaluate in vitro penetration properties of candidate drugs is desired for the most researchers. However, specimens of human skin of sufficient size, quantity and quality for penetration experiments are not highly accessible [19]. Also, they have a low standardization level due to variability related to gender, race, age and anatomical site of the donor [20]. Several previous studies reported that human SC and epidermis thickness is comparable to what is observed in porcine ear skin [21]. Furthermore, porcine ear skin and human skin also shows similarities in developed pars papillaris and abundant subdermal fatty tissue [22,23], hair follicle content, pigmentation, collagen and lipid composition [21,24]. Besides this, its use presents advantages due to their small size, the low cost and easy handling [8]. Furthermore, there is extensive scientific literature implying the similarities between human skin and animal skin, as well as indications of the OECD 428 in vitro dermal penetration guidance for use of skin from both human and animal source [25], which encouraged the use of animal skin models to replace human skin in these experiments. Reconstituted human skin models have several advantages for their in vitro application, especially because they are a tissue obtained from human cells. However, they are very expensive, difficult to standardize and have limitations, such as the membrane thickness for in vitro penetration and permeation studies (Depieri et al. 2015). For efficacy tests, this kind of skin membrane is relevant, however, for penetration and permeation in vitro studies it leads to differences of barrier function compared to human skin [8].

VERTICAL FRANZ DIFFUSION CELLS AS THE MOST USED APPARATUS TO EVALUATE DRUG DELIVERY ACROSS THE SKIN

In vitro percutaneous penetration studies have become one of the most relevant studies in the dermatological field, but not enough attention has been given to transdermal permeation of pharmaceutical transdermal forms. The United States, British and European pharmacopoeias describes the apparatus of paddle over disk, cylindrical and reciprocating holder (apparatus 5, 6 and 7 respectively) to evaluate the transdermal release of some drugs, including anti-inflammatory drugs [26]. Still, there is no consensus about the use of one effective apparatus for both release and permeation assay. Scientific literature describes Vertical Diffusion Cell (VDC) as a potential to predict the in vitro profile of both release and permeation of topical and transdermal semi-solid formulations [27-30]. It was released in 1997 the SUPAC-SS Guidance [31], which recommends in vitro VDC testing for semi-solid dosage forms at the development or improvement of topical dosage forms. In 2009, a Pharmacopeial Forum suggested the use of FDA paddle method and VCD for in vitro release and permeation of product performance quality tests for topical and transdermal dosage, respectively [32].

Franz vertical diffusion cell is the most used apparatus for drug penetration studies and it was first described in 1975 by Franz [33]. Back in that time, it consisted of a model very similar to the VDC currently available (Figure 2).

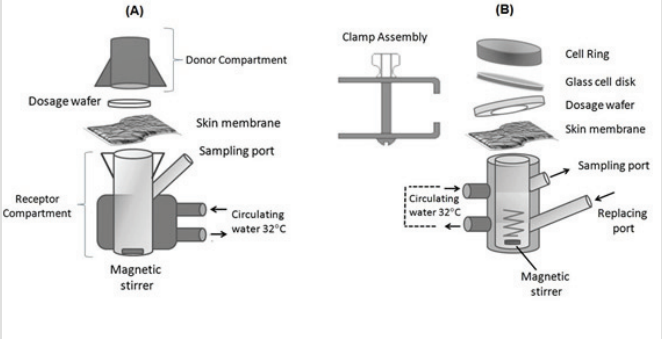

Figure 2: Schematic representation of vertical diffusion cell. (A) Franz cell first described by Franz in 1975 and (B) currently Franz cells.

The Franz cell is made from clear glass and uses a clamp to secure and isolate the donor side of the cell from the acceptor side. The clamp also ensures that all of the components and the membranes remain in place during the test. A glass disk is used to support the dosage and facilitates the observation of the donor material during the test. A magnetic stirrer is used to turn the helix and magnet, mixing the acceptor media to maintain a homogeneous mixture. The cell temperature is controlled via the water jacket ports using a bath circulation apparatus. Several variations to Franz cell model have been developed, in special related to differences in the receptor chamber volume, permeation area, stirring speed, automated collection of receptor fluid, static or dynamic flow-through diffusion cells and design characteristics [28], but the choosing the right method depends on drug solubility and sink conditions maintenance [29].

DEVELOPMENT AND VALIDATION OF THE EXTRACTING PROCESS OF DRUGS RETAINED IN SKIN LAYERS: SOLID-LIQUID EXTRACTION APPROACH

Literature reports several analytical and bioanalytical HPLC methods for anti-inflammatory skin recovery from different layers of the skin, making them very useful tools for rapid drug quantification in skin, as well as for permeation and retention studies in vitro. For that, an optimized drug extraction process from skin samples in different layers such as SC tape stripped and Epidermis (without SC) plus dermis [EP+D] should be developed and validated by selecting the most effective parameters: ideal volumes of extractor solvent (in many cases organic solvent is needed), ultrasound bath time, different centrifugation times and influence of high speed homogenization or tissue cutters [3]. The SC layers can be separated to the reminiscent [EP+D] by the simple and efficient tape stripping technique [34]. This procedure consisted to adhesive tapes (15 to 20 pieces) consecutively applied from the same treated skin area. The adhesive tape is pressed onto the skin for a defined period and quickly removed. The first tape strips contain almost a complete cell layer of corneocytes. In that way increasing the number of tape stripping applications, the numbers of corneocytes at the skin tend to diminish [34]. Finally, when tape stripping process ends, the remaining tissue is called as Epidermis and dermis [EP+D]. The [EP+D] can be processed as a unit or it may be separated by thermal procedure using 60 °C hot water during 1 minute [35]. Thus, after SC and [EP+D] or [EP] and [D] separation, the drug skin recovery by solid-liquid extraction process previously validated can be finally started.

In solid-liquid extraction, the drug is dispersed in a solid matrix and extracted to the solvent while the solid phase is then removed by filtration. Anti-inflammatory drugs skin recovery rates have been evaluated by spiking samples using three different drug concentrations (corresponding to the high, medium and low concentration standard of the analytical calibration curve) and comparing the amounts of drug extracted with the amounts of drug initially added. Satisfactory drug skin recovery rates in the range of 70 to 110% are considered. Briefly, SC tape stripped and [EP+D] should be spiked with anti-inflammatory drug solutions (in known concentrations) in a defined amount. Then, SC or [EP+D] samples containing the anti-inflammatory drug solutions should be left for some minutes to allow drug penetration on biological material followed by a complete evaporation of solvent using air flow.

An optimized drug extraction by solid-liquid process must be validated using different solvents as extraction solvents (in known volume, which was about 2 to 5 mL in many cases). Nevertheless, the extraction solvent choice depends on the drug solubility characteristics. Lipophilic drugs can be treated using methanolic solution or similar apolar solvents while hydrophilic drugs can be treated using an aqueous solution as phosphate buffer (pH 7.4). After addition of the extraction solvent, an internal standard at known concentration should be added to each sample. All samples should be shaken for a defined amount of time and maintained in ultrasound bath (to break the cutaneous cells and release the drug) followed by centrifugation process. Finally, samples should be filtered and the drug amount assessed by a quantitative method, such as HPLC.

CHALLENGES AND CRITICAL STEPS

Skin matrices samples are complex and represent the major bioanalytical challenge. The drug recovery from the skin, as well as drug separation from interferences, is a challenging factor for validation methods. So, anti-inflammatory drugs may be extracted from skin samples considering critical steps and possible difficulties. Even tape stripping technique is classified as a simple and easy assay to separate SC from [EP+D], the best condition for tape stripping procedure needs to be standardized in order to achieve reliable and reproducible results [36]. First, the uniform pressure on the skin is a basic factor influencing the amount of SC removed [34]. That may be achieved by the application of a roller to attached to the skin surface or a constant cylindrical weight applied on the skin surface, or even by using the operator finger pressure to press the tape onto the skin surface [34]. Secondly, a constant removal velocity of the stripping the tape should always be applied, once the slowing down or increase in speed could result in the amount of corneocytes adhered on the tape strip increased or decreased, respectively. At least, discarding of the first tape strip should be appropriately addressed because it represents unabsorbed drug on the skin surface. On the other hand, different amounts of SC can be removed with the first tape strips and they should be considered in the method validation [34].

To recover the drug from the tape stripped skin, the use of specific extraction solvents with different polarities is required [25]. Extraction solvent volume should be as low as possible in order to obtain a drug concentration higher than the quantification limit of the bioanalytical method. However, this volume must be enough to submerge all stripped adhesive tapes (SC samples), which could become a critical step of the extraction procedure. A mixture of organic solvent and water solution at 3mL (methanol: water, 72:28, v/v) was able to recover 90% of celecoxib from SC [3], while more hydrophobic vitamin K was 85% recovered from SC using 100% of methanol [37].

Considering drug recovery from [EP+D], critical procedures also occur when EP+D are processed as a homogenate. Ultra Turrax® tissue cutter used prior to the ultrasound bath was able to improve the recovery rate of drug from EP+D [38]. Its use must be validated case by case

considering the drug physicochemical characteristics, which may be favorable or unfavorable to increase the rate of drug skin recovery. Recently, the use of tissue cutter improved nicotine extraction, reaching 1.2-fold values [38]. On the other hand, their use did not improve celecoxib extraction values [3]. Meantime, when Ultra Turrax tissue cutter is used, care must be taken to avoid titanium sample contamination which can be released from equipment in prolonged use and increased of temperature.

Notably, care must be taken at the step of spiking samples of both adhesive tapes containing SC and remaining [EP+D]. A low volume (no more that 50 microliters) of standard drug solution (at known concentration) should be used in order to reach only the biological sample surface, considering that higher volumes can be spilled on the glass tube before penetrating the skin, and consequently, easily solubilized in extraction solvent.

TROUBLESHOOTING

In dermatological studies, the use of tape stripping is widely applied to investigate distribution profile, penetration and safety of different compounds [39]. As related above, several factors are known to affect this technique, such as the site of application, type of tape, application pressure and removal speed [34]. It is important to keep the composition and distribution of the adhesive layer on the tape strip which eventually affects the selectivity of the bioanalytical method [40]. D-Squame® discs (CuDerm, Dallas, Tex., USA) have been considered a "golden standard" for tape stripping technique. This was found to be a repeatable, quick, easy and reproducible method to evaluate protein content of tape strips. However, several adhesive tapes types with different physicochemical properties have already been used in a variety of in vitro permeation and retention studies in addition to the D-Squame®, such as Transpore® (3M, St. Paul, Minn., USA); Micropore® (3M); DurexMR (3M); Scotch® cellophane clear tape 5912 (3M) and Corneofix® [16,17,40-43]. In the last decade, Bashir and colleagues reported no significant differences in cumulative protein removal of three commercial adhesive tapes, such as the "golden standard" D-Squame® and Durex (3M) adhesives tapes [44]. On the other hand, the selectivity of the analytical method was impaired when adhesive tape DurexMR (3M) (composed by water-based acrylic adhesive) was used for glycoalkaloids drugs skin recovery studies and it was solved by replacing the tape to Durex 500MR (3M), composed by resin and rubber based adhesives [40].

In addition, the amount of SC removed by tape stripping technique can be influenced by the degree of skin hydration at the end of permeation studies [25,45]. In vitro permeation studies at a period of time greater than 24 hours probably promotes skin in an advanced state of hydration, which impairs the removal of SC by the adhesive tapes. It is believed that removal of skin parts from the epidermal layer by tape stripping procedure can be happen when hydrophilic vehicle are used. Still, to solve this problem, waiting around an hour from skin under bench absorbent paper a prior to starting the tape stripping procedure can guarantee better results, avoiding the removal epidermis layer parts in the last adhesive tapes.

Another common interfering event on the analytical method occurs when the chromatography peaks of drug are overlapped with the skin interference peaks. Tamara Angelo and colleagues reported a series of modifications in mobile phase composition (methanol: acetonitrile: water) and flow rates (1.0 to 1.5 mL/min-1) but drug and interferences peaks still eluted at the same retention time. However, when a chromatographic column with larger particle size was tested as an attempt to improve peak resolution it was able to move the drug peak to a range out of skin interferences elution [46].

METHODS APPLICATION

Several anti-inflammatory drugs were extracted from the skin samples and effectively monitored by high performance liquid chromatography (HPLC) methods with strictly validated protocols. Thus, several protocols for drug recovery from skin samples were reviewed (Table 1) and achieved high drug recovery rates as discussed below.

1. CELECOXIB

Celecoxib (CXB) is a nonsteroidal anti-inflammatory drug with selective cyclooxygenase-2 inhibitory activity and approved orally by the FDA for the treatment of rheumatoid arthritis, osteoarthritis acute pain and primary dysmenorrhea [47]. Long term oral administration of CXB causes serious gastrointestinal side effects, as oral CXB formulations require to be administered at high daily doses cardiovascular side effects are also reported [48]. Thus, the problems associated with oral administration have been minimized with development of CXB transdermal delivery systems using the skin as an alternate route of administration. A developed and validated skin recovery method of CXB was firstly reported by Praça and colleagues. The HPLC analysis procedure using RP-18 chromatographic column and mobile phase composed by methanol: water with flow rate of 0.8 mL/min at room temperature. The injection volume was 20 µL. Results after 24 h of CXB in vitro skin permeation (from a donor phase containing CXB at 2.0%) showed average values of 1.09 (± 0.65) and 8.55 (± 2.90) µg/cm2 of CXB retained in SC and [EP+D], respectively. Optimum conditions of the validated solid-liquid extraction procedure were selected by using mobile phase as extraction solvent at 5 and 3 mL for CXB extraction from SC and [EP+D], respectively; incubation samples in an ultrasonic bath for 15 minutes and centrifugation at 2500 rpm for 3 minutes were also performed. The CXB recovery after extraction from SC and [EP+D] was higher than 90% [3].

2. VITAMIN K

Several important chronic diseases with an inflammatory background have been associated with vitamin K (vitK) deficiency such bowel disease, chronic kidney disease, fibrosis, osteoporosis and pancreatitis [49]. Recently, Hodges et al., showed a review paper focused on the proposed cellular and molecular aspects of vitamin K in regulating skin inflammation [49].

Considering that vitK is a lipophilic vitamin, their solubility in oily formulations can improve their penetration into the deeper tissues of the skin, where it is supposed to exert its anti-inflammatory effect, and because of this the topical vitK application has been studied [37,50]. Lopes et al., reported a validated extraction procedure of vitK from skin samples. In the occasion, the absolute recovery of vitK from the skin was determined by spiking skin sample with methanolic solution containing vitK at 100 and 200µg/mL. The spiked skin sections were allowed to rest for 20 minutes and the skins were stripped with 15 pieces of adhesive tape; the first one was discarded, and the other tapes containing the SC were immersed in 5 mL of methanol. The remaining [EP+D] was cut in small pieces and immersed in 2 mL of methanol. Both SC and [EP+D] samples were vortex-stirred for 2 minutes, and bath sonicated for 30 minutes. The methanolic phase was filtered using a 0.45 µm membrane, and the resulting filtrate assayed for vitK by HPLC using a Lichrospher® 100 RP-18 column equilibrated at 40 °C. A mobile phase of methanol 100% and flow rate of 1.0 mL/min was used. Under these conditions, the retention time of vitK was 3.6 min. The peak of vitK was well resolved, the vitamin was quantified without the interference of other skin compounds and recovery raters of vitK from skin were found in the range of 85% for both SC and [EP+D] [37].

3. KETOPROFEN

Ketoprofen (KET) is an anti-inflammatory drug widely recommended to treat mild to moderate pain conditions such as rheumatoid arthritis, osteoarthritis, and pain after surgical operations, and others [51]. However, the skin absorption of KET is limited due to its poor water solubility.

To overcome this drawback, oil-based formulations have been reported to successfully improve both solubility of KET and its skin permeation [52]. Previously, our research group evaluated the delivery of ketoprofen into skin layers from generic and reference products in order to assess the interchangeability between topical semi-solid products concerning the drug skin penetration. In vitro skin permeation experiments were carried out using Franz-type diffusion cells and porcine ears skin. After 3 hours of permeation study, the skin sample was stripped with 20 pieces of adhesive tape in order to remove the SC and five of them were grouped per vial containing 1 mL of methanol. The remaining [EP+D] was cut in small pieces and added to vial containing 1 mL of methanol. Next, the samples were vortex-stirred for 2 minutes, bath sonicated for 30 minutes and assayed for KET by the mentioned validated HPLC method. The isocratic mobile phase consisted of acetonitrile: 0.02 M phosphate buffer (pH 7.4 ± 0.2) at a flow rate of 0.7 ml/min using a RP-18 chromatographic column. The ketoprofen skin recovery using this validated procedure showed values greater than 90% [53].

4. LIPOIC ACID (LA)

Lipoic Acid (LA) is an endogenous organosulfur compound with potent anti-inflammatory action independently of its antioxidant activity. LA is often used as a topical drug for the treatment of skin disorders. Recently, Campos and colleagues reported three sensitive and specific HPLC methods for LA using Ultraviolet (UV), Electrochemical (EC) or Evaporative Light Scattering (ELS) detection. These methods demonstrate different linearity ranges. The chromatographic separations were performed by RP-18 chromatographic column and isocratic elution using an acidic mobile phase composed by acetonitrile: dihydrogenphosphate solution (pH 2.5; 0.1 M) or acetic acid at a flow rate of 0.8 ml/min. The LA skin recovery validation was performed by spiking SC and [EP+D] skin samples with LA standard solution (1000 µg/mL) and 6 mL of acetonitrile was used as extraction solution. The samples were vortex-stirred for 2 minutes. The [EP+D] was grinded for 1 min through an Ultra Turrax® tissue cutter. Then, SC and [EP+D] samples were placed into ultrasound water bath for 15 minutes, centrifuged at 1360 × g for 5 minutes and filtered using a 0.45µm membrane. Finally, the extraction of LA from skin samples showed high recovery rates such as 81.15% (± 8.35) and 73.78% (± 4.98) for SC and [EP+D], respectively. The authors considered these optimized HPLC methods as simple, rapid and reliable for LA determination into the skin [54].

5. THYMOL

Thymol is a monoterpene widely applied to treat several skin disorders [55,56]. Recent researches have reported thymol as a promising anti-inflammatory and cicatrizing agent [57] suggesting a potential use in topical formulations. Considering the similarity of the thymol molecule and the skin components, a series of methods modifications (in mobile phase, chromatographic column and flow rate) from previous reported HPLC thymol determination was recently performed in order to avoid overlapping peaks of skin matrices interferences and thymol [46]. Optimized separation were achieved with a RP-18 column (300 × 3.9 mm; 10 µm), mobile phase composed of acetonitrile: water and , flow rate of 1.5 mL/min and oven temperature at 40 °C. This validation procedure certified that the method was selective for thymol determination even when extracted from skin matrices. Thymol recovery rates from the skin layers were higher than 90%. The method was adequate for use in skin permeation studies employing thymol topical formulations [46].

6. CYCLOSPORIN A

The peptide Cyclosporin A (CysA) is an immunosuppressive drug and has proved therapeutic potential in the treatment of skin inflammatory disorders [58]. In addition, it is a highly lipophilic peptide that presents extremely poor skin penetration, unless an associated chemical or physical strategy is used [59].

In the last decade, Lopes and colleagues have been investigating whether reverse cubic and hexagonal phases of monoolein enhance the cutaneous penetration of CysA, performing in vitro and in vivo studies using porcine ear skin and hairless mice (as animal skin model), respectively. In vitro, the cubic phase increased the penetration of CysA in SC and [E + D] at 12 h post-application while the reverse hexagonal phase increased CysA penetration in [E + D] at 6 h and percutaneous delivery at 7.5 h post-application. In vivo, both liquid crystalline phases increased CysA skin penetration. The skin was stripped with 15 pieces of adhesive tape, the first one was discarded, and the other tapes containing the SC were immersed in 5 mL methanol, and [EP+D] was cut in small pieces in 2 mL of methanol. The samples were vortex-stirred for 2 minutes, and bath sonicated for 30 minutes. The methanolic phase was filtered using a 0.45 µm membrane, and the resulting filtrate assayed for CysA by HPLC. The separation was performed by a RP-18 column equilibrated at 60°C. A mobile phase of acetonitrile and water at flow rate of 1 mL/min was used. Under these conditions, the retention time of CysA was 9.1 minutes [59].

7. DEXAMETHASONE

Dexamethasone (DXM) is a lipophilic glucocorticoid widely used in topical anti-inflammatory therapy from ointments and creams and is classified as moderately strong according to their efficacy into four potency classes [60]. Topical administration of corticosteroids has been used as an alternative of oral administration because they are highly hydrophobic and oral administration needs high doses to achieve the therapeutic level. This fact favours the occurrence of adverse effects like osteoporosis, high sugar concentration in the blood, hypertension, intestinal and stomach bleeding due to ulcers and fluid retention [61].

Recently, Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS) for determination of DXM from the ex vivo human skin revealed DXM penetrates deeper when applied in polymer nanoparticles and nanocrystals than when applied on DXM solution [35,62]. This newly and sensitive LCMS showed the limit of detection about 0.2 ng/ml. After topical treatment with different DXM formulation the epidermis and dermis were separated by heat (1 minute in 60 °C hot water) and the dermis was horizontally cut into 50 µm sections at −24 °C using a freeze microtome. Epidermis and dermis skin slices were subjected to 5 freeze-thaw cycles. Following the addition of 50 pmol Dex-d4 as internal standard, samples were extracted 3 times with 500 µl ethyl acetate. Combined extracts were exsiccated by vacuum rotation, them, the dried residues were reconstituted in 200 µl acetonitrile and DXM concentrations in the ex vivo human skin samples were quantified. Analyses were conducted with a liquid chromatography system coupled to a triple quadrupole mass spectrometer interfaced with an electrospray ion source operating in the positive ion mode (ESI+). Chromatographic separation was carried out using a RP-18 column and water:acetonitrile acidified with 0.1% formic acid as mobile phase. However, no recovery rates of DXM extracted from the skin were reported by the authors [35].

On the other hand, optimized DXM recovery from different skin layers by the ratio of the amount of drug recovery from three spiked samples to the amount of drug added were previously reported by Sugisaka and colleagues. The SC was separated by tape-stripping technique using fifteen pieces of adhesive tape (Scotch Book Tape, 3M, St. Paul, MN) and adhesive tapes containing SC or tape-stripped skin [EP+D] were spiked with sufficient quantities of methanolic solution of DXM corresponding to 5, 10 and 50 µg/mL. The SC samples were immersed in 4 mL of methanol and vortex-stirred for 2 minutes. The [EP + D] samples were cut into small pieces, immersed in 3 ml of methanol, vortex-mixed for 2 minutes and treated with cutter tissue in order to complete tissue homogenization (2500 rpm for 2 minutes). Then, all samples followed to bath-sonicated (40 KHz, continuous mode) for 30 minutes, were centrifuged (5 min at 600 x g) and filtrated through a 0.45 µm pore size membrane filter. The solutions obtained were assessed by validated HPLC with a RP-18

chromatographic column at 30 °C. The isocratic mobile phase was methanol:water and flow rate of 0.7 ml/min. DXM skin recovery rates in different skin layers using extraction conditions developed in the occasion were in the range of 96 to 85% for SC and EP+D, respectively [63].

8. TRETINOIN

Tretinoin (TT) is considered the gold standard for treatment of skin inflammatory diseases, such as acne [64] even if associated with poor tolerability, resulting in limited patient compliance [65,66]. In that way, several drug delivery strategies based on nanotechnologies have been studied to overcome the TT drawbacks [67]. One of us recently reported In vitro and in vivo topical delivery studies of tretinoin-loaded ultradeformable vesicles considering that these nanosystems are highly promising tools to enhance the percutaneous transport of tretinoin across the skin barrier and also to increase the formulation stability at absorption site and reduce the drug induced irritation. The in vitro skin permeation study was carried out through the whole skin also under non-occlusive method. For that, the formulation containing TT loaded was spread over the skin and 24 h later, the skin samples were rinsed to remove excess formulation and dried with filter paper. SC was removed using 10 adhesive tapes and a cylinder (2 kg) on a foam and an acrylic disk (both with an area of 5.73 cm2) resulting in a pressure of 349.3 g/cm2. That was used in order to improve the reproducibility of the tape stripping technique. This pressure was applied for 10 seconds for each tape. A tetrahydrofuran solution (70%) was added to SC and [EP+D] samples at 5 and 1mL, respectively. Both samples were stirred for 2 minutes and sonicated for 20 minutes and the supernatant was injected on HPLC to quantify the amount of TT skin retained in each skin layer. Chromatographic separations were performed using a RP-18 column and mobile phase composed of 0.01% trifluoroacetic acid: acetonitrile at a flow rate of 1.0mL/min. Tetrahydrofuran solution was selected as the extraction solvent due to its greater reproducibility among the samples and higher TT skin recovery rate in SC and [EP+D] such were approximately 62 and 65%, respectively [12].

Table 1: Summarized HPLC method for quantification of several anti-inflammatory drugs retained into the skin
Stratum Corneum (SC); Epidermis and dermis (EP+D); Total Skin (TS); Lower Limit of Quantification (LLOQ); Electrochemical Detector (EC); Evaporation Light Scattering Detector (ELD); Reference (Ref).

Anti-inflammatory drugs	HPLC method							Ref
	Mobile phase	Detector	Linearity (µg/ml)	Precision and Accuracy	LLOQ (µg/ml)	Extraction solvent	Drug skin Recovery	
Celecoxib	Methanol : water (72:28)	UV (251nm)	0.1 to 3.0	>92%	0.1	Methanol : water (70:30)	98% SC and EP+D	Praça et al., [3]
Vitamin K	Methanol 100%	UV (260nm)	0.03 to 500	>90%	No Mentioned	Methanol 100%	>85% TS	Lopes et al., [37]
Ketoprofen	Acetonitrile : phosphate buffer (60:40)	UV (260nm)	0.1 to 50	100 to 111%	0.1	Methanol 100%	>90% TS	Aliberti et al., [2]
Lipoic acid	Acetonitrile : dihvdrogenphosphate solution (50: 50)	UV (340nm)	0.9 to 8	>90%	0.9	Acetonitrile 100%	80% SC and 73% EP+D	Campos et al., [54]
	Acetonitrile :dihvdragenphosphate solution (60:40)	EC (+1.1V)	0.13 to 8	>93%	0.13		75% SC and 98% EP+D	
	Acetonitrile : acetic acid (60:40)	ELS	4 to 100	>93%	4.0		89% SC and EP+D	
Thymol	Acetonitrile : water (35:45)	UV (278nm)	0.5 to 15	>98%	0.14	Methanol 100%	Close to 100%	Angelo et al., [46]
Cyclosporin A	Acetonitrile : water (67:33)	UV (210nm)	0.15 to 500	>94%	0.15	Methanol 100%	89% SC and 93% EP+D	Lopes et al., [59]
Dexamethasone	Methanol : water (70:30)	UV (239nm)	0.5 to 50	>85%	0.5	Methanol 100%	96% SC and 85% EP+D	Sugisaka et al., [63]
Tretinoin	Acetonitrile : water (85:15)	UV (342nm)	No mentioned	No Mentioned	No Mentioned	Tetrhydrofuran 70%	62.4% SC and 65% EP+D	Ascenso et al., [12]

CONCLUSION

This work provided a discussion of the main skin samples processing techniques by solid-liquid extraction procedure in order to effective recover anti-inflammatory drugs retained into the skin. The extraction procedure from skin (total skin or a specific layer) is easy, fast and inexpensive, and most methods use solvents or solutions associated with ultrasound or homogenization of tissues. Technical critical steps and troubleshooting were highlighted and a series of anti-inflammatory drugs method application were reported. Finally, this review has a comprehensive approach in order to help researchers design their experiments and update the applicability and advances in this area of expertise.

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