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Quantification of 5-FU in skin samples for the development of new delivery systems for topical cancer treatment

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5-Fluorouracil (5-FU) is used for the topical treatment of pre-cancerous skin lesions. However, previous reports focus on 5-FU quantification in plasma samples, failing to provide information about drug quantification in the skin. Therefore, the aim of this work was to develop a simple and reliable analytical method employing HPLC-UV for 5-FU quantification in skin samples. Chromatographic separation was obtained using the commonly available Lichrospher RP-C18 endcapped column. Porcine ear skin matrix-based analytical curves with thymine, as internal standard, were used. 5-FU was eluted at 5.2 min and thymine at 13.0 min. The method was selective, precise and accurate in the linear range from 0.3 to 6 µg/mL. The samples were stable after three cycles of freeze/thawing and extraction efficiency rates above 95% were obtained. *In vitro* skin penetration studies of an aqueous solution and a commercial cream of 5-FU were performed. The cream improved 5-FU retention into the skin and permeation through the skin compared to the solution. Therefore, the method developed herein can be applied to the study of formulations for topical delivery of 5-FU.

1. Introduction

The drug 5-fluorouracil (5-FU) (Fig. 1) is a fluoropyrimidine available in the clinic for the treatment of a variety of solid tumors, such as skin cancer, gastrointestinal tract tumors and breast cancer (Kaiser et al. 2003; Sanson et al. 2011). Its mechanism of cytotoxicity is mainly due to the incorporation of fluoronucleotides into RNA and DNA of tumor cells and to the inhibition of thymidylate synthase, an enzyme involved in nucleotide synthesis (Longley et al. 2003). Although 5-FU has the potential to treat different malignancies by intravenous injection, it has to be applied in high doses weekly, causing gastrointestinal toxicity and myelotoxicity, (Kaiser et al. 2003).

In this context, the topical application of 5-FU is an attractive alternative and has reached the clinic. Topical creams are available on the market such as Efurix 5% (Valeant Pharmaceuticals, CA), Fluoroplex 1% (Alergan, CA) and Carac 0.5% (Dermik Laboratories, PA). The 5% dosage has been approved by FDA since the 1970s for actinic keratosis, a type of precancerous lesion known by the presence of atypical keratinocytes (McGillis and Fein 2004). The use of 5-FU, however, is not limited to this condition, and has been investigated for the treatment of basal cell carcinoma and squamous cell carcinoma (Morse et al. 2003; Petrilli et al., 2017) when incorporated in drug delivery systems.

There are many methods for the quantification of 5-FU in plasma samples (Peters et al. 1993). They usually employ HPLC using UV detection (Alsarra and Alarifi 2004; Escoriaza et al. 1999; de Mattos, Khalil, and Mainardes 2013; Sanson et al. 2011), gas chromatography combined to mass spectrometry (GC/MS) (Anderson et al. 1997) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), or not hydrophilic interaction chromatography (HILIC) (Kosovec et al. 2008; Pisano et al. 2005; Wang et al. 1998). However, in the studies where 5-FU is topically applied (Chen et al. 2013; Cosco et al. 2015), 5-FU quantification was usually performed in the receptor solution and thus, there is no information on its concentration in the skin, where the tumors are localized.

For the investigation of 5-FU skin penetration, *in vitro* experiments can be performed using Franz diffusion cells. In these experiments, the donor compartment, wherein the formulation containing the drug is placed, is separated from a receiving chamber, which mimics the systemic circulation, by a piece of skin. To mimic human skin, porcine ear skin is used due to similarities such as spare hair coat, epidermis thickness, dermis with well differentiated papillary body and large amount of elastic tissue. Furthermore, its easy obtainment from slaughterhouses and avoidance of variability related to gender, race, age and anatomical site of the donor are advantages that justify the choice of porcine ear skin as a model membrane (Barbero and Frasch 2009). To evaluate the influence of formulation components on 5-FU skin retention and permeation, the development and validation of analytical methods with adequate sensitivity and selectivity has major importance due to endogenous compounds of the skin which may interfere on the drug quantification (Bachhav and Kalia 2011).

Thus, the main objective of this study was to develop a new and simple HPLC method for quantification of 5-FU in skin samples and receptor solution of *in vitro* skin penetration studies using an

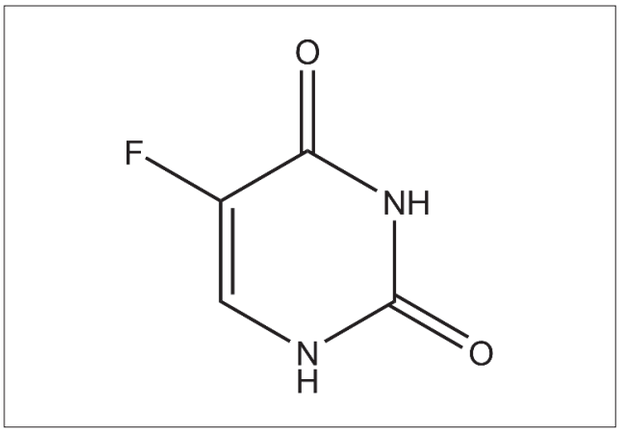

Fig. 1: Chemical structure of 5-FU

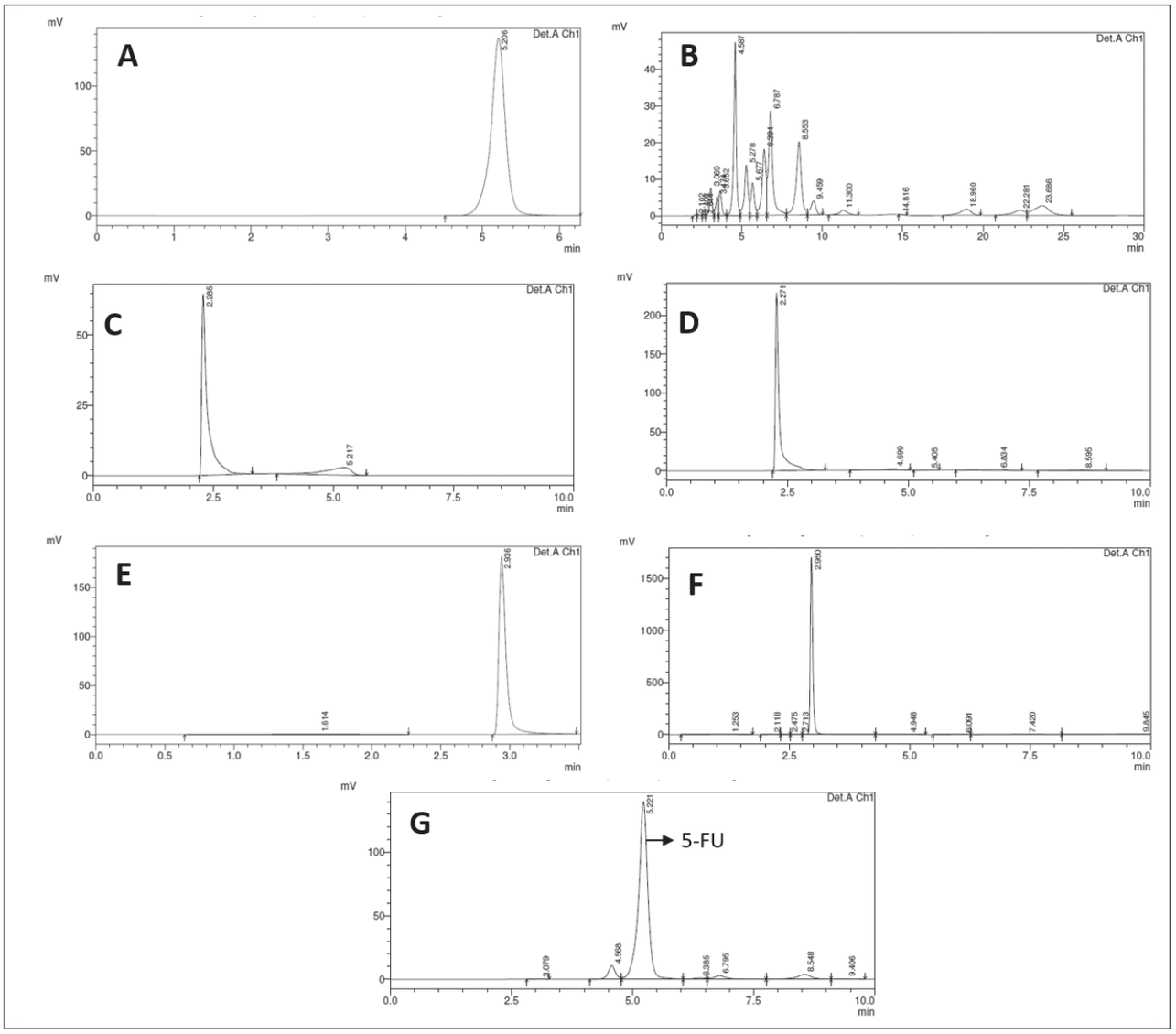

Fig. 2: Chromatograms of 5-FU solutions and skin samples in different solvents A) standard 5-FU solution in water without skin; B) skin sample in water; C) standard 5-FU solution in methanol without skin; D) skin sample in methanol; E) standard 5-FU solution in acetonitrile without skin; F) skin sample in acetonitrile and G) 5-FU in skin matrix-based sample after liquid-liquid extraction with ethyl acetate, as described in section 4.5.1.

RP-C18 column, commonly available in laboratories. Two different formulations were evaluated to explore the utility of the method; an aqueous solution and a commercial cream, both containing 5% of 5-FU. The FDA guideline for bioanalytical method validation was taken as reference for the reliability assessment of the developed method (FDA and Food and Drug Administration 2013).

2. Investigations and results

2.1. Chromatographic conditions and sample preparation

Elution of 5-FU using the mobile phases composed by acetonitrile and 0.1% phosphoric acid aqueous solution (4:96 or 2:98, v/v, pH 2.6) or methanol and 0.1% phosphoric acid aqueous solution (2:98, v/v, pH 2.6) resulted in short retention times such as 3.5 min, 4.2 min and 4.1 min, respectively. The use of 0.02% phosphoric acid aqueous solution at pH 2.6, without organic solvent was able to increase retention time to 5.2 min, without interfering peaks, and thus was selected for further studies.

Figure 2 shows the chromatograms of 5-FU solutions and skin samples prepared with different solvents (Fig. 2A to 2F) and after liquid-liquid extraction with ethyl acetate (Figure 2G), as described in section 4.4.2.

For the skin samples prepared in water without previous liquid-liquid extraction, many interfering peaks were observed that compromised 5-FU quantification (Fig. 2A and 2B). Otherwise, skin samples in acetonitrile or methanol resulted in indistinct drug and skin interference (Fig. 2C, 2D, 2E, 2F).

The extraction of the skin components with water followed by a liquid-liquid extraction with ethyl acetate before resuspension in water resulted in 5-FU elution with adequate resolution (retention time= 5.2 min) (Fig. 2G).

2.2. Method assessment

The regression of the bioanalytical matrix-based curve of 5-FU in the range of 0.3 to 6 µg/mL and submitted to the liquid-liquid extraction procedure presented r^2>0.999 with slope and intercept of 0.4072 and 0.0647, respectively. The selectivity was analyzed by the absence of interfering peaks from the biological matrix using the same extraction steps. No interference was observed in 5-FU retention time or thymine (tr= 13.0 min). The LOD and LOQ were 0.033 µg/mL and 0.3 µg/mL, respectively.

Table 1 shows intraday and interday precision, expressed as relative standard deviations (RSD), and accuracy of the developed method.

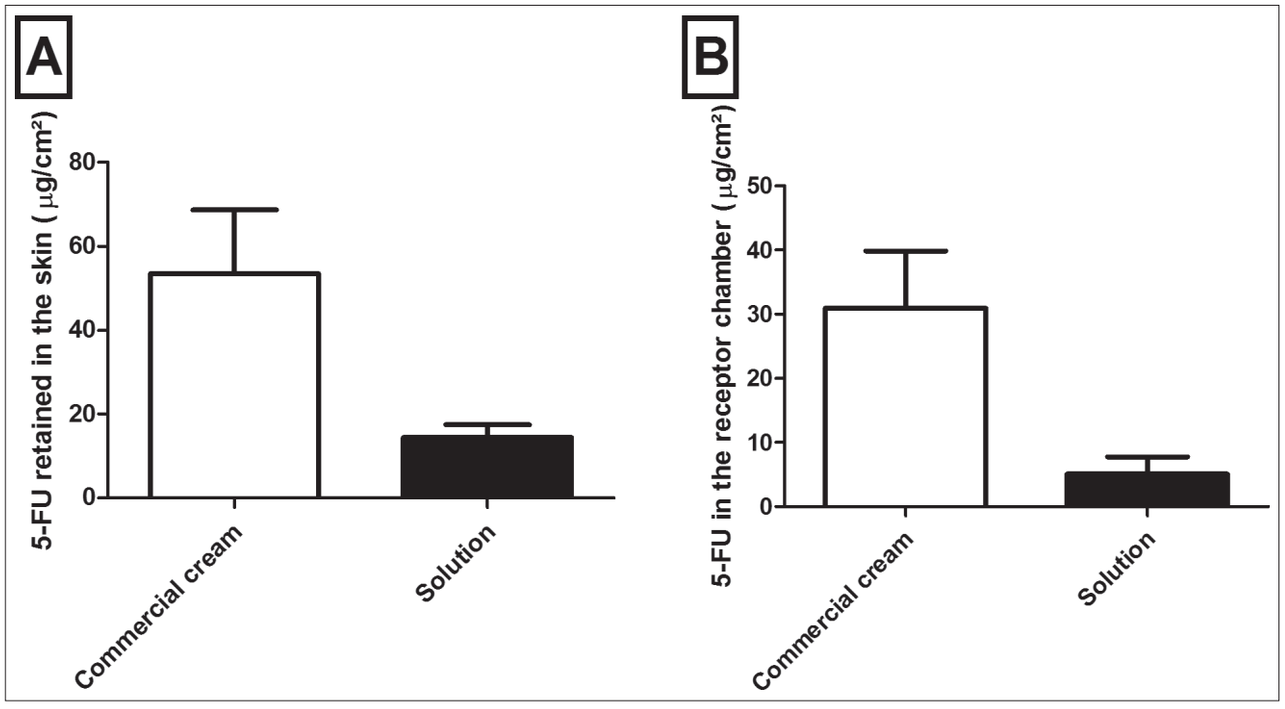

Fig. 3: 5-FU recovered from de skin (A) and from the receptor chamber (B) after 24 h of *in vitro* skin penetration of 5-FU from a commercial cream and a solution.

Table 1: Intraday and interday accuracy and precision for 5-FU quantification

Intraday (n=6)		
Concentration (μg/mL)	Accuracy* (%)	RSD (%)
0.3	108.4	0.7
0.5	93.0	6.3
1.0	96.5	3.2
2.0	100.5	1.5
4.0	102.2	0.1
6.0	99.1	0.2
Interday (n=3)		
Concentration (μg/mL)	Accuracy* (%)	RSD (%)
0.3	108.7	0.9
0.5	94.6	2.3
1.0	95.3	1.8
2.0	101.0	0.7
4.0	102.0	0.2
6.0	99.2	0.5

* shows as the percentage recoveries for 5-FU spiked blank skin samples

Table 2: Stability of 5-FU matrix-based analytical curve with the internal standard thymine after 3 cycles of freeze (-20 ºC) and thawing (25 ºC)

Concentration (μg/mL)	RSD (%)
0.3	1.02
0.5	1.02
1.0	0.95
2.0	1.00
4.0	0.99
6.0	1.00

Table 3: 5-FU skin recovery

Concentration (μg/mL)	Recovery (%)
0.3	109.9 ± 12.5
2.0	95.9 ± 2.5
6.0	95.5 ± 1.0

The stability of 5-FU samples spiked with porcine ear skin after three freeze/thawing cycles are shown in Table 2.

2.3. Extraction efficiency

Aiming for the method application in comparative skin penetration studies of 5-FU formulations, the extraction efficiency of 5-FU from the skin was evaluated in three different levels (0.3 μg/mL; 2 μg/mL and 6 μg/mL). The results were compared to the bioanalytical matrix-based curve and were expressed in percentage of drug recovery in Table 3.

2.4. Application of the bioanalytical method: in vitro skin penetration of 5-FU

Figure 3 shows 5-FU quantified in the skin and in the receptor solution after 24 h of 5-FU *in vitro* skin penetration from a solution and a commercial cream.

The amounts of 5-FU accumulated in the skin and permeated through the skin (in the receptor solution) were approximately 4-fold and 6-fold higher for the commercial cream, respectively, compared to the 5-FU solution (p<0.05, Student t-test).

3. Discussion

As known from previous reports (Alsarra and Alarifi 2004; de Mattos, Khalil, and Mainardes 2013; Sabitha et al. 2013; Zhang et al. 2012), mobile phases composed of purified water with acidic pH (near pH=3) with as without up to 10% organic solvents are appropriate for 5-FU elution. 5-FU is a weak acid (Merino et al. 1999), thus the use of acidic pH results in the non-ionized form of 5-FU,

improving its interaction with the reverse phase column, enabling higher retention times. Consequently, acidic mobile phases have been used in several studies to improve the interaction of the drug with RP-18 columns (Alsarra and Alarifi 2004; Sanson et al. 2011). In our studies, the mobile phase composed of phosphoric acid aqueous solution was the one that eluted 5-FU in adequate retention time, without interferents, and it was hence selected. The mobile phases that contained an organic phase mixture with the acidic aqueous solution resulted in very short retention times, near the column dead volume.

Using the selected mobile phase, the best skin treatment for 5-FU quantification was investigated. Sample treatments are considered methods for reducing matrix effects and improving extraction efficiency (Zhu et al. 2016). Using water, methanol or acetonitrile to prepare the skin samples followed by direct injection in the HPLC resulted in chromatograms with several interfering peaks that compromised 5-FU quantification (Fig. 2A to 2F). A liquid-liquid extraction after the use of water to extract 5-FU was able to reduce skin interference in 5-FU quantification (Fig. 2G). Liquid-liquid extraction has been extensively used previously for HPLC quantification. It involves the partition of the sample between two immiscible phases (aqueous and organic), in which the extraction efficiency is influenced by the affinity of the drug for the solvent, aqueous and organic phases ratio and number of extractions. It is easy to perform and can cause denaturation of proteins, which is useful for skin extraction, reducing the number of interferents injected into the chromatographic column (Queiroz et al. 2001). In our experiments, the extraction using water followed by a liquid-liquid extraction with ethyl acetate resulted in suitable 5-FU elution (Fig. 2G), which can be explained by the 5-FU electron donor characteristics, allowing its extraction by ethyl acetate, a hydrogen bound acceptor molecule that enables the extraction of electron donor solutes (Khot et al. 2010; Siek 1978). Furthermore, the 5-FU recovery from skin samples using the liquid-liquid extraction was higher than 95% (Table III).

The RSD values for both intraday and interday analyses (Table 1) were lower than 6.3%, which is in accordance with the FDA guideline for bioanalytical methods validation which defines values of no more than 15% of RSD, except for the LOQ which values can achieve 20% (FDA and Food and Drug Administration 2013). The values obtained for accuracy ranged from 93% to 108.7%, which are also in accordance with FDA guidelines (FDA 2013) and previous reports (Barth et al. 2011). Stability was observed for the samples, since the RSD values for the samples submitted to three freeze and thawing cycles were under 15% (Table 2) and thus are within the acceptance limits (FDA and Food and Drug Administration 2013).

Finally, the *in vitro* skin penetration of 5-FU from a commercial cream and an aqueous solution was compared. The method developed herein was used to quantify the skin retained 5-FU (Fig. 3A) and the skin permeated 5-FU (in the receptor solution) (Fig. 3B). The commercial cream significantly improved 5-FU skin permeation compared with the solution (Fig. 3). The commercial formulation is an oil-in-water emulsion. Generally, emulsions enhance drugs skin penetration when compared to simple solutions or suspensions (Sakata et al. 2014). Furthermore, the commercial cream evaluated in this work contained penetration enhancers such as stearyl alcohol ($C_{18}H_{37}OH$), a long chain saturated fatty alcohol, and propylene glycol. Long chain alcohols are able to disorganize the stratum corneum and thus improve skin penetration of drugs (Lane 2013). For instance, Andega et al. (2001) tested a variety of long chain fatty alcohols for their effect on the model drug melatonin permeability through porcine and human skin. Authors observed a parabolic relation between the length of the carbon chain of saturated fatty alcohols and the improved skin drug penetration effect on both porcine and human skin. The mechanism involved for propylene glycol acting as a penetration enhancer is not completely understood, but can be related to the cutaneous dehydration and incorporation on the polar heads of lipids (Lane 2013). Propylene glycol can also demonstrate synergistic effects with other penetration enhancers (Williams and Barry 2004).

Within this context, 5-FU creams have been used for years for the treatment of skin lesions, mainly actinic keratosis (Werschler 2008). The response rate for different concentrations of creams (0.5%, 1% and 5%) is usually high, about 87%, as found in a meta-analysis of seven 5-FU studies using topical creams with treatment duration of two to four weeks. Howevers, skin irritation is a concern and can reduce patient compliance (Gupta 2002; Werschler 2008). Loven et al. (2002) compared the efficacy and tolerability of 0.5 and 5% 5-FU creams applied topically in a single-blind, randomized study with patients that presented six or more actinic keratosis lesions. The patients were treated during four weeks with the 0.5% cream (applied once daily) and 5% cream (applied twice daily) to opposite sides of the face. The authors found a reduction of number of actinic keratosis lesions from 11.3 to 2.5 and 10.3 to 4.2 for 0.5% cream and 5% cream, respectively, which was a statistically significant difference (p= 0.044). Both treatments demonstrated similar profiles of investigator-rated irritation, however fewer patients treated with the 0.5% cream reported these symptoms. These results can be explained by the local effect of 0.5% cream compared to an extensive systemic absorption for the 5% cream (Levy 2001). In that occasion, authors compared different 0.5% 5-FU formulations in a microsphere delivery system and 5% 5-FU commercial cream for the flux and percutaneous absorption by human cadaver skin. The flux for the 5% cream was 20-40-fold higher than the 0.5% formulation, which suggests a systemic exposure for higher concentrations of the cream. However, they did not quantify the 5-FU retained into the skin to prove the efficiency of their microspheres to deliver 5-FU to skin tumors.

Herein, we assessed the permeation of 5-FU in the receptor chamber solution and found concentrations approximately 6-fold greater for the 5% cream compared to 5-FU solution, which might indicate a systemic exposure. We also determined that the higher 5-FU permeation from the cream resulted in a higher accumulation of 5-FU into the skin, which can be advantageous for skin tumors treatment.

In conclusion, we reported the assessment of a simple method for 5-FU quantification in skin samples and receptor solution. The HPLC method was developed under isocratic flow employing an RP-18 column, which is easily available in laboratories. The method was precise and accurate in the tested range. The bioanalytical curve was stable after three cycles of freeze/thawing and recovery values near 100% were obtained for the drug extracted from skin samples. The method was successfully applied for the *in vitro* quantification of 5-FU retained in the skin and permeated to the receptor solution for both 5-FU solution and commercial cream. The commercial cream improved 6-fold and 4-fold skin permeation and retention, respectively, when compared to the solution. We hope this method can be useful for the development and evaluation of new formulations containing 5-FU for topical application.

4. Experimental

4.1. Chemicals and reagents

5-Fluorouracil (5-FU≥ 99%) and thymine (≥ 99%) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Purified water was obtained from a Millipore® Direct Q 3 UV with pump (Molsheim, France). Phosphoric acid (85%) was obtained from Macron Chemicals (Pennsylvania, United States). The purchased 5-FU commercial cream 5% (Efurix®, Valeant Pharmaceuticals, CA) contains the following excipients, stearyl alcohol, white petrolatum, polysorbate 60, propylene glycol, methylparaben, propylparaben and purified water.

4.2. Chromatographic conditions

The analyses were carried out using a UFLC Prominence HPLC Shimadzu (Kyoto, Japan) equipped with a LC-20AT binary pump, a DGU-20A3 degasser, a SIL-20A auto sampler, a CTO-20A column oven, a SPD-20A UV-vis detector and a CBM-20A controller system. The chromatographic analyses were performed using an RP-C18 endcapped (250 x 4.6 mm i.d., 5 µm particle size, 100 Å, Lichrospher, Darmstadt, Germany) analytical column with an RP-18 (4 x 4 mm, 5 µm, Merck, Darmstadt, Germany) guard column operated in room temperature (25ºC).

Different compositions of mobile phases under isocratic elution were tested, such as: acetonitrile and 0.1% phosphoric acid aqueous solution (4:96, v/v, pH 2.6 and 2:98, v/v, pH 2,6); methanol and 0.1% phosphoric acid aqueous solution (4:96, v/v, pH 2.6); and 0.02% phosphoric acid aqueous solution (pH 2.6). The choice of the best composition of the mobile phase was based on peaks resolution and retention factor for 5-FU solutions in water, methanol and acetonitrile. The best chromatographic separation was obtained using 0.02% phosphoric acid aqueous solution (pH 2.6), under isocratic flow at 1 mL/min. The total run time was 16 min, using injection volume of 100 µL and UV detector operating at 265 nm.

4.3. Porcine ear skin preparation

Freshly excised porcine ears were obtained from a local slaughterhouse (Ipuã-SP, Brazil). Skin from the outer portion of porcine ears was dissected with the aid of a scalpel. Dermatomization was performed, resulting in a parallel cut of the skin surface with uniform thickness of 700 µm. The skin was stored at -80 ºC for a maximum of 30 days (Praça et al. 2011). 5-FU and thymine were absent in blank skin samples. In order to guarantee the skin barrier integrity, at the day of each experiment, the electrical resistivity was determined according to Tang et al. (2001). Skin samples with electrical resistivity under 35 kΩ/cm^2 were considered damaged and were not used in the experiments.

4.4. Method assessment

4.4.1. Linearity

A calibration curve was prepared by spiking blank samples of porcine ear skin with working solutions of 5-FU and internal standard thymine. Therefore, the porcine skin cut in small pieces (0.95 cm^2) was spiked with 5-FU and thymine solutions, resulting in 0.3, 0.5, 1, 2, 4 and 6 µg/mL of 5-FU and in 2 µg/mL of thymine (n=6 replicates per concentration), composing the matrix-based curve. Each sample was vortex mixed for 1 min in 5 mL of water, followed by ultrasound bath for 15 min. Thereafter, they were centrifuged for 10 min at 20,000 x *g* and filtered in 0.22 µm PVDF membranes. A liquid-liquid extraction was performed based on previous studies (Alsarra and Alarifi 2004; Sanson et al. 2011) by adding 1 mL of the filtered samples in 5 mL of ethyl acetate, followed by vortex mixing for 7 min and centrifugation for 10 min at 4,000 x *g*. The supernatant was collected and reserved and the extraction was repeated. The organic phase (10 mL) was evaporated to dryness at 60 ºC, reconstituted in 500 µL ultrapure water and vortex mixed for 1 min. The samples of the matrix-based curve were filtered in 0.22 µm PVDF membranes, transferred to microvolume glass inserts and 100 µL were injected into the HPLC system. Calibration curves were prepared using a least square linear regression of the ratio 5-FU peak area/thymine peak area against the concentration of 5-FU.

4.4.2. Limits of detection (LOD) and quantification (LOQ)

The detectability was determined by the LOD and LOQ. The LOD was determined using Eq. (1) (FDA 2013):

$$LOD = \text{SD} \times \frac{3}{\text{Slope}} \quad (1)$$

where SD = standard deviation and *Slope* = slope of the calibration curve. The LOQ was considered the lowest concentration obtained with precision which does not exceed 20% of the CV and accuracy within 20% of the nominal concentration.

4.4.3. Selectivity

The selectivity of the method was assessed by the investigation of the presence of interfering peaks, which could compromise the 5-FU and thymine determination. Therefore, the analyses of blank skin samples submitted through the extraction procedure in the absence and presence of 5-FU and thymine were compared.

4.4.4. Precision and accuracy

Precision and accuracy were evaluated by the analyses of all concentrations, using six replicates, prepared and injected in the same day (intraday). Furthermore, the interday assay was carried out by injecting the different concentrations in three consecutive days (using three replicates per concentration freshly prepared each day). The results of precision were calculated using the variation coefficient (CV) obtained by the ratio between the standard deviation (SD) and the mean result (Eq. 2) (FDA 2013):

$$\text{CV}(\%) = \frac{SD}{Mean} \times 100 \quad (2)$$

The results for accuracy, both intra-day and inter-day were calculated using Eq. 3 (FDA 2013):

$$\text{Accuracy}(\%) \frac{Measured\ concentration}{Nominal\ concentration} \times 100 \quad (3)$$

4.4.5. Stability

5-FU matrix-based analytical samples were submitted to three freeze/thawing cycles (-20 °C, 25 °C) and changes in the ratios of 5-FU/internal standard were monitored in order to evaluate the stability of the samples in biological matrix.

4.5. Method application in comparative in vitro skin penetration

For the *in vitro* skin penetration experiments, the skin samples (n=6) were placed on Franz diffusion cells (area 0.95 cm^2) with the stratum corneum facing the donor compartment, which was filled with 5-FU solution or 70 µg of commercial cream, both containing 5% of 5-FU, resulting in 3.5 mg total amount of 5-FU applied (OECD 2004). The receptor chamber was filled with 16 mL of phosphate buffer saline (100 mM, pH 7.4). After 24 h, the skin samples were removed, rinsed with distilled water and cut into small pieces, submitted to the extraction, filtered and injected in the HPLC system.
The results were reported as mean ± SD (n=6). Statistical analyses were performed using t-test with level of significance set as α=0.05.

4.5.1. Extraction procedure

A volume of 5 mL of water was added to total area of skin samples (0.95 cm^2), cut into small pieces, vortex mixed for 1 min followed by ultrasound bath for 15 min, added with 80 µL of the internal standard thymine (final concentration 2 µg/mL), centrifuged for 10 min at 20,000 x *g* and filtered using 0.22 µm PVDF membranes. A liquid-liquid extraction was performed based on previous studies (Alsarra and Alarifi 2004; Sanson et al. 2011). Briefly, 1 mL of the filtered samples was added to 5 mL ethyl acetate, vortex mixed for 7 min and centrifuged for 10 min at 4,000 x *g*. The organic phase was collected and a new extraction with the aqueous phase was repeated. Both supernatant fractions (organic phase) were combined (10 mL) and evaporated to dryness at 60 ºC under compressed air flow. Thereafter, the extracted fraction was dissolved by 500 µL of ultrapure water and agitated by vortex mixer for 1 min. The samples were filtered in 0.22 µm PVDF membranes, transferred to microvolume glass inserts and 100 µL were injected into the HPLC system.
The extraction procedure was evaluated by assessing the extraction efficiency of 5-FU from skin sample. The extraction efficiency involves the analyses of spiked blank skin samples (Praça et al. 2011). The skin samples with 0.95 cm^2 area were spiked with 5-FU solutions and dried resulting in final concentrations of 0.3 µg/mL, 2 µg/mL and 6 µg/mL after resuspension with 5 mL purified water. The 5-FU concentration in the spiked blank samples were calculated and expressed as percentage, that is, the obtained concentration multiplied by 100 and divided by the expected concentration (used in the fortification) in order to obtain the recovery.

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Conflicts if interest: None declared.

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