Development and Validation of a HPLC Method for 5-Fu Quantification Following In Vitro Skin Permeation Assay

Rosa J Praca and Bentley MF*

School of Pharmaceutical Sciences, University of Sao Paulo, Brazil

**ABSTRACT**

Introduction: The drug 5-Fluorouracil (5-FU) is used in various therapies as anti-metabolic including several cancers and many skin diseases. In this work, we develop a bioanalytical method to quantify the 5-FU into the porcine ear skin samples after in vitro skin permeation studies by HPLC-UV. However, skin matrices samples represent the major bioanalytical challenge due to the complexity of this matrix. This method was developed in a High-Performance Liquid Chromatography (Shimadzu LC 10AD), a C8 Phenomenex (100mm x 4.6mm, 5µm, 110A) chromatography column and detector UV-visible operated at 265nm. The mobile phase was composed by Methanol: Water (10:90) v/v, acidified with 0.05% (v/v) orthophosphoric acid (pH 4.5), injection volume was 30 µL and the mobile phase flow was optimized to 0.8mL per minute. The skin samples from in vitro permeation studies were subjected to tape stripping, to remove the stratum corneum, then dose the drug retained on this layer separately. Afterward, the remaining epidermis and dermis samples were submitted by a liquid-liquid extraction process was successfully employed, more than 80% of 5-FU was recovered from the skin, for the purpose of dosing the retained drug from the viable epidermis and dermis and 5-FU assessed by HPLC. The obtained results by HPLC agreed to the qualitative confocal laser microscopy. The proposed bioanalytical method was simple, selective, sensible, presented limits of detection and quantification were 0.07 and 0.1 µg/mL respectively. The method was also precise, accurate, robust and efficient in eliminating the interferences of the skin maintaining the bioanalytical method confidence.

Introduction

The drug 5-fluorouracil (5-FU) was developed in 1957 as a potential anticancer and was widely used in several types of cancer and considered one of the most useful drugs. Its mechanism of action has been studied for decades, and by the 1980s and 1990s its function was elucidated as anti-metabolic, acting as a pyrimidine analogue [1-4]. The 5-FU is being used as an anti-metabolic in the topical treatment of various skin diseases with emphasis on skin cancer, vitiligo and psoriasis. However, a management strategy is recommended for each treatment, as biological barriers to drug
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(stratum corneum), extension and lesion status should be considered in order for the drug to reach the site at the appropriate concentration to exert a pharmacological effect [5]. For skin diseases, it is available commercially in the form of creams for topical application, such as Carac® 0.5% (Dermik Laboratories, Berwyn, PA), Fluoroplex® 1% (Allergan, Inc., Irvine, CA) and Efudix® 5% (Valeant Pharmaceuticals, Int., Costa Mesa, CA). Due to its use in several diseases, there are already some studies that quantify 5-FU in complex matrices such as human serum and plasma and the most not followed the principles of green chemical [6-8]. In recent studies on the topical application of 5-FU in cutaneous tissue [6-9], a method that quantifies this drug in this tissue and the layers that compose it is essential. Moreover, depending on the target, it is still interesting to quantify the drug in the skin layers.

The High Performance Liquid Chromatography (HPLC) is a derivative of classical column chromatography, has the same functions of separation and quantification, but in an improved way. In addition to gas chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry, the liquid chromatography/ultraviolet (HPLC/UV) is considered an important tool of analytical chemistry for determination of several analytes in biological matrices samples since it allows monitoring its stability, adulterants of raw materials, as well as, its high specificity, sensitivity and throughput, among other applications [10]. Considering the 5-FU, some studies have already dosed the drug from skin complex matrix by HPLC [9,11-19]. Meantime, the validated methods were mostly analytical methods and the minority of these studies don't reported about the interference of the skin components and the difficulties of recovering the drug from this tissue [9,14-19]. Green analytical chemistry is a relationship between the environment and a society, where it is not only concerned with analytical factors but also with the residues generated by them [20,21]. Understanding the importance of using 5-FU for skin diseases and the small number of studies that deal with their quantification into the skin, this work aims at the development, validate and optimization of the bioanalytical methods to quantify 5-FU after permeation studies on skin samples using the minimal organic solvents as possible.

Material and Methods

1. Chemicals and reagents

5-Fluorouracil ≥ 99% was purchased from Sigma (Sigma-Aldrich, St. Louis, Missouri, EUA). Orthophosphoric acid and Methanol chromatographic grade were purchased from Merck (Merck, Germany). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Commercial Cream (5% 5-FU) was purchased from commercial pharmacy.

2. In vitro permeation studies by vertical Franz diffusion cells

In vitro permeation studies of 5-FU from commercial cream was conducted to verify its skin penetration in different layers of the skin. The 5-FU skin penetration was evaluated using ear porcine skin as animal membrane in a vertical Franz diffusion cell (Hanson Instruments, USA) with a diffusion area of 1.77 cm². Fresh porcine ears were obtained from a local slaughterhouse (Fripon, Pontal, SP - Brazil). The ear was refrigerated on ice during transportation. When they arrived they were immediately cleaned with running water. The skin samples were carefully dissected for the maximum removal of the subcutaneous tissue and fat, dermatomized to obtain a uniform tissue thickness (500 μm) (DermatonNouvag, Switzerland).

The recipient compartment of vertical diffusion cells were filled with 7 ml of 100 mM phosphate buffer (pH 7.4, ±0.2) and maintained at 32°C (temperature controlled by water-jacketed cell) and constant stirring (400 rpm) during the whole experiment period. Finally, the experiment was carried out according to Praca and colleagues [22]. Three hundred micrograms of 5-FU cream (5%) was applied in the donor compartment. After 10 minutes, 20 minutes, 30 minutes, 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 12h, 15h, 18h, 21h and 24h of permeation study the receptor solutions were collected and 5-FU amount was assessed by validated HPLC method. Calculations for quantification of 5-FU from receptor solution was based on the equation below:

\[ Q_{\text{real,t}} = C_{\text{measured,t}} \cdot V_r + V_n \cdot \sum_{n=1}^{m-1} C_{a,n} \]

where,

- \( Q_{\text{real,t}} \): Amount accumulated in the time in question,
- \( C_{\text{measured,t}} \): measured quantity of the collection in the time in question,
- \( V_r \): Diffusion cell volume,
- \( V_n \): sample volume removed,
- \( C_{a,n} \): Concentration of sample removed

3. In vitro skin sample processing techniques

To quantify the 5-FU skin retained, after in vitro permeation studies, different skin layers such as stratum corneum tape stripped (SC) and viable epidermis and dermis (EP+D) were obtained as described below. All the experiments were conducted protected from light and done in quintuplicate (n=5).

4. Skin extraction process of 5-fluouracil retained in different layers of the skin

After 30 minutes, 1, 3, 6, 12 and 24 hours of in vitro skin penetration studies, the skin samples were taken from the vertical Franz diffusion cells and the excess of the cream was carefully removed with paper. Then, skin treatment was procedure in two steps. First, solid-liquid extraction to recovery the 5-FU from the skin to aqueous solution was applied, precisely because it has good solubility in water, followed by liquid-liquid extraction process, using the minimum organic solvent, according to the green chemistry [11], in order to remove the skin interference. The 5-FU was collected in the aqueous phase.

4.1. Tape stripping: The SC was separated to the reminiscent epidermis and dermis by the conventional, simple and efficient tape stripping technique [22]. The skin permeation area (1.77 cm²) was tape stripped (Figure 1-A) with 15 adhesive tapes consecutively applied from the same skin area and quickly removed. All 15 adhesive tapes containing the SC were shaken with 5 ml of 100 mM phosphate buffer (pH 7.4, ±0.2) for a minute and the supernatant was analyzed by HPLC.

4.2. Epidermis and Dermis (EP+D): After removal of the SC, the remaining EP+D were processed together by validated treatment as describe in the (Figure 1-B). The 5-FU retained into the skin after in vitro permeation studies was extracted to the aqueous phase from a specific and innovative skin extraction process. The EP+D was cut into small pieces and triturated in a Homogenizer ultra-turrax (Marconi, MA102, Sao Paulo, Brazil) with 5 mL of phosphate buffer pH 7.4 for a minute. After then, the homogenate solution was filtrated through a 0.45µm membrane (Millipore, Burlington, Massachusetts, EUA). Exact 2 mL of Ethyl Acetate solution was mixture with the filtrated to remove the interferences substances from the skin and mixed (Phoenix, Lufereco AP56, Sao Paulo, Brazil) for 30 seconds and centrifugated (Rotofix 32, Hettich, Germany) at 600 xg for 5 minutes, thereby reaching complete separation of the organic and aqueous phases. The aqueous phase was withdrawn and 5-FU amount quantified by HPLC.

4.3. 5-FU skin recovery validation: The recovery of the drug from the skin layers is a critical step for its reliable quantification, so it is extremely important to know the efficiency of this extraction process. The optimum extraction methodology for 5-FU recovered from EP+D was obtained when 50 µL of 5-FU standard solution at
0.1 and 20µg/mL were individually added to 1.77cm² of the skin and left for 30 minutes to allow 5-FU penetration on biological material, followed by a complete evaporation of water. Then, the skin was cut into small pieces and processed as described above using phosphate buffer pH 7.4 followed by Ethyl Acetate solution as extraction solvents. In fact, a series of organic solvents were tested where 5-FU is insoluble, such were ethyl ether, hexane, ethyl acetate and methylene chloride and these results will be addressed in discussion topic of this work. The aqueous phase was withdrawn and the 5-FU amount determined was compared with those respectively 5-FU standards aqueous solutions with skin extraction process free.

4.4. Skin sectioning technique using a cryostat: After 24 h of the permeation study described in 2.2 item, the diffusion areas of skin samples were frozen in Tissue-Tek® (Pelco International, Redding, CA, USA) and vertical (n=3) sectioned skin, (20 µm) slices, was provided using a cryostat (Leica, Wetzlar, Germany). The slices were treated with nucleus dye (DAPI) and fluoromount aqueous mounting medium and observed under confocal laser microscopy LEICA – TCS SP2, (Leica, Heidelberg, Germany).

5. Quantitative and qualitative bioanalytical methods

5.1. High Liquid Performance Chromatography

5.1.1. Chromatographic conditions: The method was developed and validated in a High Performance Liquid Chromatography (Shimadzu LC 10AD), using a detector UV-visible (SPD 10A).

![HPLC profiles from the extracting process of the skin interference components](image)
The working wavelength was 265nm, with a mobile phase Methanol (10): Water (90) v/v, acidified with 0.05% (v/v) orthophosphoric acid (pH 4.5). The system contained an auto injector (SIL 10AF) and the injection volume was 30µL. The flow was 0.8 mL/min. The Column was C8 Phenomenex (100mm x 4.6mm, 5µm, 110A) placed into a thermostat (CTO 10A) controlled oven compartment with a temperature of 25°C. The software used was the CLASS VP.

5.1.2. Method validation: The method was validated using matrix overlay, where the analyte is added on a matrix solution where it will be analyzed. The assay was validated in accordance with international conference on harmonization (ICH) and European Medicine Agency (EMA) guidelines for the biological method validation [23-25] and followed the principles of green chemistry using the minimum organic solvent in the mobile phase, in the sample treatment process and solubilizing the samples in water. The matrix solution was prepared from a porcine skin, which was triturated in water and subsequently filtered. Thus, linearity, matrix effect, selectivity, sensitivity, precision, accuracy and robustness were evaluated.

(i) Linearity and matrix effect: A stock solution of 5-FU (50µg/mL) was prepared by dissolving the appropriated amount of the drug in water and the stability of this solution was assessed over 30 days. The standards solutions were obtained from stock solution in three different media such as water, complex matrix (skin sample) before and after treatment. The linearity of the three analytical curves was evaluated in order to observe the influence of the matrix (Matrix effect) and the 5-FU skin recovery rates, in front of the 5-FU in water (without interference). The three situations were evaluated in the concentrations range of 0.1µg/mL to 20µg/mL (n=5) and the residual distribution was performed by the Durbin Watson test.

(ii) Selectivity and sensitivity: The selectivity of the method was evaluated by individual analysis of possible interfering samples. Solutions containing skin matrix without 5-FU, receptor solution and mobile phase. Higher and lower limit of quantification (HLOQ and LLOQ respectively) were determined from the highest and lowest concentrations within the linearity range while Lower Limit of Detection (LLD) was determined when it was at least twice the height of the baseline. The acceptance criterion for LLD was lower than 20% [24].

(iii) Precision and accuracy: The method precision and accuracy were assessed from 9 determinations of different 5-FU concentrations: low, medium and high such was 0.5, 5 and 20µg/mL evaluated in 3 different days. Each replica was prepared independently. The Precision and accuracy were expressed as Coefficient of Variation (CV) and Relative Error in percentage (RE %), respectively. The acceptance criterion was variations values lower than 15%.

(iv) Robustness:

The method of robustness we used was the factorial planning in the Minitab Statistical Software, where small changes of the critical points of the method would be evaluated. The critical points selected were oven temperature, flow and mobile phase pH variations, and the response was retention time.

5.2. Confocal laser microscopy: The qualitative analysis of 5-FU and its distribution into the skin layers were also investigated by confocal laser microscopy using a LEICA – TCS SP2, (Leica, Heidelberg, Germany) and emissions filter of 488 nm and 638 nm was used to analyze the 5-FU and nucleus dye (DAPI). The images were recorded with identical sensitivity and exposure settings, considering the autofluorescence of the skin. These qualitative results were confirmed with those quantitative obtained by tape stripping and EP+D homogenate assessed in HPLC bioanalysis.

6. Statistical analysis

The results were expressed as mean ± Standard Deviation (SD) and analyzed using Graph Pad Prism® software. The Student’s test was used to compare two experimental groups with 95% of confidence. The Bonferroni test was used to compare multiples groups with 95% of confidence. The Durbin-Watson test was used for determinations of the residuals by the Minitab Statistical Software 2017.

Results

1. HPLC bioanalytical method
The 5-FU was extracted from the skin samples, as shown in item 2.3, and effectively monitored by HPLC and Confocal microscopy methods with strictly validated protocols. The optimized extracting the skin interference components were showed in (Figure 2). The most of the solvents tested, while removing part of the skin components from the aqueous phase, where 5-FU was present, were still substances that interfered with the chromatographic signal of the drug. Only the organic solvent ethyl acetate was able to remove the skin components from the aqueous phase.

In view of all the parameters described above, the 5-FU was analyzed and presented good resolution characteristics with approximately 3.0 minutes of retention time (Figure 2-A). In addition, the three curves of the linearity obtained ≥0.999 (Table 1 and Figure 3) and the higher and lower limit of quantification showed to be promising, presenting values of 250 and 0.1µg/mL respectively. Thereafter, the lower limit of detection was 5ng/mL for the curve in water and 70ng/mL for the other two situations.

Considering linearity data obtained in this work, the residue analysis was performed, as shown in (Figure 4). The data presented a homoscedasticity and the errors were considered random according to the Durbin Watson test. The parameters precision and accuracy were evaluated and are presented in (Table 1). The three levels of the curve were accurate and precision in the three conditions evaluated. The highest coefficient of variation (8.74) and highest % of relative error (7.52) was presented in the concentration of 0.5µg/mL in the curve 5-FU treated with the matrix in the within-day item. In the parameter robustness the variations were evaluated and presented in Figure 5, where we can observe that the variations in the oven temperature, mobile phase pH and flow rates did not influence the robustness of the bioanalytical method. The evaluation of 5-FU stability in water solution, the concentrated 50µg/mL of 5-FU was quantified from preparation day to 30 consecutive days. Thus, the 5-FU peaks area presented in the chromatogram of solution preparation day was considered 100% and it was monitored at the times: 24h, 3 days, 7 days, 14 days, 21 days and 30 days. Until the 14th day, a loss of 0.86% was evidenced while from the 21st day it was increased to 10% approximately. In this way, the 5-FU solution was considered stable until 14 days of preparation, after this time the areas were considered statistically different through the Student T test.

### 2. Method application

The 5-FU contained in the commercial product permeated across the skin and retained into the skin following in vitro permeation studies, were quantified by HPLC (Figure 6). In figure 6-A, we can see that in the time of 30 minutes there were already 17.84 µg/cm² of 5-FU in the permeated content and this was increasing as a function of time reaching approximately 140 and 350 µg/cm² of 5-FU at 8 and 24 hours respectively. The Figure 6-B shows the amount of 5-FU retained into the different layers of the skin, (SC, removed by tape striping, and EP+D) as well as the amount of the 5-FU permeate across the skin and plotted at the same test time. This quantification was performed after the treatment of the sample according to item 2.3.

At the final time of the assay, the skin samples were taken from the Franz diffusion cell and the 5-FU skin recovery was performed as well as at the times of 30 minutes, 3 hours, 6 hours and 12 hours. As a result, 5-FU present in the commercial cream penetrated all layers of the skin from the time of 30 minutes, where there were already about 50 µg/cm² of 5-FU in the SC and this was increased to non-linear manner over time. The EP+D layer also contained 50 µg/cm² of 5-FU in the time of 30 minutes.

but this retained amount did not show a pattern during the assay.

In the final time, 24 hours, a confocal microscopy analysis was performed to obtain a qualitative analysis that would complement the data obtained by HPLC. Under these circumstances the amount of 5-FU permeated across the skin after 24 hours of *in vitro* studies, as well as 5-FU skin retained into different skin layers and the respectively confocal images were summarized in (Figure 7). Consistent with HPLC analysis, confocal microscopy exposes the presence of 5-FU in both the stratum corneum and the viable epidermis and dermis. It is important emphasize that the auto fluorescence from different layers of untreated skin was removed from the background.

**Discussion**

The studies related to drugs that act topically on the skin need to be quantified in this specific tissue. In this way, we recognize the importance of a well-delineated permeation study and a method that is able to quantify the drug retained into the different layers of the skin as well as drug penetrated across the skin. Recently, our research group evaluated the critical parameters for *in vitro* skin permeation and penetration studies using different animal skin models. According to this study, the permeation study herein presented was based [22]. In fact, *in vitro* drug permeation and penetration studies

![Figure 4](image-url)
have been widely used to monitoring the drug penetration across the skin and quantifying its level in different layers of the skin [9,14,22,26]. However, the skin matrices samples remain the major bioanalytical challenge due to the complexity of this matrix. Many efforts were done to optimize the 5-FU skin recovery process by matrix effect evaluation as well as drug separation from the interfering components of the skin. Overall, the skin samples were subjected to tape striping technique, to remove the SC, and then, the drug retained on this layer was separately assessed by HPLC. Afterwards, the remaining EP+D were treated using optimized extraction process and the drug skin recovery was also quantified. These obtained results were challenged by the confocal laser microscopy.

1. Development and validation of HPLC assay

The 5-FU drug absorbs in the ultraviolet region, thus a UV-visible detector has been widely used [27] and it was considered in our work. Pascuet et al. [28] with the intention of studying the absorption spectrum of 5-FU, subjected 5-FU solutions to N2 laser beam irradiations, they observed that the absorption spectra exhibited bands in the range of 250 to 350 nm, and the main wavelength of the absorption band was 265 to 279 nm [28]. Recently, a wavelength was chosen to 265 nm [17,19] in concordance with our work. The 5-FU is a hydrophilic drug (PKa = 8.02) in this way, the use of a less hydrophobic column was chosen, C8. The mobile phase was chosen being composed in its majority for water, and thus obtains short retention times [17]. On the other hand using 100% of water as mobile phase, irregular chromatogram peaks were reported [17]. Suitable mixture of ultrapure water and organic solvent has overcome these limitations and 5-FU chromatograms have shown a symmetrical shape [29]. There are methods in the literature that use methanol and acetonitrile as the organic solvent as a mobile phase [5,9,13,16,30]. We replace the acetonitrile, due to green chemistry method [20,21,31]. Green chemistry or sustainable chemistry began in the 1990s when the United States Environmental Protection Agency (EPA) began work on pollution-reduction research programs based on the 1990 pollution prevention law. But the green chemical name was adopted in 1996 because of the intention not only to control the type of pollutants in the environment but to reduce the pollutants generated [20,32]. Paul Anastas and John Warner in 1998 defined some principles of green chemistry that have been improving and gaining space in the analytical area. In this way was introduced the term green analytical chemistry [20,21,31,32]. Lawrence [31] commented in his work how much a company can reduce an emission of polluting products in the environment and how much can be significant in the financial question, where toxic products are replaced by alternative products. And yet in this same work he comments on the prospects for 2020, where he cites a possible growth of this sustainable thinking [31]. Thus, the mobile phase chosen for us was a water methanol (90:10 v/v) mixture, in contrast to methods using up to 60% of this organic solvent [30]. Attempts were also made to decrease the volume of methanol used, but the 5-FU chromatography peak symmetry has not been reached and the tail factor increased. Hence, the mobile phase was acidified with...
**Figure 6:** Permeation and skin retention profiles of 5-FU from commercial cream. Values were expressed as mean ± standard deviation of at least five experiments. For the analysis to SC, the only one that don’t present statistical difference was 30 minutes to 3 hours; for the analysis to EP+D the only that present statistical difference was 6 hours to 12 hours (*); and for permeated content the only that don’t present statistical difference was 30 minutes to 3 hours.

**Figure 7:** Quantitative and qualitative skin distribution of 5-FU after in vitro permeation studies at 24 hours post application. Quantitative assay of in vitro permeation of 5-FU through porcine ear using a HPLC method (A) and comparative qualitative assay of in vitro permeation of 5-FU through porcine ear using a confocal microscopy (B-1) DAPI; (B-2) 5-FU fluorescence; (B-3) Merge with DAPI and 5-FU fluorescence in black background; (B-4) Merge with DAPI and 5-FU fluorescence in white background (* represents the statistical difference).
orthophosphoric acid (0.05%) and the mobile phase flow was optimized to 0.8 mL per minute, reaching a total run time about 5 minutes which allowed analyzing a large number of samples in a short period of time. Recently, chromatography method for quantify the 5FU from plasma and skin samples reported a high chromatographic run time which were 20 and 13 minutes respectively, which is considered a high retention time[17,19]. Furthermore, during the method development, the chromatography peak of the drug was overlapped with the skin interference peaks. So, different chromatography columns, wavelengths, changes in the column temperature, mobile phase compositions and flow variations were evaluated and as a result the skin components continued in the same 5-FU retention time (data not showed). Because of this, deeper investigations on Matrix Effect (ME) and treatment of the sample were conducted.

The ME occurs when substances inherent to the biological matrix sample overlapping to the drug and asymmetric chromatographic peaks are found. Because of this, investigation of the ME from biological samples extraction process has been considered mandatory during the development and validation of a bioanalytical method. However, the guidelines are still lacking indication about the most appropriate procedure to evaluate the ME during method validation [33]. Several different approaches were reported to assessment of ME each one is associated with advantages and disadvantages [22,24,25,34]. In our work a post-extraction spike method was successfully used. Briefly, this method consisted in direct injection of blank skin sample extracted, spiked after full sample preparation protocol with different concentrations of the 5-FU [34].

### Table 1: Linearity, Precision and accuracy of within-day and between-day of the 5-FU quantification by HPLC in different media.

<table>
<thead>
<tr>
<th>Parameter Linearity</th>
<th>5-FU treated with the matrix</th>
<th>5-FU in matrix (previously treated)</th>
<th>5-FU in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range (µg/mL)</td>
<td>0.1 – 20.0</td>
<td>0.1 – 20.0</td>
<td>0.1 – 20.0</td>
</tr>
<tr>
<td>Linear Equation*</td>
<td>y = 120243x + 60138</td>
<td>y = 132514x + 52417</td>
<td>y = 135307x + 13566</td>
</tr>
<tr>
<td>r²</td>
<td>0.9990</td>
<td>0.9996</td>
<td>0.9999</td>
</tr>
<tr>
<td>r</td>
<td>0.9994</td>
<td>0.9998</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter Precision and Accuracy</th>
<th>5-FU treated with the matrix</th>
<th>5-FU in matrix (previously treated)</th>
<th>5-FU in aqueous solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal concentration (µg/mL)</td>
<td>0.5</td>
<td>5</td>
<td>20</td>
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</table>

**Within-Day (n=3*)**

<table>
<thead>
<tr>
<th>Average analyzed concentration (µg/mL)</th>
<th>0.49±0.04</th>
<th>4.92±0.02</th>
<th>19.72±0.28</th>
<th>0.58±0.03</th>
<th>5.02±0.01</th>
<th>20.68±0.27</th>
<th>0.49±0.01</th>
<th>4.83±0.11</th>
<th>19.74±0.32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision (CV%)</td>
<td>8.74</td>
<td>0.51</td>
<td>1.45</td>
<td>6.74</td>
<td>0.3</td>
<td>1.32</td>
<td>2.84</td>
<td>2.31</td>
<td>1.64</td>
</tr>
<tr>
<td>Accuracy (RE, %)</td>
<td>7.52</td>
<td>0.81</td>
<td>2.04</td>
<td>1.38</td>
<td>-1.37</td>
<td>-4.78</td>
<td>2.51</td>
<td>3.19</td>
<td>1.86</td>
</tr>
</tbody>
</table>

**Between-Day (n=3**)**

<table>
<thead>
<tr>
<th>Average analyzed concentration (µg/mL)</th>
<th>0.49±0.01</th>
<th>5.04±0.03</th>
<th>19.91±0.05</th>
<th>0.47±0.02</th>
<th>4.96±0.01</th>
<th>20.10±0.27</th>
<th>0.49±0.008</th>
<th>4.87±0.05</th>
<th>19.83±0.17</th>
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</thead>
<tbody>
<tr>
<td>Precision (CV%)</td>
<td>7.72</td>
<td>0.98</td>
<td>0.96</td>
<td>6.57</td>
<td>0.31</td>
<td>1.32</td>
<td>1.88</td>
<td>1.07</td>
<td>0.87</td>
</tr>
<tr>
<td>Accuracy (RE, %)</td>
<td>5.74</td>
<td>-0.89</td>
<td>-2.57</td>
<td>1.39</td>
<td>2.41</td>
<td>-0.79</td>
<td>2.52</td>
<td>2.59</td>
<td>0.93</td>
</tr>
</tbody>
</table>

*number of determinations; **number of days; A Precision expressed as variation coefficient (CV); B Accuracy expressed as relative error percentage (RE, %)
The percentages of ME was calculated by comparing the peaks areas with the standard with those obtained by direct injection of the same concentration of the 5-FU solubilized in water, without matrix. Several trials were taken to minimize ME with different organic solvents which were ethyl ether, hexane, ethyl acetate and methylene chloride as precipitating agents. However, promising results were achieved exclusively when ethyl acetate was used, and it may be explained because it is an ester, a chemical group that can be presented interactions with a high affinity with the components of the skin mostly lipids and collagens. Consequently, the removal of skin components from the aqueous phase where 5-FU was found was important mainly at the drug lower limit quantification with accuracy and precision using this method. Similarly, the use of ethyl acetate was previously reported by Sanson et al. [35] as an extraction solvent of choice for plasma sample clean-up [35]. The bioanalytical curve of the skin matrix previously treated and spiked with 5-FU is possible to point that the skin cause a discreet decrease in the chromatographic areas of the concentrations of the curve, which can be evidenced by the angular coefficient of the curve. However, this curve has a close characteristic to those of the drug solubilized in water suggesting that there was slightly pronounced ME. To our knowledge, it is the first time that ME of skin samples containing the 5-FU skin samples was evaluated by post-extraction spiked method. A internal standard to prevent the risk of biological matrix interferences, losses in treatment processes, including 5-bromouracil and thymine, has been widely reported [19,31], but this substances make the method more complicated, running times usually increase a lot and it is very expensive, commercial standards are needed and there is no record of its practical use in compensating ME [34]. On the other hand, when the 5-FU was treated along the skin matrix extraction process evidenced drug losses less than 12% and it can be related due a partial solubility of the ethyl acetate in water in the same way, the minority portion of the drug could be carried to an organic phase. Moreover, it was efficient in eliminating the interferences of the skin maintaining the linearity of the bioanalytical method and high drug skin extraction rates for all levels of 5-FU concentrations in the bioanalytical curve. In general, the three bioanalytical curves of 5FU in different matrices were linear in the range of concentration studied. The LLOQ and LLOD were obtained with accuracy and precision using this method. The Precision and accuracy were evaluated from within-day and between-day assay and results within the values accepted by the guidelines were observed [24]. No more than 10% was evidenced as variation coefficient and relative error percentage, when three 5-FU concentrations in different matrices were assessed. In fact, they are far from the limits, proving the method confidence. Additionally, the residual values from the bioanalytical curves were analyzed statistically and according to the Durbin Watson test, the hypothesis of dependence among the residuals was rejected, admitting that the residuals were randomly distributed which was homoscedastic.

2. Method application
The in vitro skin permeation studies using Franz diffusion cells are the most widely used tools for monitoring the drug permeated/retained across the skin. This apparatus can be classified as static (mostly used for semi-solid pharmaceuticals products evaluation) or dynamic flow-through diffusion cells, commonly used for transdermal pharmaceuticals products test [26]. The 5-FU permeated across the skin after in vitro permeation studies using static vertical Franz diffusion cells have been currently explored [9,14,19] and it was optimized in our work by quantitative and qualitative bioanalytical methods. The amount of 5-FU from the commercial cream permeated across the skin was monitored until 24h and showed an increasing of drug amount exceeding the skin barriers in a time-dependent manner. These results can be better understanding when analyzed together with those of skin retention. A Higher amount of 5-FU was observed in SC, following permeated solution and finally EP+D. This lower 5-FU amount into EP+D skin layer can be explained considering their high permeation rates across the skin. When the same samples were challenging to qualitative confocal microscopy evaluations, the results were in the
agreement to those quantified in HPLC method. The drug distribution within the skin after 24 hours of its topical application displayed the 5-FU retained into the SC and EP+D and substantially lesser in the dermis layer of the skin. Thus, the characteristics of the commercial products may be related to the results obtained herein since its pharmaceutical form can affect the skin barrier integrity, hydration, and drug penetration across the skin can be increased [36]. Still, this method may be applied to various pharmaceutical forms where 5-FU is present.

**Conclusion**

The proposed HPLC method enables a simple, linear, precise, accurate and robust assay of the 5-FU in skin samples in a rapid chromatography run with good limits of detection and quantification, besides not showing a pronounced matrix effect. The liquid-liquid method of extracting 5-FU from the skin was efficient and the treatment of the sample presented excellent recoveries rates contributing to a reliable quantification. This method was applied to in vitro permeation studies after the topical administration of 5-FU. Thereby, the developed bioanalytical method was considered an advance, since that the methods in the literature are mostly analytical methods and few of these related the difficulties of recovering this drug from the skin.

**Acknowledgment**

This study was supported by São Paulo Research Foundation (FAPESP, Brazil), and Juliana dos Santos Rosa was the recipient of a FAPESP fellowship (grant #2017/04335-8).

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