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Research article

Liquid chromatography method to assay tretinoin in skin layers: validation and application in skin penetration/retention studies

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ABSTRACT

A liquid chromatography (LC) method for the quantification of tretinoin (TTN) in different matrices (adhesive tape, cotton and porcine skin layers, *stratum corneum*, viable epidermis, and dermis) was validated and applied in *in vitro* porcine skin penetration/retention studies. This study proposes, for the first time, a method for assaying TTN in separated porcine skin layers (*stratum corneum*, viable epidermis, and dermis). The skin studies were carried out using tape stripping and cutaneous retention techniques. The procedures for the extraction of TTN from dermatological formulations (creams and gels) and biological and non-biological matrices used with the tape stripping and retention techniques were also evaluated. The LC method consisted of a mobile phase composed of a mixture of methanol, water, and glacial acetic acid (85:15:1, v/v); a C18 column used as the stationary phase; a flow rate of 1.0 mL min^{-1}; an injection volume of 100 μL; and TTN detection at 342 nm. The method was linear in the range of 0.05–15.00 μg mL^{-1} (r = 0.9999), and it was precise and accurate. The limit of detection (LOD) and limit of quantification (LOQ) were 0.0165 μg mL^{-1} and 0.0495 μg mL^{-1}, respectively. TTN was extracted from different matrices, showing good precision [relative standard deviation (RSD) of <5%] and accuracy (89.4–113.9%). This method was successfully applied in the evaluation of TTN skin retention/permeation from dermatological formulations (cream and gel). A higher penetration of TTN through the skin was achieved with the gel rather than the cream, showing the influence of the dosage form. Therefore, the developed method can easily be applied in porcine skin penetration/retention studies of dermatological formulations containing TTN, and it is able to discriminate the behaviours of the different formulations.

1. Introduction

Tretinoin (TTN), also called retinoic acid, is a retinoid, and it is an active component of the metabolism of vitamin A. It has been widely used in dermatological products due to its efficacy in the treatment of cutaneous diseases, such as acne, ichthyosis, psoriasis, and skin cancer, as well as its anti-aging effects [1, 2]. The percutaneous absorption of TTN from dermatological formulations should be avoided during product development. This can be explained by its well-documented teratogenic effects after oral or topical administration [3, 4], even though it has a low percutaneous absorption of about 1% [5, 6]. On the other hand, it is important to consider that substances for the treatment of skin cancer and psoriasis, as well as those for anti-aging uses, must be able to penetrate deeper skin layers in order to reach their target sites [7].

Skin permeation/penetration studies have been widely used to evaluate the safety of dermatologic and cosmetic products [8, 9]. Several

methods have been used to evaluate drug penetration from topical and transdermal formulations, such as autoradiography [10], Franz diffusion cells [11, 12], microdialysis [13], skin biopsy [14], tape stripping [15], and cutaneous retention [16]. The tape stripping technique is a simple, efficient, and quick method to assess drug distribution through the skin layers following application of topical dermatological formulations [17]. Tape stripping, which allows for drug quantification in the *stratum corneum*, and the cutaneous retention technique have been previously used in TTN skin penetration studies [16, 18, 19, 20].

Regardless of the technique, an adequate analytical method to assay the drug, which should be specific, precise, and accurate, must be used [21]. When choosing a method, it is important to consider that a small amount of drug is expected in the different skin layers. Therefore, these analytical methods should have a low limit of quantification [22]. Moreover, dermatological formulations are formulated as semisolid

dosage forms, like creams and gels, which can be considered complex matrices [23], along with the different components of the skin layers.

To the best of our knowledge, there is still a lack of validated, simple, and suitable methods to assay TTN in different porcine skin layers for penetration/retention studies from dermatological formulations, such as creams and gels. Most studies have not evaluated TTN penetration in different skin layers, separately [24, 25, 26], or they used other skin models [27, 28]. Porcine is the most used skin model for penetration/permeation studies due to its similarity to human skin in terms of its anatomical and physiological characteristics [29, 30]. Therefore, the goal of this study was to validate an analytical method used to assay TTN in different matrices (dermatological formulations, cotton, tape, and skin) as well as to set up the protocol for TTN extraction from these matrices. This method was applied in skin penetration/retention studies from semisolid dosage forms (cream and hydrogel) using the tape stripping and cutaneous retention techniques. The influence of the semisolid dosage form on TTN distribution through the skin layers was evaluated in order to determine the feasibility of using this method to discriminate the different behaviours of the formulations.

2. Materials and methods

2.1. Materials

TTN was purchased from PharmaNostra (São Paulo, Brazil). Carbopol® Ultrez 10 NF was obtained from Fragon (São Paulo, Brazil). Imidazolydinil urea, triethanolamine, propylene glycol, methylparaben, propylparaben, glyceryl monostearate, isopropyl miristate, stearic acid, almond kernel oil, liquid paraffin, and polyethylene glycol 400 were obtained from Delaware (Porto Alegre, Brazil). Polysorbate 80 was purchased from Henrifarma (São Paulo, Brazil). Glacial acetic acid was acquired from LabSynth (Diadema, Brazil). Hydrophilic cotton was purchased from Cremer (Blumenau, Brazil). Durex™ adhesive tapes were obtained from 3M (Sumaré, Brazil). Methanol (HPLC grade) was acquired from Tedia Brazil (Rio de Janeiro, Brazil). Ultrapure water was used to prepare all formulations (Milli-Q, Millipore, USA). All reagents were of analytical or chromatographic grade. The porcine skin used in the penetration/retention studies was donated by a local slaughterhouse (Araldi, Nova Roma do Sul, Brazil).

2.2. Methods

2.2.1. Preparation and characterisation of the dermatological formulations

In this study, the dermatological formulations were prepared at our laboratory. They were designed to avoid the use of high amount of alcohol to disperse TTN and that could affect TTN skin permeation/penetration behaviour. Moreover, this approach makes easier the preparation of the respective blank formulations, prepared without TTN, for the method validation studies.

Creams were prepared (n = 3) as an O/W (oil in water) emulsion. The oil phase containing stearic acid (4%, w/v), glyceryl monostearate (6%, w/v), isopropyl miristate (2%, w/v), liquid paraffin (1%, w/v), almond kernel oil (0.5%, w/v), and propylparaben (0.2%, w/v) was heated to 70 °C in a mortar. Then, an aqueous phase composed of methylparaben (0.1%, w/v), propylene glycol (3%, w/v), triethanolamine (0.2%, w/v), and ultrapure water (83 mL) was heated to 75 °C and added to the oil phase under gentle shaking using a pestle. The homogenisation was maintained until the temperature of the emulsion reached 30 °C. TTN-loaded creams (TTN-C) were prepared (n = 3) with the addition of 0.05 g of TTN to 99.95 g of the previously prepared creams under gentle homogenisation using a mortar and pestle. Blank-creams (B-C), without any TTN, were used as control.

Gels were prepared (n = 3) through the dispersion of 0.5% (w/v) Carbopol® Ultrez 10 NF in ultrapure water. Imidazolydinil urea (0.5%, w/v) was added as a preservative. During the last step, the polymeric dispersion was neutralised with 120 μL of triethanolamine, reaching a pH of 5.5–6.0. TTN-loaded gels (TTN-G) were prepared (n = 3) by adding 0.05 g of TTN [previously dispersed in propylene glycol at 3% (w/v)] to 99.95 g of the previously prepared gel under gentle homogenisation using a mortar and pestle. In order to avoid any degradation of TTN, all procedures were carried out by protecting the samples from light. Blank-gels (B-G), without any TTN, were used as control.

The pH values, drug contents, and rheological measurements were determined for all formulations. The pH values of the formulations were measured using a calibrated pH meter (Ultrabasic, Denver Instrument, NY, USA) after dilution of the samples in water (10%, w/v). The rheological behaviour of the semisolid formulations was evaluated using a rotational viscometer (LVDV-II + PRO Digital Viscometer, Brookfield Instruments, UK) and a SC4-25 spindle. Analyses were carried out at 25 ± 1 °C. The drug content of the hydrogels was assessed using liquid chromatography (LC) by employing a previously validated method [12].

2.2.2. Preparation of the porcine skin membrane

Female abdominal porcine skin was kindly donated by a local slaughterhouse (Araldi, Nova Roma do Sul, Brazil). The adipose tissue was carefully removed. Then, the skin surface was cleaned using 1 % (w/v) sodium lauryl sulphate. Skin slices were cut into circles (3.5 cm diameter), and their thicknesses were measured using a dial thickness gage. Skin slices with 1.7 ± 0.3 mm thickness were used. Skin samples were wrapped in aluminium foil and stored in a freezer (–4 °C). They were not stored for a period longer than 90 days. Before the studies, the skin samples were left at room temperature for 30 min.

2.2.3. High-performance liquid chromatography (HPLC) system

The chromatographic system consisted of a Shimadzu® LC-20A system, a 20AT pump, a CBM-20A system controller, an SPD-M20A photodiode array detector, and an SIL-20A auto-sampler (Shimadzu, Tokyo, Japan). Chromatographic separation was obtained using an analytical column (C18, 150 × 4.6 mm, 5 μm, Phenomenex®) coupled to a pre-column (C18, 4.0 × 3.0 mm, Phenomenex®). The mobile phase consisted of a mixture of methanol and water (85:15) with 1% glacial acetic acid. The mobile phase was filtered, degassed, and pumped at a flow rate of 1.0 mL min^{-1}. The injection volume was 100 μL, and TTN was detected at 342 nm. The LC method validated in this study for application in porcine skin permeation/penetration studies was based on work by Ourique et al. [12], with some modifications.

2.2.4. Validation of the analytical method for skin penetration studies of tretinoin

Validation of this methodology for quantification of TTN was performed for samples of the following different matrices used in skin penetration/retention studies: formulations, adhesive tapes (used for the tape stripping protocol), porcine skin membranes, and cotton pieces (used to remove excess formulation on the skin membranes at the end of the experiment). The following parameters were evaluated: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), intra- and inter-day precision, and accuracy, as recommended by the guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [31].

2.2.4.1. Specificity. The specificity of the chromatographic method was evaluated for matrices without the drug (B–C and B–G), which were submitted to the extraction process proposed in this study (section 2.2.4.5) in order to demonstrate that there was no interference in the retention time of the TTN. This parameter was demonstrated by comparing the chromatograms obtained from samples containing blank matrices and samples spiked with a methanolic solution of the drug.

2.2.4.2. Linearity. The linearity was evaluated from three analytical curves (n = 3) at eight concentrations of TTN, from 0.05–15.00 μg mL^{-1}.

To obtain the analytical curve, a methanolic solution (standard solution) with 1.00 mg mL^{-1} of TTN was obtained by dissolving 0.025 g of TTN in 25 mL of methanol and sonicating for 3 min. Eight dilutions were prepared at TTN concentrations of 0.05, 0.10, 0.50, 1.00, 2.50, 5.00, 10.00, and 15.00 µg mL^{-1}. Afterwards, each sample was filtered (0.45 µm) and assayed by HPLC. The peak areas were plotted versus each drug concentration. The analytical curve and the linear correlation coefficient (r) were determined from linear regression analysis. For analysis of variance (ANOVA), Windows Excel (2010) software was used, and a 95% confidence interval was considered [31].

2.2.4.3. Limit of detection (LOD) and limit of quantification (LOQ).

The LOD and LOQ were calculated from the slope (s) and the standard deviation of the intercept of the analytical curves (S), according to the following equations [31]:

$$LOD = 3.3 \; x \; \frac{s}{S} \tag{1}$$

$$LOQ = 10 \; x \; \frac{s}{S} \tag{2}$$

Where,

  LOD means "limit of detection";
  LOQ means "limit of quantification";
  s is the slope of the analytical curves; and
  S is the standard deviation of the intercept of the analytical curves.

2.2.4.4. Precision.

The precision of the analytical method was evaluated in terms of intra-day (repeatability) and inter-day precision (intermediate precision) for TTN samples in methanolic solutions and in dermatological formulations (TTN-C and TTN-G). Nine replicates (n = 9) of each formulation were evaluated at a TTN concentration of 10 µg mL^{-1}. The intra-day precision and inter-day precision of the method were expressed as the relative standard deviation (RSD = standard deviation/mean × 100). The samples were prepared at 10 µg mL^{-1} of TTN by weighing 0.5 g of TTN-C or TTN-G in a 25 mL volumetric flask containing methanol. Afterwards, the samples were submitted to the extraction process (Table 1), filtration (0.45 µm), and the drug assay by LC using the chromatographic conditions described above.

2.2.4.5. Accuracy.

Before TTN skin penetration/retention studies, TTN recovery from the different matrices (formulations, porcine skin, adhesive tapes, and cotton pieces) was validated. The adhesive tapes were used to remove the *stratum corneum* in the tape stripping technique, while the cotton pieces were used to remove excess formulation on the skin surface. The extraction parameters used in this step are presented in Table 1. Afterwards, each sample was filtered (0.45 µm), and the amount of drug was assayed by LC according to the method described in section 2.2.3. The accuracy of the analytical method was evaluated from replicates of three different TTN concentrations and expressed as the ratio of the experimental drug concentration to the corresponding theoretical drug concentration.

The samples of the dermatological formulations were prepared by weighing 0.20 g of B–C or B–G in a 10 mL volumetric flask, while adding

a known amount of a methanolic solution of TTN to reach final concentrations of 5.00, 10.00, and 15.00 µg mL^{-1}. The samples were prepared in triplicate. The samples of the adhesive tapes were transferred to tubes and contaminated with known amounts of a methanolic solution of TTN to reach final concentrations of 2.50, 5.00, and 10.00 µg mL^{-1}. The samples were prepared in triplicate.

Moreover, porcine skins (3 cm diameter) were contaminated with known amounts of TTN methanolic solution. Afterwards, the skin remained in contact with the drug for 30 min in a dark environment. After this time, each skin was cut into small pieces and transferred to tubes, adding an adequate volume of methanol to reach 9 mL at final TTN concentrations of 5.00, 10.00, and 15.00 µg mL^{-1}. The experiments were performed in five determinations. The samples of cotton containing the formulations were prepared by weighing either TTN-C or TTN-G (0.05%, w/v) into tubes containing cotton pieces, and a sufficient amount of methanol for reaching TTN concentrations of 2.50, 5.00, and 10.00 µg mL^{-1} was added.

2.2.5. Application of the validated analytical method in skin penetration/retention studies

Skin penetration/retention studies of TTN from dermatological bases (TTN-C and TTN-G) were carried out using automated Franz-type vertical diffusion cells (Franz Microette Plus-Hanson Research® Cell) with a 7 mL cell receptor compartment and a diffusion area of 1.766 cm^2 [32,33]. The abdominal porcine skin membranes were cleaned and stored as previously described in section 2.2.2. At the beginning of the experiment, 250 ± 3.8 mg of TTN-C or TTN-G was applied on the full thickness abdominal porcine skin membranes, which was mounted in Franz diffusion cells. The receptor compartment was filled with ethanol/phosphate buffer at pH 7.4 (70:30, v/v). The experiment was carried out for 12 h. At the end of the experiment, the diffusion cells were disassembled to assay TTN in different skin layers (*stratum corneum*, viable epidermis, and dermis), as well as in the receptor compartment.

After removing excess formulation on the skin membrane with cotton pieces, the *stratum corneum* was removed by the tape stripping technique using 18 tapes (3M, Durex™), as described by Beber et al. [34]. Each tape was pressed onto the membrane and then rubbed with a spatula to remove the *stratum corneum*. The tapes were transferred to a tube containing 4 mL of methanol. After removal of the *stratum corneum* by tape stripping, the viable epidermis was separated from the dermis by immersion of the porcine membrane in ultrapure water heated at 60 °C for 45 s [31,32]. Subsequently, the epidermis was removed by scalpel blade scraping, while the dermis was cut into small fragments [31, 32]. Both layers were transferred to a tube containing 9 mL of methanol. All samples were submitted to the extraction process (Table 1) and then filtered (0.45 µm), and TTN was assayed by the LC method described in section 2.2.3. The analyses were performed in sextuplicate.

Skin penetration/retention analyses were performed by comparing the amount of TTN that penetrated (µg/cm^2) through the different layers of the skin (*stratum corneum*, viable epidermis, and dermis). Excess TTN on the skin discs (removed using cotton pieces) was quantified at the end of the experiment in order to verify the percent recovered (%) after part of the drug penetrated the skin.

2.2.6. Drug release studies

Drug release studies from the semisolid formulations were carried out using the same conditions described in the skin permeation/penetration studies (section 2.2.5), replacing the skin discs with membrane discs (MW of 12,400 kDa; Sigma-Aldrich, USA).

2.2.7. Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Data were evaluated by one-way ANOVA and/or the *Student*-t test. For the *in vitro* penetration/retention studies, the data were assessed by ANOVA followed by Tukey's post-tests, considering a significance level at p <

Table 1. Parameters used in the tretinoin extraction from different matrices.

Sample	Vortex mixing	Sonication		Centrifugation	
	Time	Time	° C	Time	g
Formulation	—	30 min	34	30 min	4500
Porcine skin	2 min	30 min	34	30 min	4500
Adhesive tapes	2 min	4 min	34	30 min	4500
Cotton pieces	2 min	30 min	34	15 min	4500

0.05. All analyses were performed using GraphPad Prism software (version 5.0).

3. Results and discussion

The aim of this study was to validate a method to assay TTN in different matrices for its application in skin penetration/retention studies. Methods designed for this purpose, which are able to evaluate the amount of TTN in the *stratum corneum*, viable epidermis, and the dermis, separately, through skin penetration studies, are lacking. Reliable data from these studies are essential to improving the efficacy and safety of dermatological formulations containing TTN. Therefore, this study was organised into the following three steps: a) preparation and characterisation of two semisolid formulations used as models in this approach; b) validation of the analytical method comprising different matrices involved in the skin penetration/retention studies; and c) application of the method in real *in vitro* skin penetration/retention studies using porcine skin as a model.

3.1. Physicochemical properties of the formulations (creams and gels)

Creams and gels were prepared using excipients approved for cutaneous use and widely employed in the preparation of semisolid dosage forms. Formulations containing TTN or formulations not containing TTN were produced. Table 2 shows their physicochemical and organoleptic properties. Regarding their appearance, the presence of TTN makes the creams and gels slightly yellow due to its own colour. The presence of the drug did not affect the pH range of the formulations, regardless of their semisolid type (cream or gel). Moreover, both formulations containing TTN (TTN-C and TTN-G) showed a drug content near the expected value of 0.50 mg/g.

In studies of the skin permeation/penetration behaviour of semisolid formulations, considering the influence of their rheological profiles on drug release and, consequently, drug penetration is highly recommended. Figure 1 shows the rheological behaviour for all the formulations developed in this study. All showed non-Newtonian pseudoplastic rheological behaviour since there was no linear correlation between the shear stress (Pa) and shear rate (s^{-1}) [35], and the viscosity decreased with increasing shear rate. This behaviour is recommended for dermatological formulations since the flow resistance tends to be low for the application of medium to high shear forces [36]. On the other hand, as can be visualised in Figure 1, the shear stress at the same shear rate (0.6 s^{-1}) was higher for the gels compared to the creams, which means that these creams have a lower consistency compared to the gels, regardless of the presence of TTN.

3.2. Validation of the analytical methodology used to assay tretinoin in different matrices

In order to develop an analytical method that meets the requirements necessary for application in medicine, as well as to ensure the reliability of the data obtained, the methodology must be validated through experimental studies [37]. According to Ribani et al. [38], the specificity
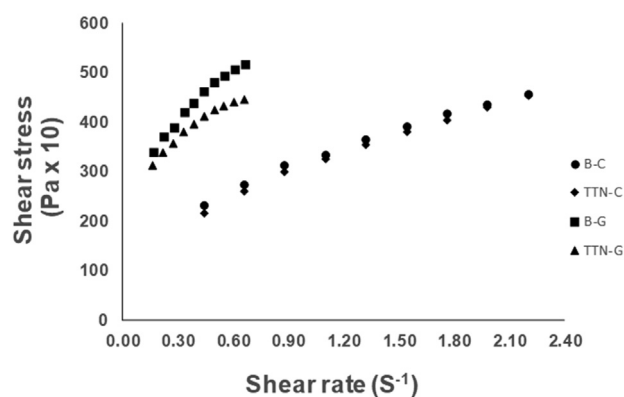

Figure 1. Rheological profiles (viscosity *versus* shear rate) of creams and gels produced containing or not TTN (n = 3). B-C: blank cream; TTN-C: tretinoin-loaded cream; B-G: blank gel; and TTN-G: tretinoin-loaded gel.

should be the first parameter evaluated. For *in vitro* permeation/penetration studies, different matrices must be considered, such as the drug vehicles (formulations), cotton pieces, adhesive tapes, and skin. In this study, the mobile phase, composed of methanol, water, and glacial acetic acid (85:15:1, v/v), was able to separate the TTN from the components of the different matrices, as shown in Figure 2A–F. The matrices did not show any chromatographic peaks at the retention time for TTN (approximately 15 min). The proposed method was specific for TTN since no interferences were observed when the chromatograms obtained from the blank matrices were compared to that of the drug (Figure 2A).

In the next step, three independent analytical curves were prepared and analysed in the concentration range of 0.05–15.00 µg mL^{-1}. The regression equation was y = 626420x – 2126.2 (r = 0.9999). These results indicate that the response of the method was linearly proportional to the analyte concentration. A good quality standard curve was obtained due to the correlation coefficient (r) value being greater than 0.99 [37]. In addition, the calculated LOD and LOQ values were 0.0165 and 0.0495 µg mL^{-1}, respectively, reflecting the sensitivity of the method. The concentration determined for the LOQ was very low, which is one advantage if considering this method for quantification of a drug with low skin penetration, facilitating evaluation in different skin layers [39]. Intra-day (1.29%) and inter-day precision (0.99%) for samples of TTN in methanolic solution were less than 5%, as required by ICH for these assays [31, 37]. Moreover, the intra-day precision and inter-day precision of the samples of TTN extracted from the semisolid formulations were also evaluated (Table 3). A suitable precision for the assay of the drug in the formulations was obtained (RSD<5%).

The accuracy of the method was evaluated using the recovery test. Data are presented in Table 4. The recovery (%) ranged from 89.4 to 114.3% of TTN from the different matrices. The TTN extraction results from the different matrices were within the range specified (80–120%) by the official agencies [31] for complex matrices. Therefore, the method was accurate enough to assay TTN in different matrices.

3.3. Application of the validated analytical method in skin permeation/retention studies

After validating the method, its applicability was demonstrated in a real experiment. Therefore, the skin permeation/retention of TTN from a gel and a cream was evaluated using porcine skin. TTN did not permeate to the receptor compartment from any formulation. Figure 3 shows the results of TTN skin penetration/retention studies from TTN-C and TTN-G. A higher concentration of TTN was found in the *stratum corneum* for both formulations, followed by a decrease in drug flow to the adjacent skin layers, evident by the higher amount of TTN in the *stratum corneum* and the smaller proportion in the viable epidermis and dermis (p < 0.05). The

Table 2. Physicochemical properties of the semisolid formulations (mean ± SD). B-C: blank cream; TTN-C: tretinoin-loaded cream; B-G: blank gel; and TTN-G: tretinoin-loaded gel.

Formulation	pH	Drug content (mg/g)	Aspect
B-C	7.50 ± 0.01	——	Opalescent, white
TTN-C	7.30 ± 0.08	0.52 ± 0.08	Opalescent, slighly yellow
B-G	5.70 ± 0.11	——	Transparent, colorless
TTN-G	5.90 ± 0.08	0.53 ± 0.03	Slighly opalescent, slighly yellow

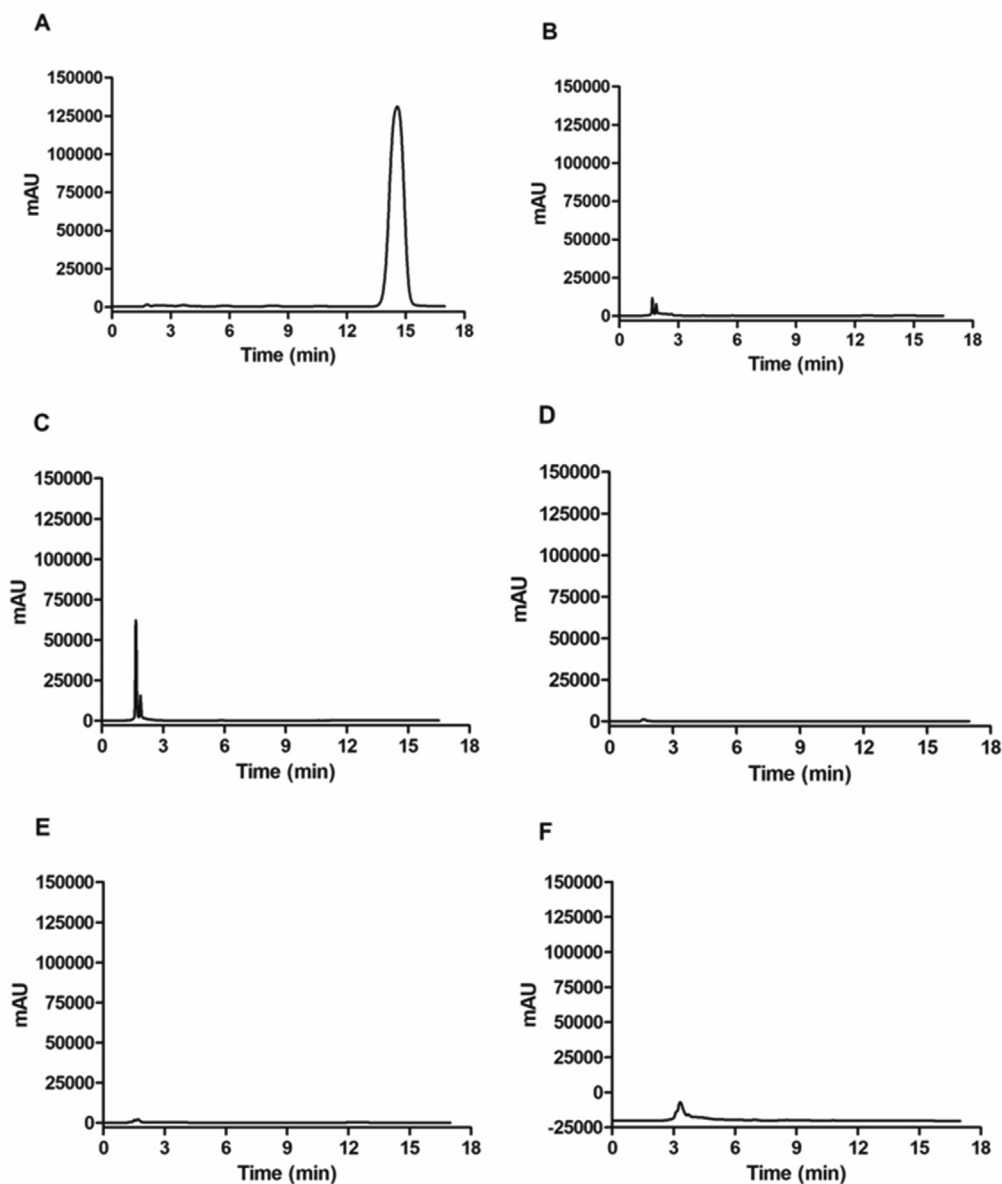**Figure 2.** Chromatograms of the tretinoin solution (10 µg mL^{-1}) (A), blank cream (B), blank gel (C), cotton (D), adhesive tape (E) and porcine skin (F) assessed during the analytical validation of the method.

Table 3. Values of intra- and inter-day precision of tretinoin (TTN) assay in the semisolid formulations.

Formulation	Intra-day precision RSD (%)	Inter-day precision RSD (%)
TTN-C	2.11	2.95
TTN-G	0.81	0.84

TTN-C: tretinoin-loaded cream; TTN-G: tretinoin-loaded gel; RSD: relative standard deviation.

amount of drug in the viable epidermis and dermis from TTN-C was below the LOQ, and it could not be quantified.

However, a significant difference (p < 0.05) between the formulations was observed for the amount of drug penetrating all skin layers. The penetration of TTN to the skin layers was higher for the gel than the cream. This can be explained by the greater affinity of TTN (Log P = 5.66) for the lipid components of the cream [40]. On the other hand, lipophilic TTN has no affinity for the hydrophilic gel, facilitating its release to the

skin. The influence of the viscosity on this result can be refuted since the creams showed a lower consistency, as previously discussed. This lower consistency could improve drug release and, consequently, the skin penetration of TTN. To confirm the hypothesis that the affinity of TTN for

Table 4. Accuracy of the method to assay TTN in different matrices. Data are showed as R (%) and RSD (%) for B-C and B-G at 5.00; 10.00 and 15.00 µg mL^{-1}; porcine skin, adhesive tape and cotton (+ TTN-C or TTN-G) at 2.50; 5.00 and 10.00 µg mL^{-1} of drug as low, middle and high concentration, respectively.

Formulation	Low concentration		Middle concentration		High concentration	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
B-C	90.74	1.64	91.33	1.61	97.13	1.18
B-G	92.36	1.55	92.72	1.02	96.30	0.57
Porcine skin	89.41	5.40	89.40	5.55	89.47	4.88
Adhesive tape	95.25	4.18	100.31	5.40	96.81	3.97
Cotton + TTN-C	106.80	2.18	107.70	1.95	114.30	4.01
Cotton + TTN-G	113.90	3.40	103.65	2.19	102.47	2.19

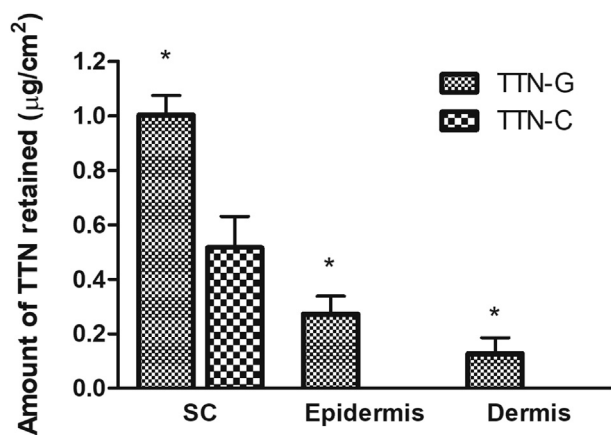R: recovery; RSD: relative standard deviation.

Figure 3. Amount of tretinoin (TTN) penetrated in different layers: *stratum corneum* (SC), epidermis and dermis from TTN-C (TTN-loaded cream) and TTN-G (TTN-loaded gel). Data represent the mean $\pm$ SD (n = 6). *Significant difference (p < 0.05) compared with the TTN-C.

the semisolid formulations explains the differences in skin penetration, a release study was carried out using the same conditions used in the skin permeation/penetration studies, replacing the skin discs with a membrane disc. The experiment was carried out for 12 h. After this time, the percentage of TTN released from the gel (33.42 $\pm$ 6.62%) was higher than the percentage released from the cream (2.42 $\pm$ 0.72%) (p < 0.05). These data confirmed our hypothesis, explaining the deeper penetration of TTN from the gels (semisolid formulation) due to the faster release of TTN to the *stratum corneum* surface compared to the cream.

4. Conclusion

In conclusion, a simple and reliable analytical method for quantification of TTN in different matrices comprising porcine skin permeation/penetration studies was validated. The method was specific, linear, precise, and accurate. Moreover, the method was applied in a real skin permeation/penetration study, and it was able to assay TTN in the different porcine skin layers (*stratum corneum*, viable epidermis, and dermis), as well as differentiate the different skin penetration behaviours of the formulations (gel and cream). Skin penetration of TTN was dependent on the type of vehicle since the hydrogel resulted in deeper penetration compared to the cream.

Declarations

Author contribution statement

Dileusa de Oliveira: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Diego Fontana de Andrade: Designed and performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Edilene Gadelha de Oliveira: Analyzed and interpreted the data; Wrote the paper.

Ruy Carlos Ruver Beck: Designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

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