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Abstract: Transdermal patches and medicated plasters are designed to sustain efficacious systemic or loco-regional drug concentrations, respectively. In both cases, drug skin permeation is a critical attribute from the early stage of the pharmaceutical development. In 2014, the EMA introduced the guideline "on the quality of transdermal patches", in which the importance of equivalence of drug fluxes in vitro skin permeation study was particularly emphasized to generic or abridged applications for the marketing authorization or manage dossier variations during the product cycle life. Moving from experimental data, this work provides information on the set-up of such studies and on the statistical evaluation of obtained fluxes. In particular, the impact of the inter-sample variability on the equivalence assessment was deeply investigated by using two formulation pairs containing propranolol, diclofenac or nitroglycerine. The main outputs of the work were attributable to the definition of the acceptability interval and number of replicates to be performed. As an example, the equivalence of two propranolol patches (flux variability lower than 25%) can be assessed using six replicas and a confidence limit with-in the 0.8-1.25 range (alfa=0.05; power 90%). In contrast, the equivalence of diclofenac plasters, which exhibit a variability near the 50%, can be demonstrated increasing the number of replicas (i.e., 20 skin samples) for each formulation and widening the acceptance range according to a statistical approach proposed in the work.

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Prof. M. Brandl,
Editor-in-Chief of EJPS

Dept. of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, DK-5230, Odense M, Denmark (D)

Milan, September 7nd 2018

Dear Prof. Brandl,

I am pleased to submit the revised version of the manuscript titled "Design of in vitro skin permeation studies according to the EMA Guideline on quality of transdermal patches" by Francesco Cilurzo, Umberto M. Musazzi, Silvia Franzé, Guido Fedele, Paola Minghetti. A point-to-point reply to reviewers was provided. The main modifications of the text are evidenced in red.

I declare under my responsibility that the manuscript, or its content in some other form, has not been published previously by any of the Authors and/or is not under consideration for publication in another journal at the time of submission.

All authors have seen and approved the revisions of the manuscript.

Best regards,

Francesco Cilurzo

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Abstract

Transdermal patches and medicated plasters are designed to sustain efficacious systemic or loco-regional drug concentrations, respectively. In both cases, drug skin permeation is a critical attribute from the early stage of the pharmaceutical development. In 2014, the EMA introduced the guideline "on the quality of transdermal patches", in which the importance of equivalence of drug fluxes in vitro skin permeation study was particularly emphasized to generic or abridged applications for the marketing authorization or manage dossier variations during the product cycle life. Moving from experimental data, this work provides information on the set-up of such studies and on the statistical evaluation of obtained fluxes. In particular, the impact of the inter-sample variability on the equivalence assessment was deeply investigated by using two formulation pairs containing propranolol, diclofenac or nitroglycerine. The main outputs of the work were attributable to the definition of the acceptability interval and number of replicates to be performed. As an example, the equivalence of two propranolol patches (flux variability lower than 25%) can be assessed using six replicas and a confidence limit with-in the 0.8-1.25 range (α=0.05; power 90%). In contrast, the equivalence of diclofenac plasters, which exhibit a variability near the 50%, can be demonstrated increasing the number of replicas (i.e., 20 skin samples) for each formulation and widening the acceptance range according to a statistical approach proposed in the work.

***Response to Reviewers**

Reviewers' comments:

Reviewer #1: The topic is highly important to researches in this field.

The manuscript is well structured and to my opinion no further modifications are necessary.
In some phrases English could be improved - for example - "Indeed, the same biological membrane cannot be used to assay both the formulations (page 4).
[a] The sentence was reworded and, more in general, the English revised.

Reviewer #2: The authors discussed a very important issue regarding the standardization of the in vitro skin permeation studies as a mean to assure the quality of transdermal patches.
Up to now, the issue is rather neglected and often the conditions applied in the evaluation are not standardized.

I found the work well presented and have only a minor comments.

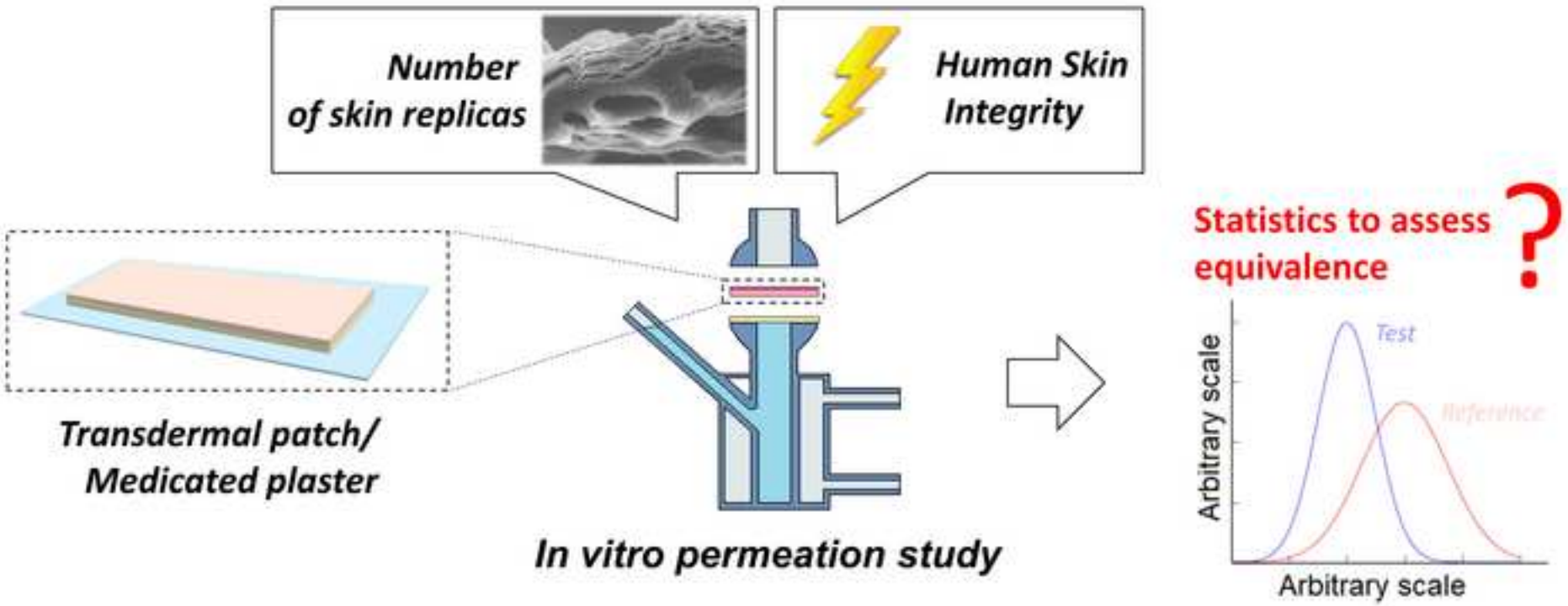
In in vitro studies using the human skin: I fully agree that the integrity is a crucial parameter, but I would also recommend to discuss the thickness of the skin,
[a] Basing on our previous experiences, we noticed that the epidermis thickness significantly influenced the amount retained by the human epidermis. Conversely, we did not find any relationship between thickness and the drug permeated amount for the same anatomical site. Thus, in this work we did not check the weight of the samples and we cannot discuss this topic. However, we'll took in consideration your comments for further works.

as well as the age of the donor.
[a] The donors were 30-50 years old. Therefore, considering the data available in literature, this factor should not influence the skin permeation profile. The material and method section was improved with this information.

One should also consider that mostly it is a female skin used in the experiments.
[a] Respectfully, we re-checked the literature (on the bases of Scopus database) and we didn't find any information concerning the influence of the gender in in vitro skin permeation experiments.

In the discussion, it would be beneficial to address the medium used in the receptor compartment.
[a] Thanks for the suggestion. We selected the composition of the receiver medium merely to guarantee the sink conditions as reported in the materials and methods. We never consider the possible impact on the variability of the experimental data. This topic that will be surely an object for further investigations. As sentence was added in the discussion (page 11).

*Graphical Abstract (for review)



Design of in vitro skin permeation studies according to the EMA Guideline on quality of transdermal patches

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Keywords

In vitro skin permeation studies; Franz's cell; equivalence; Highly variable drugs; transdermal patch; medicated plaster.

Abbreviations

C_t: drug concentration at the same time point;

D-plaster: diclofenac epolamine-loaded plaster;

ESI: electron spray ionization;

GMR: geometric Mean Ratio;

HVDP: highly variable drugs or drug products;

J: maximal permeation flux;

J_{tot}: permeation flux calculated on the basis of real-life application area;

L: lower limit of acceptability interval;

MRM: multiple reaction monitoring;

N-patch: nitroglycerine-loaded patch;

P-patch: propranolol-loaded patch;

Q_t: permeated amount at the time t;

R: electrical resistance;

U: upper limit of acceptability interval;

V: volume of the Franz cell.

1 Introduction

Transdermal patches and medicated plasters are well-known prolonged release dosage forms designed to guarantee efficacious drug concentrations to the systemic circulation or in the tissues beneath the application site, respectively. The interest in these adhesive dosage forms resides in the possibility to prolong the drug release over a period ranging from 12 hours to 7 days and to pre-determine the administered dose.

To achieve these goals, the adhesion to the skin surface and the ability to release the loaded drug through the *stratum corneum* over the required period of time are key requirements in the design of these formulations. Both of these aspects should be carefully evaluated in the pharmaceutical development taking into consideration that all the formulation variables can influence both the drug release/permeation and the adhesion properties.

The design of the *in vitro* skin penetration experiments, the selection of the diffusion cell and the relative operative conditions, as well as the biological membrane, are crucial. The most widely used diffusion cells are based on the design proposed by Franz even if some modifications have been introduced (Ng et al., 2010). The diffusion surface and the volume of the receiver chamber of these cells have to be chosen in order to guarantee detectable concentrations of the permeant in the receiver medium. Moreover, the agitation system should be validated (Ng et al., 2010) to assure a uniform concentration of the permeant in the receiver medium, whose composition has to be suitable to guarantee the sink conditions and avoid any significant alteration of the barrier properties of the biological membrane. The last aspect is obviously the most critical variable. Usually, the human skin or the isolated human epidermis are used even if the porcine skin is often used as a surrogate (Campani et al., 2016; Heylings et al., 2018). Regardless of the source of the biological membranes used in the permeation study, the demonstration of their integrity has to be demonstrated and, in consideration that a compendium method is not available, the suitability of the adopted method has to be accurately studied (Davies et al., 2015). Moreover, the whole procedure should be periodically controlled using standard molecules (i.e., caffeine benzoic acid or testosterone) as suggested by the OECD(OECD, 2004). It should be also kept in mind that the suitability of the diffusion cell system for the determination of the skin permeation of a drug has to be accomplished by an appropriate statistical approach which allows to discriminate the similarity or dissimilarity of two formulations containing the same active pharmaceutical ingredient.

Recently, the EMA introduced a new guideline “on the quality of transdermal patches” which reports the critical attributes of a transdermal patch and establishes the requirements necessary to assess its quality (EMA, 2014). The *in vitro* permeation data are particularly emphasized since they have a key role not only in the development and optimization of the formulation but are also supportive of the stability data. Finally, when the *in vitro* permeation assay is discriminative of the critical variables, it results helpful in the management of the variations that can occur during the cycle life of the drug product.

Interestingly, the guideline reports the general concept that *in vitro* skin permeation studies are a valuable measure of the patch biopharmaceutical performances since they allow to estimate the thermodynamic

activity of the drug in the adhesive matrix even if the "*in vitro permeation studies are not expected to correlate with in vivo release*". Indeed, the thermodynamic activity of the drug is the main driving force in the partition process occurring between the patch and the *stratum corneum* that is the first step for the drug absorption. Hence the *in vitro* skin permeability studies can be considered as a surrogate of the *in vivo* studies and the need to perform bioequivalence studies might be waived in certain cases (Cilurzo et al., 2015a). Indeed, although the *in vitro* permeation data do not fit always the expected one to one relation with the *in vivo* data (Franz et al., 2009), a certain proportionality between the *in vitro* and *in vivo* fluxes is maintained, therefore a direct *in vitro-in vivo* correlation of the biopharmaceutical performances has been postulated and well established, either for local (Minghetti et al., 2000) and systemic administration (Rohr et al., 1998).

The European Guideline on the quality of transdermal patches proposes the comparison of the experimentally determined fluxes upon a null hypothesis of non-equivalence. According to the guideline for the assessment of *in vivo* bioequivalence, the 90% confidence interval for the ratio of the two products should be determined and comprised within the ratio of 0.8 to 1.25 to support a claim of equivalence, unless justified. However, unlike the *in vivo* bioequivalence study, the guideline suggests the number of replicas without considering that the variability of the permeation process may be strongly dependent on the characteristics of the permeant (Farahmand and Maibach, 2009). Nevertheless, it is well-known that the diffusion process through the skin is determined by both the physiological variability of the excised biological sample and the physicochemical properties of the permeant. As widely demonstrated in the literature, the permeation pattern of hydrophilic drug substances is more variable than that of hydrophobic ones. For example, Akomeah and co-workers demonstrated that the intra- and inter-skin donor variability of caffeine resulted higher than the variability of methyl and butylparaben (Akomeah et al, 2007). Thus, unlike the *in vivo* bioequivalence studies, the establishment of the number of replicas for the *in vitro* skin permeation studies could be critical taking also in consideration that these experiments have to be considered as parallel experiments. Indeed, once exposed to the formulation and the acceptor medium, the epidermis sample cannot be re-used since the features of the biological membrane at the end of experiment could be modified and a certain amount of drug would have been retained in the tissue.

Basing on these considerations, this work aimed to evaluate the effect of the variability of the permeant in the diffusion process through the epidermis, to provide suggestions helpful to rationalize the design of bioequivalence assessment experiments of two transdermal patches. On the basis of the literature data, propranolol and diclofenac epolamine were selected as model drugs having a low and a high skin permeation variability, respectively. Indeed, the propranolol exhibits an intra-subject variability lower than 30% (Cilurzo et al., 2014a; Casiraghi et al., 2016), whilst the diclofenac salts have generally a larger variability (Cilurzo et al., 2015b; Dehghanyar et al., 2015). Thus, in the design of the experiments, we decided to follow the European Guideline indication (6 replicas) in the case of propranolol patches and to increase the number of replicas up to 20 in the case of diclofenac plasters. Moreover, since transdermal patches can claim the same

in vivo performances but different surface, two commercially available patches of nitroglycerine having different strength and patch surface were also tested in terms of *in vitro* performances. In this case, the number of replicas was reduced to that proposed by OECD guideline, because of the low variability of the skin permeation behavior of nitroglycerine (OECD, 2004).

2 Materials and methods

2.1 Materials

Propranolol-loaded patches: Propranolol was obtained by SIMS (I); Eudragit NE 40D was purchased by Evonik (D); N-methyl pyrrolidone was obtained by Sigma–Aldrich (US) and DURO-TAK 87-608A (Henkel); backing layers: polyethylene membrane and silicon polyester release liner were gently provided by Bouty (I).

Propranolol-loaded patches (1.49 mg/cm^2) were prepared according to Cilurzo et al. (Cilurzo et al., 2014a) and have the composition reported in the previous paper: in particular, P-Patch no. 1 corresponds to the formulation 7 and P-Patch no. 2 to the formulation 11.

Diclofenac epolamine-loaded plasters: D-plaster 1 (Flector®; 1.29 mg/cm^2, Bayer S.p.A, I) was purchased in a Community pharmacy, whereas D-plaster 2 was provided by MIAT S.p.A. maintaining the same qualitative composition of D-plaster 1.

Nitroglycerine-loaded patches: the nitroglycerine patches used in the study have the following specifications. N-patch 1: drug content 37.4 mg; patch surface 18 cm^2 (Deponit 10 mg/24h, UCB Pharma GmbH, D); N-patch 2: drug content 44.8 mg; patch surface 14 cm^2 (Triniplas 10 mg/24h Novartis S.p.A, I). Both products were purchased in a Community pharmacy.

2.2 In vitro skin permeation studies

The Franz diffusion cells are used to investigate the kinetics of drug release rate in vitro. This cell comprises two compartments, one containing the active component (donor vehicle) and the other containing a receptor solution, separated by a slice of human epidermis. The vertical cells used in the actual set of experiments had a wider column than the original Franz-type diffusion cell, and the bowl shape was removed. They had a diffusion area of 0.636 cm^2 and a 3.0 mL (approx.) receptor compartment. The receptor volume of each cell was equipped with a magnetic stirrer and individually calibrated.

2.2.1 Human epidermis sample preparation

The skin samples were obtained from surgical abdominal reduction (Eurasian female, 30-50 years old) with the informed consent of the patient.

Skin samples were prepared following an internal standard procedure (Cilurzo et al., 2014b). The full thickness skin was sealed in evacuated plastic bags and frozen at -20°C within 6 h after removal. After

removing the subcutaneous fatty tissue, the skin was kept frozen at -20°C (Labor 2T 500 ECT-F Touch, Fiocchetti, Italy) until use.

Prior to experiments, the skin was thawed at room temperature, and the excess of fat was carefully removed. The skin sections were cut into squares of about 5 cm^2 and, after immersing the skin in water at 60°C for 1 min, the epidermis was gently separated from the remaining tissue by forceps.

The integrity of each human epidermis samples was assessed measuring their electrical resistance (voltage: 100 mV, frequency: 100 Hz; Agilent 4263B LCR Meter, Microlease, Italy). The specimen was used in the permeation experiment if the values were higher than 20 $k\Omega cm^2$. This acceptability criterion was assessed on a series of skin permeation studies performed by using caffeine, benzoic acid and sodium dodecyl sulphate as model compounds (**Musazzi, 2018**).

2.2.2 Mounting the Franz cell

A 2.0 cm^2 circular sample, obtained from medicated plaster by a precision die cutter, was gently applied to the epidermis specimen. Then, the assembly was mounted on the receiver compartment of the Franz diffusion cell filled with saline solution, containing sodium azide (100 µg/mL), as a preservative and maintained at 34±1°C, so that the skin surface temperature was 32±1°C. Special care was taken to avoid air bubbles between the medium and the epidermis in the receptor compartment that was continuously stirred with a small magnetic bar at 1800 rpm to assure a uniform concentration of the permeant. The upper and lower parts of the Franz cell were sealed with Teflon (VWR International, I) and Parafilm® (Pechiney Plastic Packaging Company, USA) and fastened together by means of a clamp.

At predetermined times (1, 2, 4, 8, 24 h), 200 µL samples were withdrawn from the receiver compartment and replaced with fresh receiver medium. Sink conditions were maintained throughout the experiments.

Samples were analyzed by HPLC according to the methods described below.

2.2.3 Data analyses

The following parameters were calculated starting from the concentrations withdrawn from the receiver compartment of the Franz cell:

a) the <u>cumulative amount</u> permeated through the cell per unit of area was calculated according to the equation:

$$Q_t\left(\frac{\mu g}{cm^2}\right) = \frac{C_t\left(\frac{\mu g}{mL}\right)\times V(mL) + C_{t-1}\left(\frac{\mu g}{mL}\right)\times 0.2(mL) + C_{t-2}\left(\frac{\mu g}{mL}\right)\times 0.2(mL) + \ldots}{0.636\ cm^2} \quad (1)$$

where Q_t is the permeated amount at the time *t*, C_t is the drug concentration at the same time point, V is the volume of the Franz cell, 0.2 mL is the volume withdrawn from the receiver compartment at each time point and 0.636 cm^2 is the surface area of the Franz cell.

The Q_t values were plotted as a function of time. Such plot should yield a straight line;

b) the <u>maximum flux</u>, *J* ($\frac{\mu g \times h}{cm^2}$), of the drug permeated through the skin was calculated as the slope of the linear portion of the cumulative curve.

2.3 HPLC analyses

2.3.1 Propranolol

The quantification of propranolol permeated amount was performed using the analytical method described by Cilurzo and co-workers (Cilurzo et al., 2014). Briefly, an Agilent 1100 HPLC system equipped with an 1100 autosampler, an 1100 quaternary pump with degasser, an 1100 thermostated column compartment, and 1100 diode array detector was used (Agilent, US). A reverse-phase column was used as the stationary phase (Bondclone C18, 10 mm, 3.9x300 mm, Phenomenex, US) and a combination of acetonitrile with 0.2% phosphoric acid at the ratio of 30:70 at 25 °C was used as the mobile phase. The flow rate was set at 1.0 mL/min. The injection volume was 20 µL. The retention time of PR was 7.1 min. The drug concentration was determined from three standard calibration curves in the range 0.005–5, 1–40 and 10–200 µg/mL. The limit of quantification was determined at 0.004 µg/mL.

2.3.2 Diclofenac epolamine

Experiments were performed on an LC-320 with an electron spray ionization (ESI) source and a 320-MS triple quadrupole mass spectrometer, equipped with two 212 LC chromatographic pumps and a 410-tray cooled autosampler (Varian, US). The system was managed by MS Workstation software Version6.9.1 (Varian, US). The ESI-triple quadrupole mass spectrometer was set to perform collision-induced dissociation experiments in positive ionization mode, using argon as a collision gas. Particularly, multiple reaction monitoring (MRM) experiments were conducted by the continuous injection at a rate of 20 µL/min of the standards of interest (2 µg/mL) into the mass spectrometer set in positive ionization mode, in methanol solutions. In order to enhance ion formation and to improve conductivity, the ESI parameters and the chromatographic conditions were set up according to preliminary studies. The method was optimized for the research of the ions of interest, for example, the specific MRM transitions of each standard. In detail, once recognized the molecular ion of each standard (M + H+), MRM experiments were performed to study the characteristic fragmentation pattern of the analytes. ESI source settings and mass spectrometer parameters used for compound identification were Needle Voltage: +5000V; Shield Voltage: +600V; Nebulizing Gas (N_2) Pressure: 30.00 psi; Drying Gas (N_2) Pressure: 30.00 psi; Drying Gas (N_2) Temperature: 250 °C; Q0 Offset: +3.0V;L4 Offset: +2.000V; Housing Temperature: 50°C; CID Gas (Ar); Pressure: 2.00 mTorr; Electron multiplier: 1750.0V; Scan time: 0.9 sec; Dwell time: 0.1 sec.

Chromatographic column: Raptor Biphenyl Restek 100 x 2.1 mm i.d. particle size 2.7 µm; (Restek, US); pre-column: Security Guard Cartridges Raptor Biphenyl (Restek, US); column temperature: 40 °C; manifold temperature: 42 °C; housing temperature: 50 °C; flow rate: 200.0 µL/min; injection volume: 20 µL; injection mode: partial loopfill; solvent A: formate buffer 3mM + 0.1% formic acid; Solvent B: Methanol; mobile phase: solvents were filtered under vacuum on 0.45 µm membrane filters and degassed by immersion in ultrasonic bath for 15 minutes before column conditioning; linear gradients: 0.0-1.0 min, 60% B; 2.0-6.0

min, 90% B; 7.0-9.0 min 60%. Retention times: diclofenac 5.0±0.2 min; IS (Indomethacin) 5.9±0.2 min. The linearity of Diclofenac base was in 20 to 2000 ng/mL range.

2.3.3 Nitroglycerine

The quantification of nitroglycerine in the receiver medium was determined using an Agilent 1100 HPLC system equipped with an 1100 autosampler, an 1100 quaternary pump with degasser, an 1100 thermostated column compartment, and 1100 diode array detector was used at 220 nm (Agilent, US). A reverse-phase column C18 was used as the stationary phase (Spherisorb ODS2, 5 μm, Shandon HPLC, UK). A combination of methanol, water and tetrahydrofuran (54:43:3) was used as the mobile phase. The flow rate was set at 1.0 mL/min. The injection volume was 10 μL. The drug concentration was determined from three standard calibration curves in the range 0.001–0.1 mg/mL.

2.4 Statistical analyses

The possible outliners within each series were checked by Dixon's Q test and eventually discarded.
The comparison was performed on the bases of fluxes (*J*) since the flux is the most relevant permeation parameter (EMA, 2014). Indeed, considering that both the *J* and the C_{max} can be considered kinetic descriptors, the same statistically approach for assessing bioequivalence *in vitro* can be also applied for calculating the acceptance interval to assess the equivalence between the permeation fluxes of two products.
The statistical analysis of the skin permeation experiments was upon a null hypothesis of non-equivalence considering the treatment of parallel experiments:

Hypothesis testing:

H_0: $Mean_{Reference} - Mean_{test} \leq L$; $Mean_{reference} - Mean_{test} \geq U$

H_1: $L < Mean_{Reference} - Mean_{test} < U$

The choice for the parallel design has been done considering the asset of the experiment: two treatments assigned randomly at each experimental unit, constitute by Franz cells plus skin patch, exposed univocally and independently to each treatment.
In general, the acceptability interval for the difference of the means in the log scale is 0.8-1.25 for bioequivalence study. However, in the case of data presenting a high variability, it could be computed using the total between-experimental unit variance. Thus, the U and L of the acceptability interval were therefore determined according to Eq. (2) and (3):

$$U = e^{k\,\sigma_r} \quad (2)$$

$$L = e^{-k\,\sigma_r} \quad (3)$$

where *k* is constant set to 0.760 (EMA, 2010) and σ_r is the standard deviation of the log-transformed values of *J* of the reference product. CV is the coefficient of variation of the log-transformed values of *J* of the reference product. For small estimates of σ_r^2, the σ_r can be approximated to CV (Julious, 2004).

Therefore, the Eq. (2) and (3) can be written as:

$$U = e^{k\,CV} \quad (4)$$

$$L = e^{-k\,CV} \quad (5)$$

For assessing the equivalence of the fluxes of two formulations in the logarithmic form the Two One-Sided Test Procedure (TOST; $\alpha = 0.05$) was used in agreement with Schuirmann (Schuirmann, 1987). The SAS 9.4 TS Level 1M3 (SAS Institute Inc., USA) procedure used was reported in the **Supplementary materials**.

3 Results

3.1 Propranolol

The propranolol permeation profile and calculated fluxes are shown in **Fig.1** and **Table 1**, respectively. The coefficient of variation of both the formulations resulted lower than 30% and therefore to assure the equivalence between the two patches the limits of the confidence intervals should be included in the 0.80-1.25 as suggested by the EMA guideline on quality of transdermal patches (EMA, 2014).

The application of the SAS procedure (see **Supplementary materials** for details) for calculating the interval of confidence assuming both the patches as a reference gave the following results:

P-patch 1 vs P-patch 2: 0.8932 - 1.1843

P-patch 2 vs P-patch 1: 0.8457 - 1.1210

This data revealed that when the skin permeation variability is low, the equivalence between two drug products can be easily demonstrated using a limited number of replicas.

3.2 Diclofenac epolamine

The permeation fluxes of diclofenac through the epidermis of five different donors from the two plasters under evaluation are summarized in **Table 2**. As shown in **Fig.2**, the in vitro permeation profiles through the human epidermis of the reference and the test overlapped and the Geometric Mean Ratio (GMR) was close to 1 (GMR: 1.002). Nevertheless, as supposed on the basis of literature data, the fluxes showed a wide variability since the overall coefficient of variation resulted 55.50% and 47.31% for D-plaster 1 and D-plaster 2, respectively.

The average permeation profiles of diclofenac epolamine were represented in **Fig.2**.

This high variability impeded the application of the 0.80-1.25 range (Zhang et al., 2013). However, considering that the flux (*J*) calculated in the *in vitro* permeation study is a kinetic descriptor as C_{max} retrieved by the *in vivo* pharmacokinetic studies, the same methodological approach reported in the EMA guidance on the investigation of bioequivalence for assessing the bioequivalence of highly variable drugs or drug products (HVDP) could be applied (EMA, 2010). In accordance, the acceptance intervals determined

using Eq. (4) and (5) reported in **Table 4** could be established. It should be underlined that according to the EMA guideline the maximum acceptable interval was 69.84 – 143.19% (CV% ≥ 50%) and, therefore, such range must be used for assessing equivalence D-plaster 1 vs D-plaster 2 instead of the calculated range (**Table 3**).

The 90% confidence interval of the ratio between the experimentally determined *J* (Two One-Sided Test (TOST; $\alpha = 0.05$) procedure calculated by SAS, see ***Supplementary materials***) resulted:

D-plaster 1 vs D-plaster 2: 0.7740 – 1.2982

D-plaster 2 vs D-plaster 1: 0.7703– 1.2920

Both the confidence intervals are included in the U, L limits reported in **Table 4** confirming the equivalence of the two diclofenac epolamine plasters.

3.3 Nitroglycerine

The fluxes of N-patches per cm^2 and normalized for the area of application of the patch are shown in **Table 4**. As expected, the analysis of the equivalence performed by SAS procedure evidences that the two patches cannot be considered equivalent when the fluxes are calculated by the surface unit ($\mu g/cm^2/h$), whilst the confidence interval falls within the range indicated by the EMA guideline if the entire surface of the patch is considered (**Table 5**).

4 Discussion

The introduction of *in vitro* human skin permeation experiments in the EMA guideline (EMA, 2014) opens new opportunities in the development of transdermal patches and medicated plasters providing the possibility to rationalize the reduction of the number of *in vivo* studies. Nevertheless, the experimental set up has to be clearly designed and the critical variables validated (Ng et al., 2010) for their applicability to generic or abridged applications for drug products containing active pharmaceutical ingredients with a high skin permeation variability. This variability is determined not only by the physicochemical properties of the permeant (Franz et al., 2009), but also by the intrinsic physiological variability, which is related to a different composition of the stratum corneum lipids and/or keratins as well as their spatial organization (Gennari et al., 2016). On the bases of these premises and the results obtained in the present experiment, the following remarks can be withdrawn.

First, the validation of the method used to assess the skin sample integrity remains one of the main critical issues in the experimental set up of an i*n vitro* skin permeation test. Even if the skin integrity control is a routine procedure which is usually performed before each permeation experiment, the available data is still limited and the values reported in the literature are not uniform (**Table 6**).

This could be related to different conditions or methods to isolate the biological membrane as well as the operative conditions to measure the skin integrity. The inter-laboratory comparability is still difficult and the obtained results in term of skin sample acceptability can be different.

The defined operative conditions concerning the evaluation of skin integrity as well as Franz cell set up should be validated in the laboratory and monitored over time using molecules having different physicochemical characteristics and an in-house well-known permeation pattern (**Musazzi, 2018**).

Secondly, the suitability of all operative conditions should be monitored over time using permeant with well-known permeation patterns determined by intra-laboratory experiments. Indeed, the overall data revealed that the establishment of the number of replicas in *in vitro* skin permeation experiment is strictly dependent on the variability of the diffusion process which is obviously related to the physicochemical characteristics of the permeant. The caffeine and benzoic acid could be used (Musazzi, **2018**), since it is expected that the former should permeate in a lower extent and with a higher variability (Van de Sandt et al., 2004). This dependence of the permeation pattern on the physicochemical properties of the permeant is well known by literature data. As mentioned before, for hydrophilic solutes like caffeine, *in vitro* skin permeation is much more sensitive to inter and/or intra-subject variation in skin barrier properties, compared to more lipophilic molecules like the parabens (Akomeah et al., 2007).

Besides performing in vitro drug permeation studies, it is essential to consider the variability in the absorption of the tested permeant according to its physicochemical properties, mainly when these data are used to normalize/standardize or compare any generated data. The cases of propranolol and nicotine (permeants with a very low intra-individual variability) revealed that six or fewer replicas could be sufficient to demonstrate the equivalence between two patches and, therefore, in agreement with the EMA guideline a wider number of replicas are required merely to exclude a possible inter-donor variability. Conversely, in the case of diclofenac, a high number of replicas (n=20), which exceeds the number suggested by all the compendial guidelines [the OECD guideline reported at least four replicas per formulation (OECD, 2004), whereas the EMA guidelines on Transdermal Patch at least six replicas (EMA, 2014)] are needed. This large variability of the skin permeation profile of diclofenac epolamine, regardless of the vehicle used, was also reported in the literature (Minghetti et al., 2007) confirming the empirical relationship between the hydrophilicity and permeability of compounds. However, considering that the solubility of a compound in the *stratum corneum* environment changes as function of the pH of the human skin which is in turn influenced by the pH of the receiver medium (Wagner, 2003), the study of this parameter could be performed to reduce the variability of the permeation data.

Thirdly, the analyses of the skin permeation data of medicated plaster or cutaneous patches (i.e., the formulation intended for a loco-regional administration) should be partially distinguished from those obtained by transdermal patches. Indeed, in the latter case, the total flux can be modulated by changing both the patch dimension and the drug content while in the former case the dimensions cannot be modified since they determine the area of the treatment. As a consequence, in the case of transdermal patches, fluxes should

be normalized for the effective patch area rather than the Franz cell permeation area. In this work, the results obtained with nitroglycerine confirm the ability of the designed experiments to discriminate different fluxes and meantime the *in vitro-in vivo* correlation in consideration that the two types of patches, qualitatively and quantitatively different but bioequivalent, gave the same fluxes per patches area.

Fourth, in a regulatory perspective, the following aspects should be highlighted. The 90% confidence interval comprised within the ratio of 0.8 to 1.25 required by the EMA Guidelines to claim equivalence is an adequate range when the skin variability is low (i.e., CV lower than 25-30%). For permeants that exhibit a wider variability, the acceptable range could be extended using the Eq. (4) and (5) reported in this work. Accordingly, the number of replicas should be also increased. Nevertheless, the number of replicas cannot be easily predicted using the commonly used statistical approaches for parallel clinical studies since they are mainly based on the estimation of the intra-subject variability, which cannot be determined for *in vitro* skin permeation studies. As an example, when no difference between the flux means is hypothesized, the following equation can be applied (Julious, 2004):

$$n_t = \frac{(r+1)\sigma^2\left(Z_{1-\beta/2}+Z_{1-\alpha}\right)^2}{rd^2} \quad (6)$$

where $Z_{1-\beta/2}$ and $Z_{1-\alpha}$ are the Z-values of Standard Normal Distribution calculated for Type I (α) and Type II error (β). In the case of $\beta = 0.10$ (Power: 90%) and $\alpha = 0.05$, the Eq. (6) becomes:

$$n_t = \frac{21.87\sigma^2}{d^2} \quad (7)$$

Even considering a low variability, i.e. a permeant with a CV = 25%, the number of replicas, calculated using Eq. (7), resulted in 34 replicas, which is six-fold higher than the number indicated by the EMA guideline and used in this work for propranolol patches.

In conclusion, the skin penetration studies could be designed following the well-established approach defined for *in vivo* bioequivalence study. Nevertheless, the most significant output of the experiment, namely the flux of the permeant through the skin should be treated as the C_{max} since it is obviously a kinetic parameter. Furthermore, in the case of a high variable permeant, the number of replicas should be established on the basis of the experience since, to the best of our knowledge, no suitable model to predict them is available. Further studies may permit to relate the CV with an acceptable range of replicas.

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Figure captions

Figure 1 In vitro propranolol skin permeation profiles obtained by propranolol patches (mean±Dv.st; n=6).

Figure 2 Permeation profiles of D-plaster 1 and D-plaster 2 plasters (mean±Dv.st; n=20).

Tables

Table 1 – Permeation fluxes (*J*) of propranolol obtained by P-patch 1 and P-patch 2.

Epidermis Donor	*J* (μg/cm^2/h)	
	P-patch 1	P-patch 2
A	12.43±1.50	12.03±1.20
B	10.93±1.51	10.67±1.59
All	11.70±0.80	11.40±1.57

Table 2 – Permeation fluxes (*J*) of Diclofenac obtained by D-plaster 1 and D-plaster 2.

Epidermis Donor	*J* (μg/cm^2/h)	
	D-plaster 1	D-plaster 2
C	0.240±0.049	0.207±0.129
D	0.287±0.075	0.245±0.075
E	0.145±0.051	0.178±0.048
F	0.151±0.069	0.150±0.064
G	0.198±0.118	0.214±0.069
All	0.205±0.081	0.198±0.080

Table 3 – Upper (U) and lower limits (L) of the acceptability interval calculated on the bases of overall *J* data of D-plaster 1 or D-plaster 2.

Plaster	Limits	Calculated Value
D-plaster 1	U	1.5247
	L	0.6559
D-plaster 2	U	1.4327
	L	0.6980

Table 4 – Permeation fluxes of nitroglycerine obtained by the tested patches and calculated on the basis of the permeation (J) and application (J_{tot}) area, respectively.

	J (μg/cm^2/h)	J_{tot} (μg/patch area/h)
N-patch 1	28.35±4.85	510.30±87.27
N-patch 2	35.88±3.04	502.32±42.61

Table 5 – Confidence intervals of the fluxes (J and J_{tot}) of the two commercial nitroglycerine patches

	Confidence interval	
	(μg/cm^2/h)	(μg/patch area/h)
N-patch 1 vs N-patch 2	0.6448-0.9505	0.8291-1.2221
N-patch 2 vs N-patch 1	1.0520-1.5508	0.8181-1.2062

Table 6 – Intervals of electrical resistance (R) available in the literature for assessing the integrity of human skin membranes.

Permeation membrane	R acceptability interval	Reference
Human cadaver skin	≥ 15-20 kΩcm^2	(Chantasart et al., 2013)
Human cadaver skin	≥ 35 kΩcm^2	(Kasting and Bowman, 1990)
Human epidermis	> 50 kΩcm^2	(Mitragotri, 2000)
Human full-thickness skin	12-120 kΩcm^2	(Baroli et al., 2007)
Human epidermis	20 kΩcm^2	Current study

Figure 1

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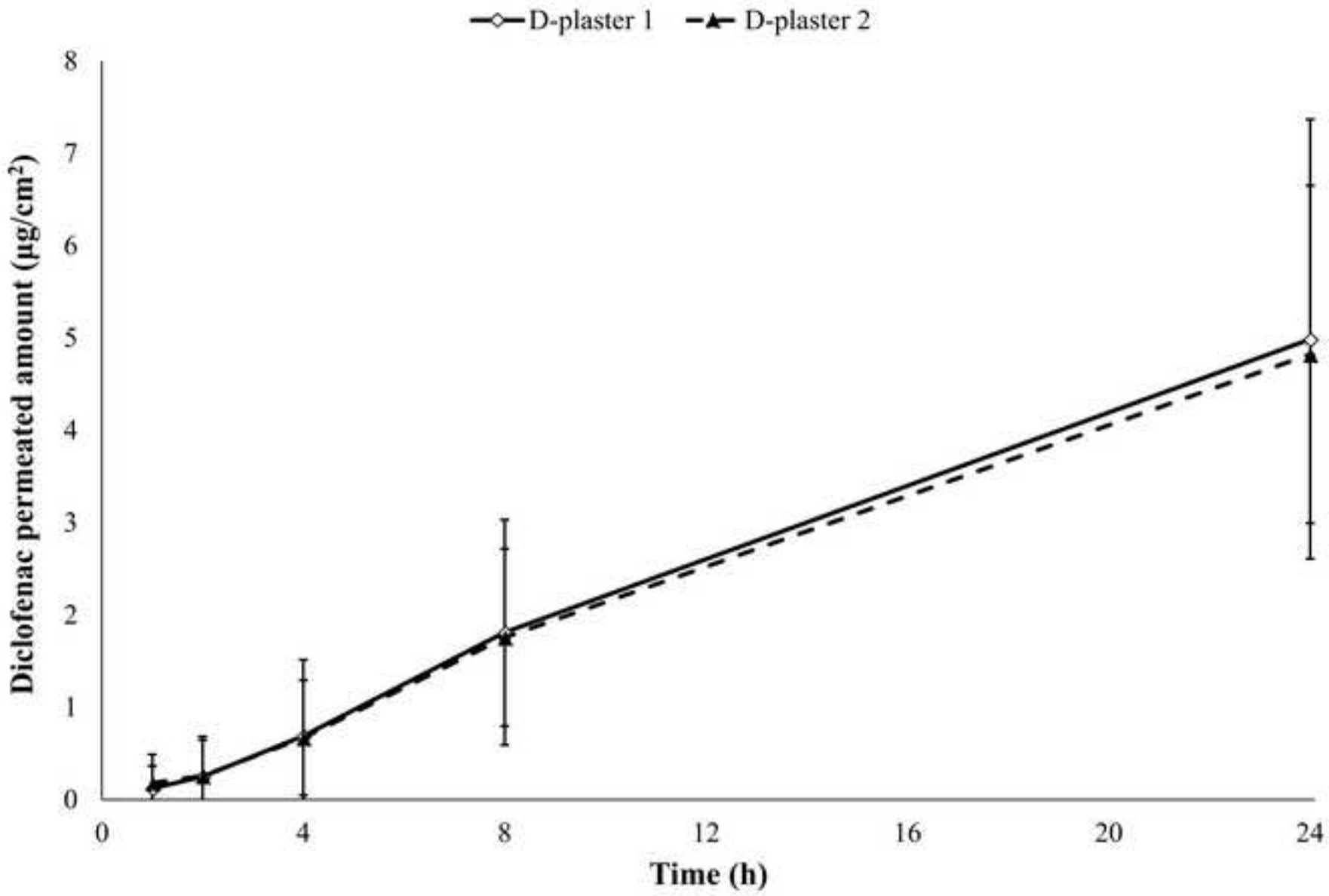

Figure 2

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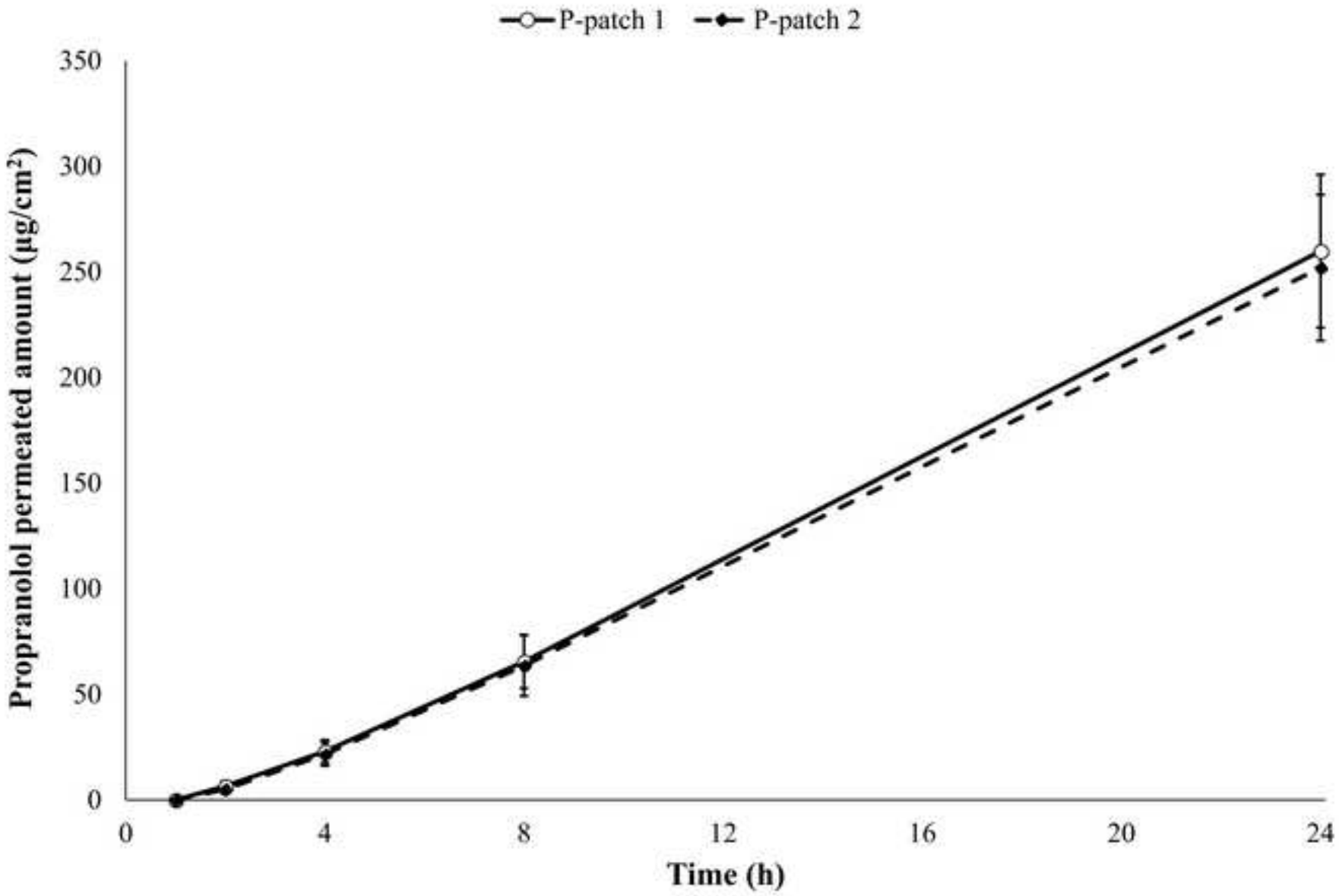

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