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Comparison of skin permeability for three diclofenac topical formulations: an *in vitro* study

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Diclofenac is a hydrophilic non-steroidal anti-inflammatory drug (NSAID) widely used in humans and animals. There are limited published studies evaluating diclofenac's skin permeation following topical administration. The aim of our study was to evaluate and compare the *in vitro* permeation of three different diclofenac-containing formulations (patch, gel, solution) over 24 hours. These formulations were applied (n = 6 per formulation) to pig skin sandwiched between the two chambers in a static Franz diffusion cell and aliquots from the receptor medium were sampled at pre-defined time points. An HPLC method with UV detection was developed and validated with the aim of characterizing the transepidermal penetration in the *in vitro* system. Using this assay to determine the permeation parameters, results at 24 hours showed that the Flector® patch released the highest drug amount (54.6%), whereas a lower drug amount was delivered with the Voltaren® Emulgel (38.2%) and the solution (34.4%). The commercial gel showed the highest flux (39.9 ± 0.9 μg/cm^2/h) and the shortest lag-time (1.97 ± 0.02 h). Based on these *in vitro* results using pig skin, the transdermal patch resulted in a long-lasting controlled release of diclofenac, while the gel had the shortest lag-time.

1. Introduction

Diclofenac (2-(2,6-dichloranilino) phenylacetic acid) can be administrated topically to treat pain and reduce inflammation due to its analgesic, antipyretic and anti-inflammatory effects (Gardner 1994; Skoutatis et al. 1988; Zacher et al. 2008). Diclofenac is available in topical and oral formulations, including a transdermal patch. Advantages of transdermal drug delivery include ease of administration and less frequent dosing due to continuous drug delivery. Local drug delivery serves as a mode to deliver drug directly to the site of action; potentially reducing the risk of systemic side effects and improving patient compliance (Nokhodchi et al. 2002). However, the continuously renewing epidermis is the major limiting factor to topical absorption and makes the transdermal drug delivery challenging. To overcome the low permeability through the skin many strategies have been developed in the realm of transdermal drug delivery during the three last decades (Hussein et al. 2012; Prow et al. 2011; Schoeter et al. 2010; Subedi et al. 2010).

Available topical diclofenac formulations (e.g., patch, gel, spray, lotion) likely differ significantly in their permeation properties. A recent study (Klein 2013) investigated, for quality control purposes, the influence of different test parameters (type of membrane, pH of acceptor media, impact of degassing, influence of the stirring speed and stirrer type) that can impact the *in vitro* drug release from topical diclofenac formulation in a vertical diffusion cell setup.

The objective of our *in vitro* study was to compare the permeation profile for three diclofenac formulations and determine the related pharmacokinetic parameters (i.e., lag-time, flux) using pig skin placed on a static Franz diffusion cell. Moreover, a high performance liquid chromatography (HPLC) method with ultraviolet detection was developed and validated in order to quantify the permeation rate through the skin.

2. Investigations, results and discussion

2.1. Method validation

A rapid, simple and specific method using HPLC with ultraviolet detection was developed and validated by using linearity, stability, precision and accuracy parameters according to the FDA guideline (2007).

The LLOQ was determined to be 10 ng/mL. Over the concentration range of 19.5 ng/mL - 5 μg/mL, the six calibration curves were linear. The coefficient of determination (expressed as mean ± SD) was 0.9997 ± 0.0002 (n = 6). The %RSD values were low for both the intra-day and inter-day precision demonstrating the adequate precision of the developed method. Intra-day and inter-day precision values for QC samples are presented in Table 1. The accuracy was assessed for the QC samples and all the results were within the 85–115% range. No instability problems were detected following short and long-term storage (Table 2).

2.2. Determination of the drug amount in an initial piece of patch (n = 6)

The drug concentration was calculated for each sample using the equation obtained from the calibration curve. The mean content of each piece was 566.1 μg with a standard deviation of 38.9 μg.

Table 1: Intra- and inter-day accuracy and precision at three concentration levels

Nominal concentration (μg/mL)		Intra-day (n = 6)			Inter-day (n = 6)		
		Concentration Mean ± SD (μg/mL)	CV(%)	Accuracy (%)	Concentration Mean ± SD (μg/mL)	CV (%)	Accuracy (%)
LQC	0.025	0.025 ± 0.003	13.3%	100.9%	0.022 ± 0.002	7.3%	87.3%
MQC	2.5	2.54 ± 0.06	2.5%	101.5%	2.54 ± 0.09	3.7%	101.5%
HQC	4	3.94 ± 0.03	0.8%	98.4%	4.11 ± 0.07	1.8%	102.8%

Table 2: Stability results for quality control samples (n = 6)

Nominal concentration (μg/mL)		% RSD			
		Bench top (22 °C - 24 h)	Auto-sampler (4 °C - 48 h)	Freeze/thaw (3 cycles)	Long term (-72 °C - 30 days)
LQC	0.025	2.46%	3.06%	2.55%	1.72%
MQC	2.5	1.39%	1.65%	1.45%	1.68%
HQC	4	1.45%	1.47%	1.22%	1.20%

2.3. Permeation study results

Six permeation studies (i.e., replicates) were performed for each formulation under the same experimental conditions. A mean thickness of 1.3 ± 0.3 mm and a transepidermal water loss (TEWL) of 12.5 ± 2.5 $g.m^{-2}.h^{-1}$ were obtained. Figure 1 shows the cumulative amount of diclofenac, expressed as mean ± SD, that permeated through pig skin over 24 h when using a patch, gel, and solution. The rank for the cumulative amount of diclofenac permeating through pig skin at 24 h was: solution < gel < patch.

Using the linear portion of each curve, the highest flux was obtained for the gel as compared to that for the patch or the solution. Regarding the lag-time, the permeation occurred faster for the gel than with the solution or the patch (Table 3). A significant formulation, time, and formulation*time effect was observed ($p<0.001$).

2.4. Drug content in different compartments after 24 hours permeation

As shown in Fig. 2, the greatest permeation was observed with the patch, where 80.4% diclofenac permeated from the donor chamber through the skin into the receptor medium within 24 h. Permeations of 45.5% and 44.0% were observed for the gel and the solution, respectively.

After 24 h, residual diclofenac was extracted out of the skin for each dosage form. The rank of degree of cutaneous retentions was patch (32.7 ± 0.5 μg, 8.5%) < solution (72.4 ± 0.7 μg, 16.4%) < gel (79.7 ± 0.7 μg, 16.7%). For the solution (39.6%) and the gel (37.8%), a high diclofenac quantity remained in the donor chamber after 24 h, whereas a lower fraction for the patch (11.1%) was not able to permeate through the skin.

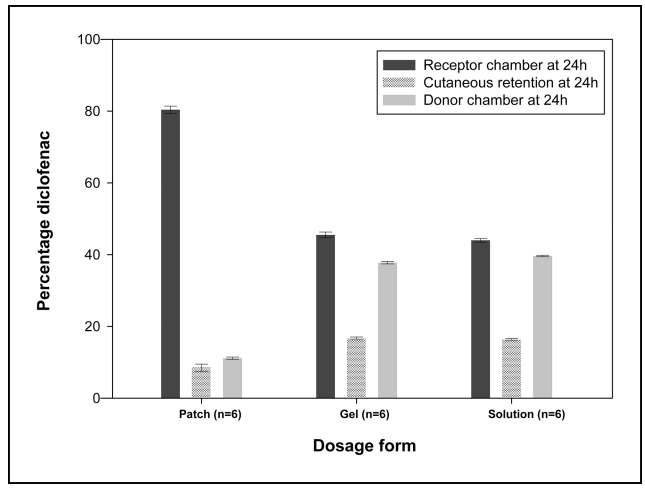

Fig. 2: Percentage of total diclofenac present in the receptor chamber, cutaneous skin, and donor chamber (expressed as a %mean, n = 6) at 24 h for the three different formulations.

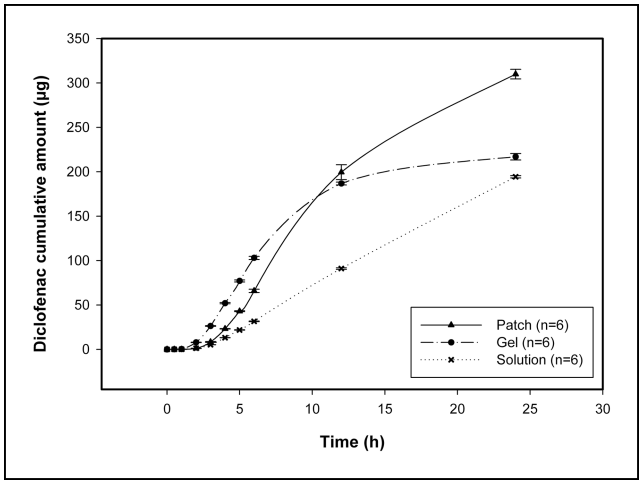

Fig. 1: Permeation profile over 24 h for the three investigated forms (triangle-solid line = patch, circles-dash line = gel, crosses-dotted line = solution) using pig skin and a Franz diffusion cell. The skin temperature was maintained at 31 ± 1 °C during the 24 h. A drug amount of 566 μg was applied to the donor chamber for each permeation study (n = 18 in total). Each point represents the mean cumulative amount in μg (for 6 experiments) ± standard deviation expressed for the investigated dosage form.

Table 3: Cumulative amount of diclofenac permeated after 24 hours through pig skin (0.64 cm^2) maintained at 31 ± 1 °C, flux and lag-time expressed as mean ± SD for the patch, the gel and the solution

Sample name	Cumulative amount (μg) after 24h	Flux (μg/cm^2/h)	Lag-time (h)
Patch (n = 6)	309.9 ± 5.9	33.3 ± 1.6	2.93 ± 0.07
Gel (n = 6)	216.9 ± 3.9	39.9 ± 0.9	1.97 ± 0.02
Solution (n = 6)	194.3 ± 1.4	14.2 ± 0.1	2.45 ± 0.02

3. Discussion

There are a plethora of commercially available diclofenac formulations, which may be topically applied, but an *in vitro* comparison of patch, gel, and solution has not been previously evaluated.

Characterizing the permeation of topical products is an important goal in the drug development process. Available techniques to study skin permeation *in vitro* include excised human skin, animal skin and synthetic systems. Excised human skin is clearly a favorable option due to its ability to mimic *in vivo* skin conditions. Unfortunately, its availability is limited and inter-individual differences due to covariates (e.g., age, race) can result in vastly different permeation profiles. Frequently, use of animal skin is a feasible alternative. Within animal species, monkey and pig skin have been proven to be suitable models of human skin (Moser et al. 2001; Schmook et al. 2001). Numerous descriptive or qualitative comparisons between animal and human skin have been made. Studies have shown that pig skin is a good animal model of percutaneous penetration based on the similarities between porcine and human skin (Meyer et al. 1978, 2007; Schmook et al. 2001). For example, spare hair coat, a thick epidermis with a well differentiated under sculpture, a dermis with well-differentiated papillary bodies, and a large content of elastic tissue.

In the present study, the permeation of diclofenac through pig ear skin for three different formulations was investigated. Independent of the dosage form (patch, gel and solution), diclofenac concentrations could be quantified in the receptor medium following 24 h of application. However only a portion of the total drug amount contained in the formulation permeated after 24 h of application. The rank order for the permeation based on drug permeated at 24 h was: solution (44.0%) < gel (45.5%) < patch (80.4%). On the one hand, the limited permeation over time for the gel may be explained by the fast evaporation of solvent contained in the gel. Furthermore, the use of excipients that control the release of diclofenac in the patch (e.g., solvent, complexing agents and polymers) resulted in a better and a more consistent release over time.

Despite differences in transdermal flux between the gel and the patch, the observed values were close to published human data from the literature (Inayat et al. 2008) ($F = 36.66\ \mu g/cm^2/h$). The low flux for the solution ($14.2 \pm 0.1\ \mu g/cm^2/h$) could be explained by the lack of permeation enhancers such as propylene glycol or isopropyl alcohol.

To achieve a fast pain relief, permeation enhancers are included in the formula of the commercial gel and this led to a short lag-time (1.97 ± 0.02 h), whereas the polymer matrix present in the patch resulted in a longer lag time (2.93 ± 0.07 h). However, the lag-time could have been shortened for the gel, if it had been spread on the skin as indicated on the medical insert.

Considering the observed results, the gel appeared to be less ideal for long application times because of the high solvent content, which evaporates quickly after application. The patch is the most appropriate medication form due to the high drug delivery and the controlled release.

The developed method for diclofenac determination by HPLC with UV detection is simple, precise, and accurate. The precision and accuracy met acceptability requirements and no stability concerns were identified. When comparing the extent of permeation between the three formulations *in vitro*, the patch resulted in the greatest absorption, followed by the gel and solution. In contrast, the gel resulted in the fastest flux and shortest lag-time. These results support the use of the topical gel for a more fast-acting effect, whereas the patch may be useful for more prolonged analgesic and anti-inflammatory effects. The *in vitro* bioavailability of diclofenac and the permeation properties of three different dosage forms were compared and these results may aid in the selection of the most appropriate dosage form for a desired effect.

4. Experimental

4.1. Chemicals, products studied and apparatus

Sodium diclofenac, ammonium bicarbonate salt, acetic acid, formic acid and HPLC-grade methanol were purchased from Sigma (St. Louis, MO, USA). Flector® 1.3% patches (180 mg diclofenac epolamine/patch corresponds to 126.9 mg diclofenac sodium/patch) Lot # 1001062, Expiration 01/2013, Package opened on 06/07/2011 (King Pharmaceuticals®, USA) and the Voltaren® Emulgel 1% (11.6 mg diclofenac diethylamine/g gel, corresponds to 10 mg diclofenac sodium), Lot # W8056 Expiration 02/2015, Package opened 16/04/2012 (Novartis®, France) were obtained directly from retail pharmacies. A 0.0566 mg/mL diclofenac solution in water was prepared for each experimental run, when needed. The drug solution was stirred for 2 h and sonicated for 15 min before each use.

The buffer, ammonium bicarbonate 0.1 N acidified at pH 7.4 with a 10% acetic acid solution in water, was prepared daily in the lab before each experiment. As the diluent, a mixture of methanol and 0.1% v/v formic acid was used in a ratio of 75:25 (v/v). Water was prepared by triple distillation using a borosilicate triple distillation apparatus.

The diffusion cell *(#4G-01-00-09-05*, Fisher, USA) has a side arm, a flat ground and is made of clear glass. The jacketed receptor volume is 5.1 mL and the membrane surface area of the cell is 0.64 cm^2.

4.2. Pig skin

The International Animal Care and Use Committee (IACUC) at the University of Florida approved the study protocol. The ears were purchased from a local Meat Processing Center (University of Florida, Gainesville, FL, USA). During transport from the slaughterhouse, the fresh porcine ears were kept at 4 °C. The whole ears were then cleaned with cold water, placed in zip bags, labeled and stored at -72 °C until the time of the experiments. Selected skin samples were taken from pigs of the same breed (white pigs) and age (about 24 weeks). Moreover no skin samples with wounds, warts or hematomas were utilized. Skin samples were used within three months. The day before conducting the permeation study, one ear was taken out of the freezer and defrosted at 4 °C until next day. On the experimental day, after cleaning the pig skin with cold water, a skin square sample (2.5x2.5 cm) from the central region of the dorsal side of the auricle was excised using scissors and a surgery scalpel (Meyer et al. 2007). The skin was sandwiched in the Franz diffusion cell and 2 hours lapsed before the start of the experiments. This time allowed the skin temperature to reach 31 ± 1 °C. The thickness of the skin was measured and a range of thicknesses between 1.0 and 2.0 mm was accepted. The skin integrity was characterized using a Delfin Wireless VapoMeter (Delfin Technologies, Stamford, USA).

4.3. Diclofenac HPLC determination

4.3.1. Chromatographic conditions

The HPLC system consisted of an Agilent series 1100 quaternary pump with an online degasser, auto sampler and diode array detector (Agilent Technologies, Santa Clara, CA, USA). Sample aliquots (100 µL) were injected onto an Eclipse XDB-C18 column (4.6 × 100 mm, 3.5 µm) (Agilent Technologies, Santa Clara, CA, USA) and an absorbance of 280.16 nm was monitored. The system was run in an isocratic mode with a mobile phase consisting of methanol: 0.1% v/v formic acid in a ratio of 75:25 (v/v). A flow rate and injection volume of 0.8 mL/min and 100 µL were used, respectively. The observed retention time of diclofenac was 3.9 ± 0.5 min. Integration of chromatographic peak was done using ChemStation version A10.02 (Agilent Technologies, Santa Clara, CA, USA). The mobile phase was filtered through a 0.22 µm Nylon filter (Millipore, Billerica, MA, USA) and degassed in an ultrasonic bath for 30 min prior to use. The column was maintained at 38 °C during the run and subsequently washed with water followed by acetonitrile following each use. The carryover into the auto sampler was negated by the injection of blank mobile phase following injection of the highest standard curve concentration. No carryover was detected.

4.3.2. Calibration curves

Quantification of diclofenac was based on plotting the peak area versus the diclofenac concentration obtained from each calibration curve. The calibration curve standards for diclofenac were prepared by spiking diluent with the working solution to achieve concentrations in the range of 19.5 ng/mL (CS9) to 5 µg/mL (CS1). Nine different calibration standards were prepared in total for each calibration curve. In addition, to assess the linearity, precision and

accuracy, three quality control samples (QC) at three different concentrations (HQC; 4 μg/mL, MQC; 2.5 μg/mL and LQC; 25 ng/mL) were prepared separately and analyzed with each calibration curve. The calibration curves were obtained by plotting the concentration of diclofenac *versus* the peak area. The calibration range was chosen to reflect the diclofenac concentration contained in the receptor medium after drug delivery.

4.3.3. Method validation

The linearity of the method was evaluated by analyzing six replicates for each of the nine calibration standards (CS1-CS9). The linearity in peak area for concentrations up to 5 μg/mL was assessed. The coefficient of determination (R^2) of the calibration line, and the percentage residuals for each calibration standard (% difference of back-calculated concentration from the nominal concentration) were calculated. The desired standards, which needed to be met, included a coefficient of determination (R^2) greater than 0.999, and the standard deviation (%RSD) to be within ± 15% for all the concentrations tested. The lower limit of quantification (LLOQ) was determined by evaluating the precision for 6 replicates (i.e. the standard deviation (%RSD) needed to be within 20%).

The intra-day precision was determined by analyzing six replicates of QC samples at three concentrations (4 μg/mL, 2.5 μg/mL and 25 ng/mL). The inter-day precision was determined on five separate days by analyzing QC samples at three concentration levels. The intra- and inter-day precision was calculated as the coefficient of variation (%CV), whereas the accuracy (% accuracy) was expressed as the relative error. To meet acceptable precision requirements, measured values must be within ± 15% for all the QC samples except LLOQ (±20%). Similarly the accuracy should not exceed ± 15% deviation for QC samples (±20% for LLOQ).

The stability of diclofenac under different conditions was established. Autosampler stability (48 h at 4 °C), bench-top (24 h at room temperature), long-term (30 days at -70 °C), and freeze-thaw stability (3 cycles) were evaluated. Each stability test was conducted with six aliquots of the quality control samples at three concentration levels (6xHQC, 6xMQC and 6xLQC). A 15% or less difference between the mean concentration of the stored samples (n = 6) and nominal concentration was used as a benchmark to conclude that there are no instability concerns.

4.4. Patch, gel, solution and skin extraction

4.4.1. Determination of the drug amount in an initial piece of patch

The initial diclofenac content was assessed in six separate 0.64 cm^2 round pieces of Flector® patch. The average of the six pieces was calculated and used as the initial content for all future studies conducted with the transdermal patch. Briefly, after removing the plastic adhesive strips, each piece (n = 6) was transferred into a labeled 15 mL Corning tube. Then 5 mL of methanol was added to each tube as an extracting agent. The sealed extraction tubes were shaken for 2 h using a Vortex machine (Maxi Mix II, Thermo Scientific, IL, USA), at a speed of 10, and then sonicated for 15 min. Next, 50 μL of the obtained solutions were transferred into microcentrifuge tube and 950 μL of diluent was added. Each tube was vortexed for 30 s. Last, a 500 μL aliquot from each sample was transferred into a labeled 0.3 mL vial to lead the HPLC-analysis as described above.

4.4.2. Determination of diclofenac content remaining in the patch following each permeation study (n = 6)

The patch was carefully removed from the pig skin after the 24 h of application. Each patch piece was directly placed into a 15 mL Corning tube containing 5 mL methanol. The sealed extraction tube was shaken for 2 h as described above and then sonicated for 15 min. The sample was analyzed after a 10-fold dilution.

4.4.3. Determination of diclofenac content remaining on residual gel (n = 6)

Residual gel layer remaining on the skin was carefully removed from the skin using a spatula and added to 5 mL methanol. The same procedure (vortex, dilution and analysis) described for the patch was applied after each study (n = 6) to quantify the diclofenac content in the residual gel.

4.4.4. Quantification of the amount of drug remaining in the solution after permeation (n = 6)

The remaining liquid present in the donor chamber was transferred into a 15 mL Corning tube containing 5 mL methanol. The sealed extraction tube was shaken for 2 h. After a 10–fold dilution, the sample was analyzed using HPLC.

4.4.5. Determination of the amount of drug remaining in the skin after permeation; characterization of the cutaneous retention (n = 18)

At the end of each permeation study, the skin was removed from the Franz diffusion cell. The skin was directly blotted dry and the permeation area of the skin was then excised with scissors and its diclofenac content was extracted with 5 mL methanol. The tube was vortexed for 2 h and then sonicated for 15 min. The resulting solution was diluted 10-fold and the diclofenac level was measured by HPLC.

4.5. Permeation studies

The permeation studies were performed using the diffusion cell described above. Dorsal pig ear skin samples were placed between the donor and receptor chambers of the cell with the dermal side in contact with the receptor medium. The receptor chamber was filled with 5.1 mL of ammonium bicarbonate buffer (pH = 7.4) and kept at 37 ± 0.5 °C using a circulating water jacket; the goal being to maintain a skin temperature of 31 ± 1 °C. The receptor sampling side arm opening was carefully covered with Parafilm® to avoid unnecessary evaporation. The studied formulation was applied on the epidermis of the sandwiched skin. Then either a 0.8 cm round piece of patch, 0.0566 g gel or 1 mL of a 0.0566 mg/mL diclofenac solution were applied on the skin. Samples of 500 μL were withdrawn from the middle of the receptor compartment, using a 1000 μL-syringe, at 0.5, 1, 2, 3, 4, 5, 6, 12 and 24 h, and replaced with the same volume of ammonium bicarbonate buffer maintained at 37 °C in a temperature controlled water bath. Sink conditions were maintained in all cases and the presence of no air bubbles was carefully checked after each buffer replacement. Six different permeation studies per formulation were performed on different days using the dorsal parts of pig ears. Samples were stored in the freezer (-72 °C) until HPLC analysis. The cumulative drug amount permeated at each sampling time (Q_t) was calculated using Eq. (1) (Gonsho et al. 1990)

$$Q_t = V_r * C_t + \sum_{i=0}^{t-1} V_s * C_i \quad (1)$$

where C_t is the drug concentration of the receptor medium at each sampling time, C_i the drug concentration of the *i*th sample, and V_r and V_s are the volumes of the receptor medium and of the sample, respectively.

4.6. Data analysis

The permeation of diclofenac from the three different formulations was measured over 24 h and plots of the cumulative amount of diclofenac (μg) in the receptor fluid against time (h) were created using SigmaPlot (Version 12, Systat Software Inc., San Jose, CA, USA). For each study the abscissa intercept of the linear region (Lag-time, h) was determined by linear regression. In addition, the slope of the linear portion of the graph divided by the surface area (0.64 cm^2) provided maximum flux values at steady state (Flux, μg/cm^2/h) (Baert et al. 2011). A two way ANOVA analysis to study the statistical significance between formulations was conducted with the software R (version 2.15.2).

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