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A miniaturized flow-through cell to evaluate skin permeation of endoxifen

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ABSTRACT

Endoxifen, an anti-estrogenic agent, has been recently implicated in the use of breast cancer. Its physicochemical properties make it a good candidate for transdermal delivery. However, as an investigative drug, its limited supply makes it difficult to conduct extensive pre-formulation studies. To address this issue, a miniaturized flow-through diffusion cell has been fabricated that utilized minimal amounts of the drug for *in vitro* skin permeation studies. The novel flow-through cells have been validated against horizontal diffusion cells and shown to cause no noticeable damage to the applied skin, as observed by histological sectioning. The cells were also demonstrated to be useful in search of suitable enhancers for endoxifen. Endoxifen permeation using permeation enhancers was tested by using this new device and limonene was found to achieve highest flux, attaining the requirement for clinical applications. The fabricated cells can thus be useful in carrying out pre-formulation studies for expensive, new drug entities, both in industrial as well as academic research.

1. Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide with about 1.38 million newly diagnosed cases each year (Jemal et al., 2011). Nearly 70% of breast cancer patients are hormone-receptor positive (Del Re et al., 2011). For these patients, tamoxifen has been the most widely used adjuvant endocrine therapy (EBCTCG-Secretariat, 2005). While tamoxifen is effective, it is a pro-drug that requires extensive CYP2D6 metabolism into active metabolites such as endoxifen (ENX) (Fig. 1) (Desta et al., 2004; Holmes and Liticker, 2005; Johnson et al., 2004). Recently, it has been reported that genetic polymorphism in CYP2D6 can impair the biotransformation of tamoxifen into its active metabolites (Hoskins et al., 2009; Singh et al., 2011). To overcome the poor outcomes associated with breast cancer therapy for patients with reduced CYP2D6 activity, direct administration of endoxifen has been advocated (Ahmad et al., 2010a,b; Wu et al., 2009). Clinical trials are currently being conducted on the oral use of endoxifen as the hydrochloride salt form (NIH, 2011a,b).

Apart from the oral route, transdermal drug delivery of the endoxifen has also been explored. However, such studies showed a limited drug flux through skin. Based on the required daily dose of endoxifen, it cannot achieve the therapeutically relevant concentrations (Ahmad et al., 2010a; Lee et al., 2011). Therefore, further studies are needed for its effective delivery through skin. Moreover, a transdermal gel of 4-hydroxy metabolite of tamoxifen is currently under phase 2 clinical trials, indicating the potential for transdermal administration of active metabolites like endoxifen in the management of breast cancer (NIH, 2009).

Conventionally, a variety of transdermal diffusion cells were developed for the evaluation of *in vitro* permeation characteristics of transdermally delivered drugs. In principle, some are based on the static, non flowing cells (Bartosova and Bajgar, 2012) in which the donor and receptor compartments may be placed either vertically (Franz type) (Windheuser et al., 1982) or horizontally (side-by-side) (Bellantone et al., 1986; Tojo et al., 1987) and others are the in-line, flow through cells, that offer the advantage of continual replenishment of receptor fluid and hence aid in maintaining a condition similar to microcirculation in the *in vivo* setting (Bronaugh and Stewart, 1985; Selzer et al., 2012).

Several modified versions of these diffusion cells have also been fabricated and validated against the conventional apparatus. Sanghvi and Collins compared the permeation characteristics of hydrocortisone using the "enhancer cell", which is a modified version of USP type II dissolution apparatus to serve as a diffusion cell (Sanghvi and Collins, 1993). Modified automatic sampling apparatus have been developed (Akazawa et al., 1989; Hanson, 2012; Martin et al., 1989; Permegear, 2012). These static and flow-through cells have been compared and validated (Cordoba-Diaz et al., 2000; Ng et al., 2010; Rapedius and Blanchard, 2001).

However, a major drawback of these cells is the requirement of relatively large amounts of drug owing to their inherent design. Investigational new drug entities, such as endoxifen, are prohibitively expensive for such studies. This motivated us to

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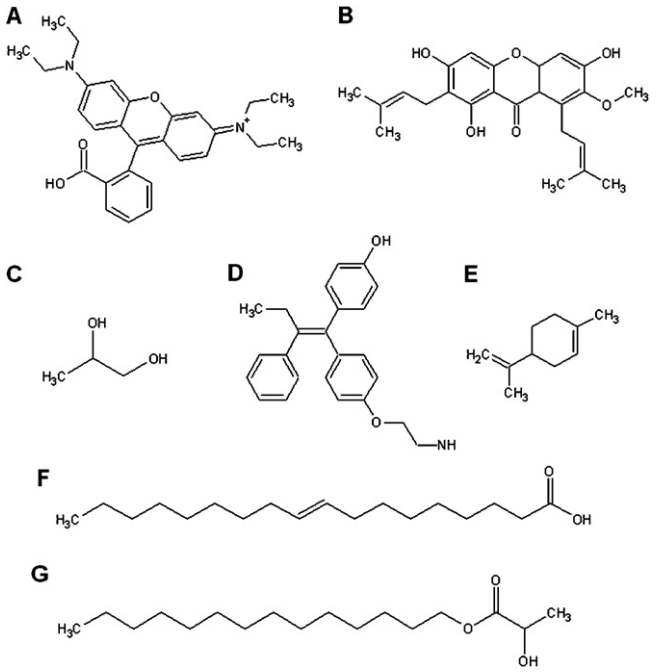

Fig. 1. Chemical structures of (A) mangostin (MW = 410.46, log *P* = 6.64), (B) rhodamine B (MW = 479.02, log *P* = 2.43), (C) propylene glycol (MW = 76.09, log *P* = −1.00), (D) endoxifen (MW = 373.49, log *P* = 4.94), (E) limonene (MW = 136.2, log *P* = 4.83), (F) oleic acid (MW = 282.46, log *P* = 7.42) and (G) myristyl lactate (MW = 286.45, log *P* = 6.08).

develop a miniaturized testing system that utilizes minimum amount of the drug.

Microfluidic platforms which are miniaturized fluid flow systems have recently received significant attention in the drug discovery and development horizon, due to their abilities to reduce the amount of reagents necessary for assays and pre-clinical development (Kang et al., 2008). These microscale systems fabricated with biomaterials such as polydimethylsiloxane (PDMS), may provide a useful model to develop miniaturized flow-through cells. We envisaged a PDMS-based, miniaturized flow-through cell to minimize the consumption of candidate drugs. With the economic environment in pharmaceutical firms becoming more tenuous and pharmaceutical cost containment being the main focus, the need to develop pre-formulation testing systems that utilize minimum amount of the drug is the need of the hour.

In this study, we fabricate a miniaturized flow-through cell for *in vitro* skin permeation studies. The system was compared and validated against a static, horizontal diffusion cell (HDC) using two model drugs, namely, rhodamine B and α-mangostin. We also conducted histological sectioning of the skin 24–48 h post-application in both diffusion cells to test for skin damage. Subsequently, the skin permeation of endoxifen was assessed with several skin permeation enhancers (PEs). One of the enhancers was found to be able to deliver enough endoxifen for its clinical applications.

2. Materials and methods

2.1. Materials

Rhodamine B and sodium azide were obtained from Alfa Aesar, UK. Phosphate buffered saline (PBS) (10×) was obtained from Vivantis, Malaysia. Propylene glycol was obtained from Chempure, Singapore. Polydimethylsiloxane (PDMS) (Sylgard 184 Silicone Elastomer Kit) was obtained from Sylgard, USA. Methanol for HPLC was purchased from Tedia, USA. Endoxifen hydrochloride, (R)-(+)-limonene and oleic acid were obtained from Sigma–Aldrich, USA. Myristyl lactate was a gift from Chemic Laboratories, USA. α-mangostin standard was supplied by Dr. Prachya Kongtawelent from Chiang Mai University, Thailand. All PBS solutions used in the permeation experiments contained 0.005% of sodium azide as anti-microbial agent (Sznitowska et al., 2001). Ultrapure water (Millipore, USA) was used in the preparation of aqueous solutions.

2.2. Fabrication of miniaturized flow-through cell (MFtC)

The fabrication process involved two simple PDMS molding steps. Firstly, for fabricating the receptor compartment (16 mm tall, 22 mm wide), a specially designed borosilicate glass mold (16 mm wide) was inserted into a single well of a 12-well plate, (Cellstar, Greiner Bio-One), carrying 0.9 mm poly vinyl chloride tubing, (B. Braun, Germany) bore through its axis. The borosilicate glass sits firmly in a small split created in the tubing. PDMS was then filled into the cavity between the glass mold and the well plate (Fig. 2A) and was subsequently cured at 70 °C for 2 h. The glass mold was then removed to create a hollow cavity for donor compartment to sit in.

The donor compartment (13 mm tall, 16 mm wide) was fabricated with a similar process in a single well of a 24-well plate. A 6 mm hollow lumen was first created with a metal mold (Fig. 2B). The mold was placed in the well of the 24-well plate and PDMS was used to fill the space between the external wall of the mold and the 24-well plate and was similarly cured at 70 °C for 2 h. The metal mold was removed to create a hollow cavity, to serve as the donor liquid compartment. As part of the property of MFtC, donor compartment was designed to hold up to 283 μl of drug solution with an area of 0.283 cm^2. The assembled donor and receptor compartments are shown in Fig. 2C.

2.3. Assembly and operation of MFtC

MFtC was assembled by connecting the tubing of the fabricated cell to an infusion pump system (Terufusion, UK) at one end and sampling tubes at the other end (Fig. 2D). The fabricated diffusion cell was then placed in a water bath maintained at 37 °C using a hot plate. Drug solution is placed in donor compartment. Flow rate of the receptor solution through the fabricated diffusion cell was controlled by the infusion pump that delivers the solution from a syringe (Fig. 2D).

2.4. Validation of MFtC against horizontal diffusion cell

To evaluate the performance characteristics of the MFtC, permeation of model compounds (rhodamine B and mangostin) using a horizontal diffusion cell (TK-6H1, Shanghai Kai Kai Technology, China) and MFtC were compared.

Rat abdominal skins were obtained from National University of Singapore Animal Centre and kept at −80 °C until use. Prior to permeation studies, the skins were thawed and hair was completely removed with an electrical shaver and hair remover cream (Veet) (Varshney et al., 1999). Subcutaneous fat and connective tissues were also lightly trimmed off. All animal experiments were approved by Institutional Animal Care and Use Committee, National University of Singapore.

Rat abdominal skin of 2.0 cm × 2.0 cm was mounted between the donor and receptor compartments of the horizontal diffusion cell, with stratum corneum side facing the donor compartment. The effective diffusion area was 1.13 cm^2. Each donor cell contained 4.5 ml of each model compound in propylene glycol (PG) and the receptor cell contained the same volume of PBS. Mangostin was used at a concentration of 2.3 mg/ml and rhodamine B at concentrations of 1 mg/ml and 5 mg/ml. Both compartments

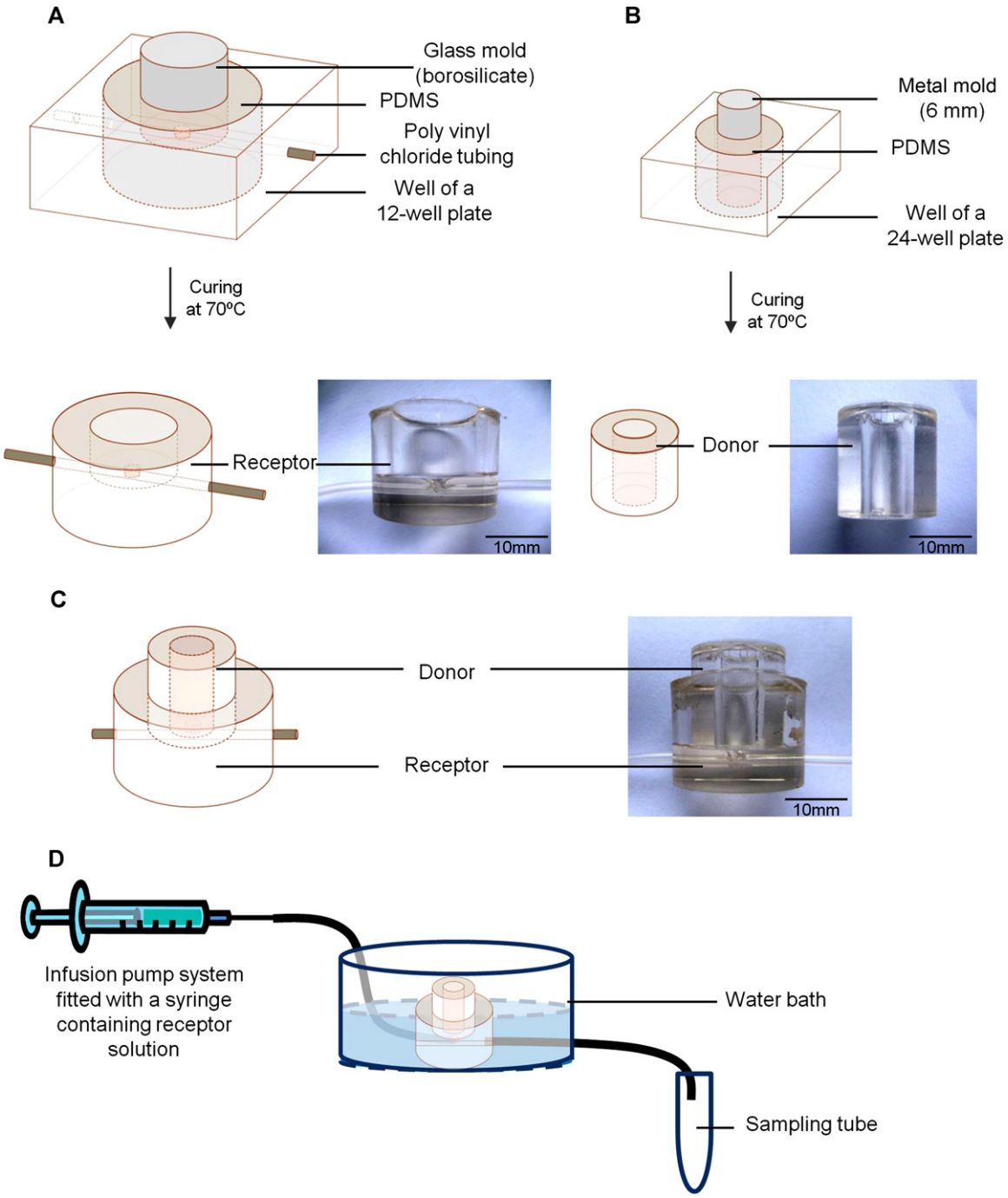

Fig. 2. Schematic diagram of fabrication process of (A) donor compartment and (B) receptor compartment. (C) Full assembly of fabricated diffusion cell. (D) Schematic diagram of full assembly of miniaturized flow-through cell (MFtC).

were thermostated at 37 °C by means of a surrounding temperature controlled water jacket. In order to minimize evaporation, all cell openings were occluded with parafilm. The fluids in both compartments were maintained in a stirred state by a Teflon coated magnetic stirrer at a speed of 250 ± 1.25 rpm. Samples (1 ml) were withdrawn from the receptor compartment for analysis at specific time intervals. Upon each sample withdrawal, the receptor compartment was immediately replaced by equal volume of fresh solution. The experiments were performed in triplicates or more.

Similar conditions were used in the MFtC setup. Rat abdominal skin of 1.0 cm × 1.0 cm was mounted between the donor compartment and the receptor compartment with the stratum corneum side facing the donor compartment of this apparatus. High vacuum grease (Dow Corning, USA) was applied to the donor compartment on unexposed stratum corneum side, in contact with receptor compartment, to minimize leakage from the donor compartment. Application of grease prevented the leakage of donor solution, even at the end of 48 h study, which could be observed when no grease was applied (SI 1). The effective diffusion area was 0.283 cm^2. 70 μl of the donor solution was added into the donor compartment. Degassed PBS solution constituted the receptor fluid. Flow rate of each diffusion cell was controlled by an infusion pump at 0.20 ml/h. MFtC were placed in a water bath maintained at 37 °C using a hot plate. In order to minimize evaporation, the donor compartment and sampling microfuge tubes were occluded with parafilm. The sampling tubes were collected at specific time

intervals and replaced by empty tubes for subsequent collections. The experiments were performed in triplicates or more.

Skin samples from the same rat were used for comparisons between the horizontal diffusion cell and MFtC setups to minimise inter-animal variability. All experiments were performed at least three times. The samples were collected at the same time intervals and stored at 4 °C until analysis. During analysis, samples were first centrifuged at 13,000 rpm (Sorvall Biofuge Pico, UK) for 5 min. The supernatant was obtained and analyzed according to their respective assay methods as reported below.

Concentration of rhodamine B was determined by fluorescence spectroscopy with a microplate reader (Tecan, Switzerland) (Park et al., 2005; Tokumoto et al., 2005) at an excitation wavelength (λ_{ex}) = 554 nm and an emission wavelength (λ_{em}) = 586 nm at ambient temperature. All samples were protected from light to prevent possible light quenching of fluorescence during the assay (Lakowicz et al., 1994).

Mangostin concentration was determined with a reversed phase HPLC (Hitachi, Japan) using a C_{18} column (5 μm, 4.6 mm × 250 mm; ODS Hypersil, Thermo Scientific) maintained at ambient temperature. The mobile phase comprised of methanol and ultrapure water (90:10, v/v) delivered at a flow rate of 1 ml/min. The UV detector (L-2400, Hitachi, Japan) was operated at a λ = 320 nm. Under these conditions, the mangostin peak appeared at a retention time of 6.8 min.

Comparison of change in skin's properties when applied on a diffusion cell for a particular period of time was done by histological examination of the skin applied to MFtC and horizontal diffusion cell. For this purpose, defatted rat skin was clamped in both the diffusion cells in a manner as described above. PG was applied to the donor side and receptor comprised of PBS. Histological examination of skin applied to both the diffusion cells was carried out at 0, 24 and 48 h post application by cutting the skin longitudinally into 20 μm sections using a microcryostat (Leica, Germany). Subsequently the sections were fixed in absolute ethanol and stained with hematoxylin and eosin and imaged using a Nikon AZ100 (Nikon, Japan) microscope.

2.5. Endoxifen fluorescence assay

A fluorescence assay for endoxifen was established whereby endoxifen was converted to highly fluorescent phenanthrene derivatives following exposure to ultraviolet (UV) irradiation (Aranda et al., 2011; Šalamoun et al., 1990). A UV transilluminator (Bio Rad, USA) at a λ = 302 nm and an intensity of 866 μW/cm^2 was used for the conversion of endoxifen to its phenanthrene derivatives. The fluorescence emitted from the phenanthrene derivatives of endoxifen after various durations of UV exposure was determined with a microplate reader at a λ_{ex} = 260 nm and a λ_{em} = 380 nm. Fluorescence measurements from non-UV exposed samples served as a control. The optimum duration of UV irradiation was determined with 10 μg/ml solution of endoxifen. For all subsequent experiments, this duration of UV irradiation was fixed at the optimal time.

The linearity and sensitivity of the assay were determined by spiking endoxifen in PBS at eleven concentrations (0.78–25.00 μg/ml). The fluorescence, obtained post UV irradiation, was plotted against endoxifen concentrations. Linear regression was performed to obtain the slope and intercept. The limit of detection (LOD) and limit of quantification (LOQ) were set as three and ten times the standard deviation of the blank respectively (ICH, 1994).

The intra-day accuracy and precision of the assay method were determined by spiking receptor solution collected from a permeation study with PG as the donor solution with four concentrations (1.56–12.50 μg/ml) of endoxifen. Aliquots of these samples were analyzed on three occasions on the same day. Triplicates were prepared for each analysis.

2.6. Endoxifen permeation studies

The validated MFtC mounted with rat abdominal skin was employed to determine the permeation profile of endoxifen. Donor solutions consisting of endoxifen (2 mg/ml) in PG with and without permeation enhancers (PEs) namely limonene, myristyl lactate and oleic acid at 0.5% (w/v) were prepared. All solutions were sonicated for 3 min to ensure dissolution of endoxifen and PEs (Aranda et al., 2011). Each donor compartment was filled with 200 μl of donor solution. Endoxifen was allowed to permeate through the rat abdominal skin over 48 h. The experiments were performed in triplicates. Samples of permeated solutions were collected at specific time intervals and stored at −20 °C until analysis. The flux at steady state (J_{ss}) and lag time were obtained from the cumulative plots. The effect of the PEs on the flux was evaluated by calculating the enhancement index (EI).

2.7. Statistical analysis

Independent sample *t*-test (IBM SPSS PASW Statistics 18) was used to compare the permeation parameters obtained from experiments involving horizontal diffusion cell and MFtC. For endoxifen permeation experiments, one-way analysis of variance (ANOVA) with Scheffe post hoc test was used for the comparison of the permeation parameters of endoxifen with or without the different PEs. For all tests, $p < 0.05$ was considered significant.

3. Results

3.1. Validation of MFtC against horizontal diffusion cell

For the receptor liquid, an average flow rate of 0.18 ± 0.01 ml/h was measured (SI 2). The choice of low flow rate was selected to achieve adequate drug to be present in the samples for detection and quantification. This is particularly important in the case of low flux. It was reported that flow rate of the receptor solution does not affect the numerical value of the flux of drug but the time to achieve steady state instead (Cordoba-Diaz et al., 2000). Therefore, any small fluctuations in the flow rate would not influence the flux significantly.

The different nature of the two model substances (rhodamine B and mangostin) and varied concentrations were chosen to ensure the reproducibility of permeation parameters in the presence of different test substances. No significant difference in J_{ss} ($p > 0.05$) was found between the horizontal diffusion cell and the MFtC for the three different donor solutions (Fig. 3 and Table 1). While the design of the MFtC varies significantly from that of the horizontal diffusion cell, the results obtained confirmed that permeation profiles from both the set-ups were comparable, thereby confirming that the MFtC fabricated is a suitable platform for reproducible results for scaled-down permeation studies.

Histological examination of the skin from both diffusion cells revealed that there were no apparent changes in the skin structure over a period of 48 h (Fig. 4). The structure of stratum corneum obliterated minimally, particularly for the first 24 h of the permeation study. However, shrinkage of the skin thickness was observed in both the diffusion set-ups. This may be attributed to the continuous shredding of the skin as it is in contact with the donor and receptor fluids. Also, the excised skin loses its inherent water content, leading to transepidermal water loss and the resultant shrinkage.

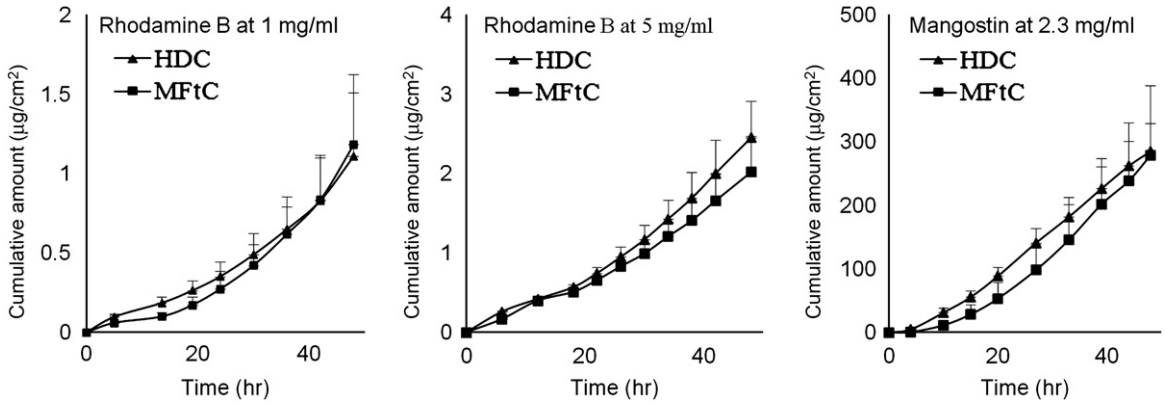

Fig. 3. Time course of cumulative amount permeated through rat abdominal skin for rhodamine B at 1 mg/ml, rhodamine B at 5 mg/ml and mangostin at 2.3 mg/ml. Each point represents mean ± S.D.

Table 1
Comparison of lag time and fluxes between HDC and MFtC across rat abdominal skin using rhodamine B at 1 mg/ml, rhodamine B at 5 mg/ml and mangostin at 2.3 mg/ml. N denotes number of replicates. Error bars denote S.D. between replicates. Flux comparisons between the setups showed no statistical difference.

	Rhodamine B (1 mg/ml)			Rhodamine B (5 mg/ml)			Mangostin (2.3 mg/ml)		
	N	Lag time (h)	Flux (μg/cm²/h)	*N*	Lag time (h)	Flux (μg/cm²/h)	*N*	Lag time (h)	Flux (μg/cm²/h)
HDC	5	17.7 ± 3.47	0.04 ± 0.03	4	11.4 ± 3.31	0.07 ± 0.01	5	7.19 ± 0.47	7.06 ± 1.06
MFtC	3	22.1 ± 4.07	0.05 ± 0.02	5	8.35 ± 4.75	0.05 ± 0.02	7	14.5 ± 2.71	8.34 ± 3.40

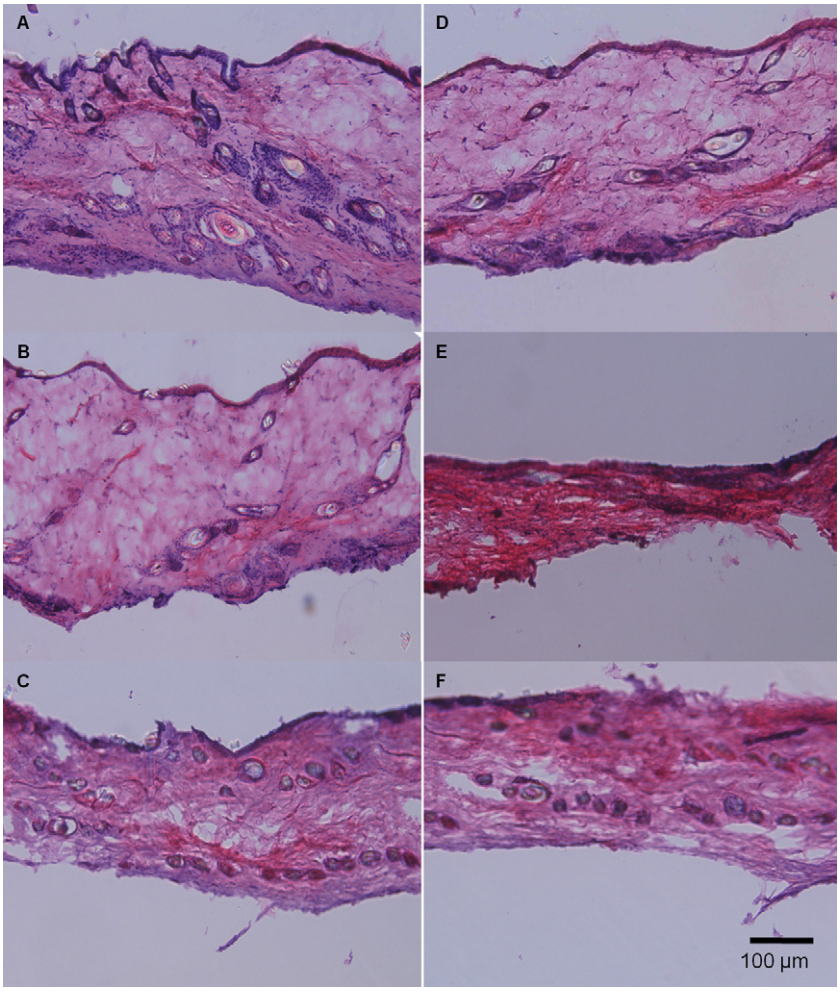

Fig. 4. Histological images of the skin mounted on MFtC (A) at 0 h, (B) at 24 h, (C) at 48 h, as well as horizontal diffusion cell (D) at 0 h, (E) at 24 h and (F) at 48 h. The images demonstrate no apparent damage to the skin was caused by MFtC and skin exhibited similar properties as compared to horizontal diffusion cells.

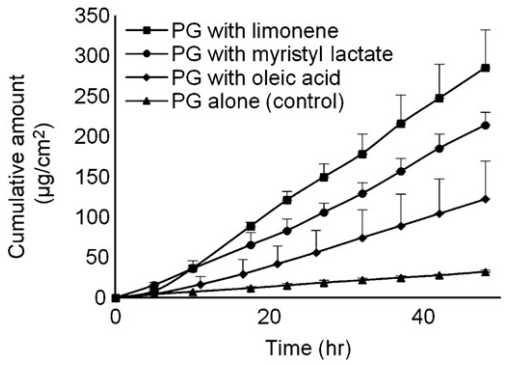

Fig. 5. Time course of cumulative ENX permeated through 0.283 cm^2 of rat abdominal skin with or without enhancers using MFtC. ENX donor concentration = 2 mg/ml. Each point represents mean ± S.D.

3.2. Endoxifen fluorescence assay

Endoxifen, without irradiation, emits minimal fluorescence. Following UV irradiation, the phenanthrene derivatives of endoxifen emitted fluorescence, which is dependent on the amount of UV exposure (Aranda et al., 2011). The optimum duration of UV irradiation of 15 min, which correspond to maximum fluorescence value, was used for all subsequent experiments (SI 3).

In order to ensure accurate quantification of endoxifen, the linearity and sensitivity of the fluorescence based assay was determined using calibration experiments. The regression curve was obtained and the limits were:

Range: 0.78–3.13 μg/ml, $A = (510.7 \pm 90.7)$ [endoxifen]–(422.4 ± 93.4), $r^2 = 0.97$

Range: 3.13–25.00 μg/ml, $A = (1226.5 \pm 38.8)$ [endoxifen]–(2663.4 ± 149.1), $r^2 = 0.98$.

N = 4, LOD = 0.31 μg/ml, LOQ = 0.78 μg/ml, where A is in arbitrary units and the concentration is in μg/ml.

Accuracy and precision were assessed using four concentrations, i.e., 1.56 μg/ml, 3.13 μg/ml, 6.25 μg/ml and 12.50 μg/ml. An accuracy of +2.05%, +18.86%, +11.41% and +19.06% with an inter-day CV of 2.99%, 5.21%, 1.68% and 4.82% was respectively observed (SI 4).

3.3. Endoxifen permeation studies

Cumulative permeation plots and permeation parameters of endoxifen in PG with and without PEs are shown in Fig. 5 and Table 2. All PEs significantly increased ($p < 0.05$) the endoxifen flux in comparison with the PG alone. Endoxifen in PG with 0.5% w/v oleic acid, myristyl lactate and limonene achieved an EI of 6.26, 8.17 and 9.99 respectively, when compared to endoxifen in PG alone. The highest J_{ss} was achieved using limonene as a PE, with an EI of about ten times more than PG alone. Lag time of permeation for endoxifen in PG alone was however lower than those achieved with the use of PEs.

Table 2
Permeation parameters of ENX in various donor solutions. Data was expressed as mean ± S.D. PE concentration = 0.5% (w/v). ($N = 3$). Enhancement index (EI) = J_{ss} (with enhancer)/J_{ss} (without enhancer).

Donor solution	Lag time (h)	Flux (μg/cm^2/h)	EI
PG alone (control)	1.03 ± 1.40	0.65 ± 0.01	–
PG with oleic acid	7.58 ± 4.04	4.09 ± 1.07*	6.26
PG with myristyl lactate	7.62 ± 2.18	5.33 ± 0.13*	8.17
PG with limonene	3.75 ± 2.37	6.52 ± 1.41*	9.99

* $p < 0.05$ compared to control.

Table 3
Comparison between MFtC and commercial flow-through cells.

Mechanical elements	MFtC	Commercial
Donor area	0.283 cm^2	0.785 cm^2
Donor volume	70–200 μL	100–1000 μL
Receptor volume	10 μL	230–855 μL

4. Discussion

4.1. Choice of PDMS for the fabrication of MFtC

PDMS was selected for the fabrication of MFtC because of its advantageous properties. Firstly, the total cost of such a device was reduced substantially, thereby making such a setup readily affordable. The fabrication process is simple and can be easily adapted by individual research labs to customize their diffusion cells as per their specific requirements. A single diffusion cell made of PDMS approximately costs 1 USD (material cost) as compared to commercial equivalent that costs around 440 USD.

Secondly, the rheological properties of PDMS allow shaping of any desired design due to its flowability into any pre-formed mold. Owing to the flexible nature of PDMS, miniaturization of the whole assembly could be made possible. MFtC had significantly lower donor area, donor and receptor volumes as compared to the current flow-through cells (Table 3) (Brand et al., 2003; Cordoba-Diaz et al., 2000; Kang et al., 2007). The low receptor flow rate of 0.20 ml/h is in accordance with the general rule that flow rate should be at least ten times the receptor volume (10 μl) (Bronaugh, 1993).

In addition, the optical clarity of PDMS allows a clear view of the area below the skin. This in turn facilitates the ascertainment of the absence of air bubbles which is especially important as these air bubbles can adversely affect the accuracy of permeation results (COLIPA, 1997). Lastly, PDMS being an inert material, allows for prolonged shelf life of the diffusion cell and making them reusable.

Moreover, it has been reported the adsorption of PDMS is comparable to glass, especially for hydrophilic compounds while it is four times higher than glass for hydrophobic compounds (Li et al., 2009). While most of the compounds used in our study were relatively hydrophobic, we did not observe significant loss of drug due to adsorption. The diffusion cells made of PDMS were made reusable by washing with acetone and isopropanol. If needed, the surface of PDMS may be modified physically or chemically, to reduce the adsorption of hydrophobic drug molecules (Wong and Ho, 2009).

4.2. Validation of MFtC against horizontal diffusion cell

While validating the newly fabricated MFtC against the established permeation equipment, horizontal diffusion cells, we chose to consider two factors, namely, the varied concentrations and the log P of the compounds. First, we chose to validate two different concentrations of rhodamine B to investigate the validity of flux at low and high concentrations of the donor solution. Rhodamine B is a fluorescent molecule, with a suitable log P (2.43) and molecular weight (479.02) for skin permeation testing. Its pink color aids in easy detection of leakage of the donor solution as shown in SI 1. It was observed that the flux achieved was comparable for both the concentrations between MFtC and the horizontal diffusion cells. Second, we compared the permeation profiles of rhodamine B and mangostin, a molecule similar to rhodamine B, in terms of molecular structures and molecular weights (Fig. 1), but with a higher log P value (6.64) than rhodamine B. log P is an important parameter to consider for skin permeation, as it will affect the partition of the drug inside stratum corneum and viable layers of epidermis.

It was observed that the permeation parameters of MFtC and the horizontal diffusion cell were in close correlation to each other, signifying the validity of our newly fabricated diffusion cells.

The device was also adaptable for thicker skin samples, such as those from pig cadaver, which closely resemble human skin. Pig skin could be supported on the MFtC set-up, with the application of vacuum grease and no leakage was detected from the donor compartment when a rhodamine B solution in PG was applied (SI 5).

4.3. Endoxifen permeation studies

To achieve a plasma concentration of endoxifen that is comparable to those achieved on administration of an oral daily dose of 2–4 mg (Ahmad et al., 2010a), an ideal flux of 2.0–4.0 $\mu g/cm^2/h$, assuming an application area of 40 cm^2 would be required from endoxifen transdermal drug delivery system. The reported transdermal endoxifen study was not able to achieve this flux (Lee et al., 2011). In the study, the highest flux reported was 0.22 $\mu g/cm^2/h$ for endoxifen dissolved in 60% (v/v) ethanol–phosphate buffer with 0.5% (w/v) oleic acid.

In the search of a suitable vehicle and PEs for endoxifen in transdermal drug delivery system, we used three different permeation enhancers. It has been reported that high skin flux of tamoxifen can be achieved by using limonene as a PE with PG as the vehicle (Zhao and Singh, 2000). Because of the molecular structural similarities between tamoxifen and endoxifen, in our study, endoxifen was incorporated in PG while limonene was selected as one of the PEs. In addition to limonene, oleic acid and myristyl lactate were also selected as PEs in this study.

It was found that oleic acid, myristyl lactate and limonene in PG enhanced the permeation of endoxifen by 6.26, 8.17 and 9.99 folds respectively as compared to PG alone (Table 2). Oleic acid has been reported to increase drug transport by coexisting as pools in the stratum corneum lipids structure (Williams and Barry, 1992). Myristyl lactate may act by disrupting ceramide-cholesterol or cholesterol-cholesterol interaction and increase permeation of endoxifen (Brain and Walters, 1993). As myristyl lactate ($\log P = 6.08$) has a shorter carbon chain than oleic acid ($\log P = 7.42$), the higher flux achieved by myristyl lactate can be explained by lower partitioning of drugs into stratum corneum as compared to oleic acid.

Our results showed that limonene delivered the highest flux among the three PEs tested. Limonene belongs to the class of terpenes which are constituents of essential oils (Kang et al., 2007). Their ability to enhance drug flux could have been attributed to partial extraction of stratum corneum lipids, (Krishnaiah et al., 2002) phase separation within the SC lipid lamellae (Moghimi et al., 1997) and limonene-PG synergy (Barry, 1991).

Besides, our results using oleic acid as a PE in PG has shown significantly better endoxifen delivery with a J_{ss} of 4.09 ± 1.07 $\mu g/cm^2/h$ compared with the J_{ss} of 0.22 $\mu g/cm^2/h$ as reported by using ethanol–phosphate buffer as the vehicle (Lee et al., 2011). A plausible explanation for this observation is the different effects of various vehicles on the skin. It has been reported that PG can affect the transdermal permeability by altering thermodynamic activity of drug and/or barrier nature of skin (Panchagnula et al., 2001). Moreover, it is also been known that activity of PEs can be significantly increased when applied in combination with PG (Aungst et al., 1986). Overall, all three PEs chosen in this study were able to achieve higher flux than control. The best one was limonene, which attained a flux of 6.52 $\mu g/cm^2/h$ through rat skin, which can be translated to 2.17 $\mu g/cm^2/h$ through human skin (Godin and Touitou, 2007). Therefore, the target flux of 2–4 $\mu g/cm^2/h$ through human skin can be achieved with this limonene formulation.

5. Conclusion

We have developed and validated a miniaturized flow-through cell. It utilizes a small amount of donor solution (70–200 μl) and membrane (0.283 cm^2) for skin permeation studies. The device had no damaging effect on the skin as compared to the established models like horizontal diffusion cell. A novel fluorescent spectroscopic method was also developed to quantify endoxifen in a fast and convenient manner. Permeation studies of endoxifen attained the targeted flux. The miniaturized diffusion cell is demonstrated to be useful for investigative drugs with limited supply during the pre-formulation studies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2012.11.011.

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