ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 1015–1023

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Novel diffusion cell for in vitro transdermal permeation, compatible with automated dynamic sampling[1]

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Received for review 10 September 1995; revised manuscript received 2 November 1995

Abstract

The development of a new diffusion cell for in vitro transdermal permeation is described. The so-called Kelder cells were used in combination with the ASPEC system (Automatic Sample Preparation with Extraction Columns), which is designed for the automation of solid-phase extractions (SPE). Instead of SPE columns, 20 Kelder cells were placed in the racks. This allowed automatic sampling of up to 20 cells for 24 h in a dynamic mode. The cells consist of an inlet compartment, a donor compartment and a receptor compartment. The size and the depth of the inlet compartment were important to avoid entrapment of air bubbles in the receptor compartment. The Kelder cells mimic blood flow beneath the skin by replacement of the permeating drug every 2 min. Hence sink conditions are more easily maintained than with the static Franz diffusion cell. The performance of the cells was tested with permeation experiments using atropine as a model drug permeating through an artificial membrane (Silastic). The use of this skin model minimized the variability in permeation of atropine as compared with human skin.

Keywords: ASPEC system; Atropine; Automated dynamic sampling; Diffusion cell; In vitro; Kelder cell; Silastic

1. Introduction

Recently, there has been increased interest in drug administration via the skin both for local therapeutic effect on diseased skin (topical delivery) and for systemic delivery (transdermal delivery). The permeation of chemicals throught the skin can be measured by in vivo and in vitro techniques. Frequently this has been done by in vitro techniques because of the simplicity of the experimental conditions. Although many variations exist, there are two basic approaches to measure skin permeation in vitro: the static or non flowing cell and the flow-through cell [1–3].

One of the most widely used static designs for studying in vitro permeation is the Franz diffusion cell. This cell has a static receptor solution reservoir with a side-arm sampling port. In previous experiments, we used these static cells to measure the in vitro permeation of anticholinergics

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[1] Presented at the Fifth International Symposium on Drug Analysis, September 1995, Leuven Belgium

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SSDI 0731-7085(95)01717-8

through pig skin [4]. Permeation was monitored by sampling the stirred receptor chamber solution. This manual procedure is labour intensive, and large variations were found between the experiments.

To permit automatic sampling of the receptor solution in a dynamic mode, we developed new diffusion cells (Kelder cells) which can be used in combination with the ASPEC system (Automatic Sample Preparation with Extraction Columns). This liquid handling system has been designed for the automation of solid-phase extractions (SPE) [5]. Instead of SPE columns, 20 Kelder cells were placed in the racks and experiments can run for 24 h automatically. These cells mimic blood flow beneath the skin since the receptor solution is replaced every 2 min (discontinuous flow-through).

In this paper, we describe the development of Kelder cell designs for in vitro transdermal permeation. The aspects of the designs emphasized were compact, small diffusion area, small dead volume, air bubble free, variable rinsing of the receptor compartment, flexible and reproducible collection of receptor solution and adequate temperature control. Experiments were performed with atropine as a model drug permeating through an artificial membrane (Silastic) which was used as a model skin. Atropine in the receptor solution was determined by radioreceptor assay. The results are discussed and compared with those obtained using the static Franz diffusion cell.

2. Experimental

2.1. Materials

[*N-methyl*-^{3}H]Scopolamine methyl chloride ([^{3}H] NMS, 80.4 Ci mmol^{-1}) was obtained from Du Pont NEN (Du Pont, Wilmington, DE). 1-Dodecylazacycloheptan-2-one (Azone) was kindly supplied by Nelson Research (Irvine, CA). Sigmacoat was obtained from Sigma (St. Louis, MO, USA). Plexiglas and Delrin were obtained from Vink Kunststoffen (Didam, Netherlands). Viton O-rings were obtained from Eriks (Alkmaar, Netherlands). Atropine base was obtained from Merck (Darmstadt, Germany). Propylene glycol was purchased from Brocacef (Maarssen, Netherlands). All other chemicals and solvents were of analytical grade and obtained from Merck. Polyethylene tubes (12 ml) were obtained from Greiner (Alphen a/d Rijn, Netherlands). The GF/B glass-fibre filters were from Whatman (Maidstone, UK). Rialuma was used as a scintillation liquid, obtained from Lumac (Olen, Belgium), in combination with mini-scintillation counting vials from Packard (Groningen, Netherlands).

2.2. Preparation of solutions

Isotonic phosphate-buffered saline (pH 7.4) (PBS buffer) was prepared by dissolving 8.00 g of NaCl, 0.20 g of KCl, 0.20 g of KH_2PO_4 and 1.44 g of $Na_2HPO_4 \cdot 2H_2O$ in 1 l of distilled water. PBS buffer was used as the receptor solution.

Sodium phosphate buffer 50 mM (pH 7.4) was prepared by dissolving 1.38 g of $NaH_2PO_4 \cdot H_2O$ and 7.12 g of $Na_2HPO_4 \cdot 2H_2O$ in 1 l of distilled water.

The drug solution consisted of 15 mg ml^{-1} atropine in ethanol–propylene glycol–PBS buffer–Azone (60:20:15:5, v/v/v/v) [6]. An atropine stock standard solution of 1.23×10^{-3} M was prepared in ethanol and stored at -20°C.

The tissue suspension was prepared by dissolving 5 mg of lyophilized receptors [7] in 1 ml of sodium phosphate buffer.

2.3. Preparation of human skin

Human skin was obtained from females having undergone breast surgery (University Hospital Groningen, Department of Pathology). The subcutaneous fat was removed and the whole skin membranes were frozen in liquid nitrogen and stored at -80°C until used. Before experiments were performed, the membranes were thawed and epidermal membranes were prepared by soaking the whole skin membranes in water for 2 min at 60°C, followed by blunt dissection [8,9].

2.4. Preparation of Silastic membranes

Non-reinforced silicone membrane (Silastic, polydimethylsiloxane, type 500-1; Laboratoire Perouse Implant, Bornel, France) of 0.125 mm thickness was extensively rinsed in hot distilled water (60°C) until all sodium hydrogencarbonate (on the surface to facilitate handling) was removed. This was followed by a thorough rinse in distilled water (20°C) for 1 h [10].

2.5. Experiments with Franz diffusion cells

Permeation experiments with Franz diffusion cells were performed using human epidermal membranes and Silastic membranes. The Franz diffusion cells (Fig. 1) were made of glass with a contact area of 1.35 cm^2 (University Centre for Pharmacy, Groningen, Netherlands) and pretreated with a silanizing agent (Sigmacoat). The Franz diffusion cell consisted of a donor compartment (A) and a receptor compartment (B). The membrane (C) was mounted between the cell compartments and an O-ring (D) was used to position the membrane. The two cell compartments were held together with a clamp. The receptor compartment had a volume of 4.3 ml and was filled with PBS buffer. It was kept at 37°C by circulating water through an external water-jacket

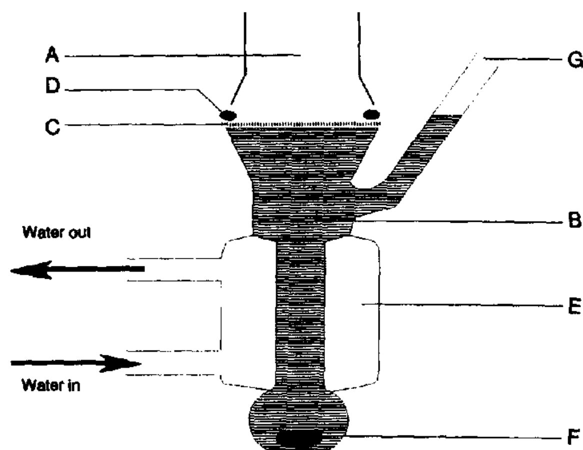


Fig. 1. The static Franz diffusion cell. A = donor compartment; B = receptor compartment; C = membrane; D = O-ring; E = water-jacket; F = stirring bar; G = sampling port.

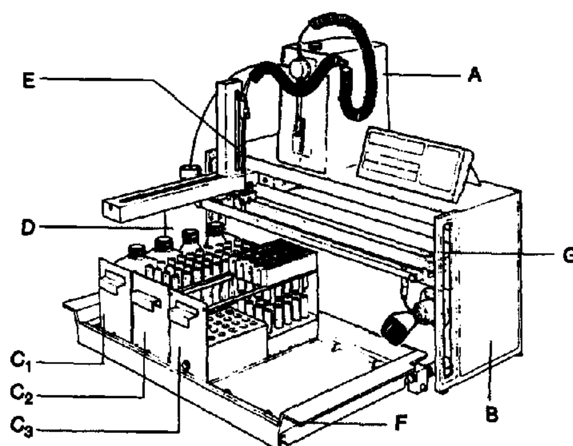


Fig. 2. The ASPEC system. A = Model 401 dilutor; B = sample processor; C1 = solvent rack; C2 = sample rack; C3 = SPE rack; D = reservoir; E = needle; F = polypropylene tray; G = rinsing station.

(E). After 30 min of equilibrium of the membrane with the receptor solution, 200 μl of the drug solution were applied in the donor compartment by means of a pipette. The donor compartment was then covered with Parafilm to prevent evaporation of the solvent. The receptor solution was continuously stirred by means of a spinning bar magnet (F) at 400 rpm (Multipoint HP 15, Variomag, Munich, Germany). Aliquots of 2.0 ml of receptor solution samples were withdrawn through the sampling port (G) of the receptor compartment at various time intervals. The cells were refilled with receptor solution to keep the volume of receptor solution constant during the experiment. The experiments were run for 25 h.

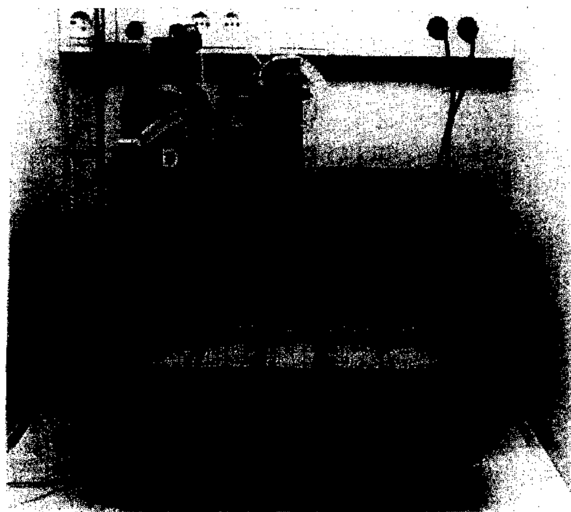
2.6. Experiments with Kelder cells

2.6.1. ASPEC system

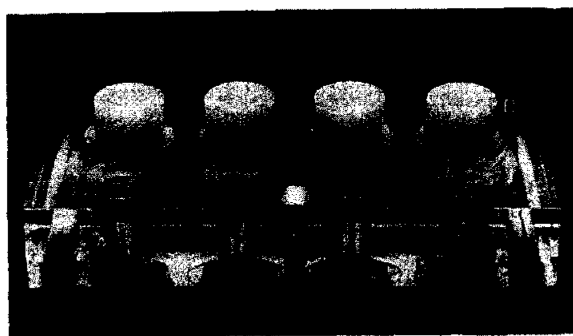
The newly developed Kelder cells were made compatible with the ASPEC system (Gilson Medical Electronics, Villiers le Bel, France). As shown in Fig. 2, the ASPEC system consists of three components: a Model 401 dilutor (A), a sample processor (B) and a set of racks and accessories to handle SPE columns and solvents (C). The dilutor allows the transfer of a specified volume of solvent from a reservoir (D) through the needle (E) into a container. The latter can be an SPE column

or one of the cell designs. It is also possible to aspirate air or liquid from a container into the needle. The needle of the ASPEC system is able to move blocks with SPE columns to a programmed position. These three features of the ASPEC system were used to perform permeation experiments.

Five racks were placed in the polypropylene tray (Fig. 2, F) and, instead of blocks with SPE columns, five blocks each containing four cells, were placed in these racks (Fig. 3). Polyethylene tubes (12 ml) were positioned below the cells, to collect the receptor solution buffer flowing through the cells. For each cell, 12 collection tubes were available. This means that for an experiment of 24 h, samples were collected every 2 h.



(A)



(B)

Fig. 3. (A) The ASPEC system. A = Model 401 dilutor; C = rack with block of four cells; D = reservoir containing PBS buffer; E = needle. (B) Block containing four Kelder cells. A = inlet compartment; B = donor compartment; J = polypropylene cap.

2.6.2. Cell designs

Three designs of Kelder cells were developed and permeation experiments were performed at room temperature using Silastic membranes. The designs all consisted of an inlet compartment (A), a donor compartment (B) and a receptor compartment (C) (Fig. 4). The main difference between the three designs is the size and the depth of the inlet compartment.

The cells were made from Plexiglas to permit viewing of air bubbles inside the receptor compartment. A circular piece of membrane (D) with a diameter of 12 mm was cut and placed in the receptor compartment, which was filled with PBS buffer. A Viton O-ring (E) was used to position the membrane between the donor and receptor compartment. The donor compartment was fabricated from Delrin and screwed on to the receptor compartment. After 30 min of equilibration of the membrane with the PBS buffer, 75 μl of the drug solution were applied in the donor compartment by means of a pipette. The donor compartment was covered with Parafilm to prevent evaporation of the solvent. The receptor solution entered the cell via the inlet compartment (A), flowed through the inlet channel (G). An outlet tube (H) made of stainless steel was fixed at the end of the outlet channel to make the outlet hole smaller. The inlet compartment of the cell was sealed with a polypropylene cap (J) to force the buffer to flow through the cell when fresh buffer was injected. The receptor solution with permeated drug was collected in polyethylene tubes, placed below the cells. The entire cell was very compact with a contact area of 0.51 cm^2, defined by the internal dimensions of the O-ring, and a receptor compartment volume of 77 μl.

2.6.3. Programming

The ASPEC system was programmed to run permeation experiments automatically for 24 h (ASPEC system user's guide). The inlet compartments of the cells were defined by their x–y-posi-

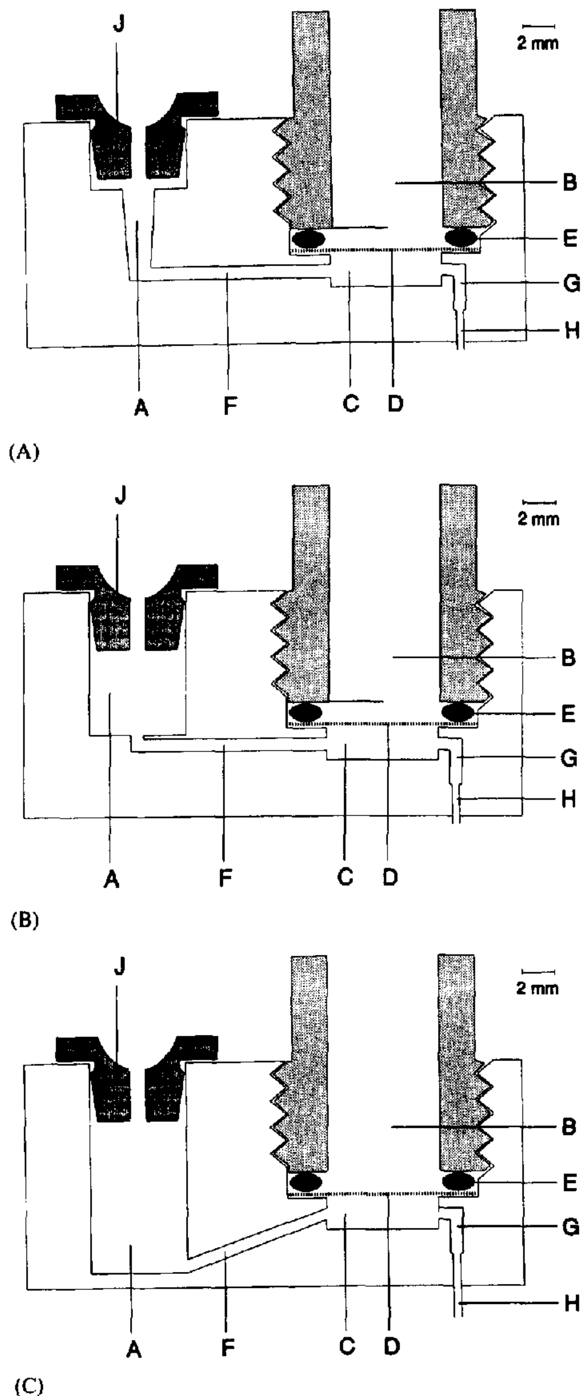


Fig. 4. Cross-sections of the (A) first, (B) the second and (C) the third Kelder cell designs. A = inlet compartment; B = donor compartment; C = receptor compartment; D = membrane; E = O-ring; F = inlet channel; G = outlet channel; H = outlet tube; J = polypropylene cap.

tion in the polypropylene tray (Fig. 2, F). The needle was programmed to move to the first cell and inject PBS buffer in the inlet compartment. Then the needle moved to the second cell and injected again. After 20 cells, the needle moved back to the first cell and the procedure was repeated.

The interval time between subsequent rinsings, the dispensing volume and flow rate of the buffer can be varied. In these experiments, aliquots of 150 μl were injected into the cells sequentially with a flow rate of 50 $\mu l\ s^{-1}$ and the interval time between subsequent rinsings of one cell was of the order of 2 min. The dilutor syringe capacity was 10 ml, which means that after injecting 150 μl in to 20 cells three times, the syringe had to be refilled from the reservoir, which took about 30 s. Before the needle moved back to the first cell, 250 μl were dispensed into the rinsing station (Fig. 2, G). After 2 h, the needle shifted the blocks of cells in the y-direction above the next row of polyethylene tubes. In 2 h, 9 ml (60 times 150 μl) of receptor solution were collected in each polyethylene tube. In the programme, wait commands were used to postpone the operating procedure until the desired time had elapsed.

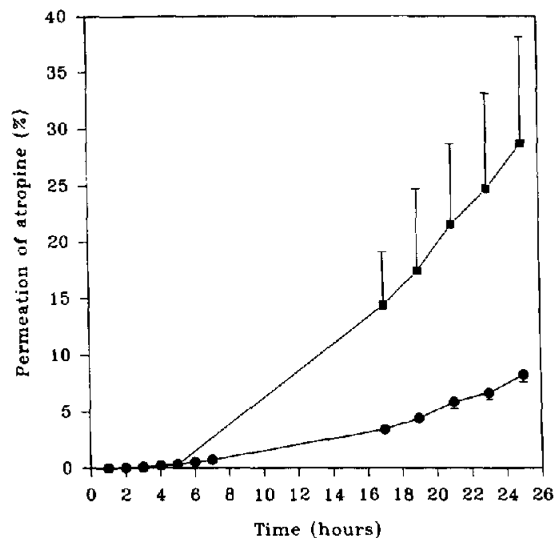


Fig. 5. Permeation of atropine through human epidermal membranes ($n = 4$) (■) and Silastic membranes ($n = 6$) (●) using Franz diffusion cells.

2.6.4. Optimization of the ASPEC procedure

First, the programming of the ASPEC system was optimized to prevent the injection of air. Before buffer was injected into the cells, 2 μl of buffer were dispensed at a flow rate of 12 $\mu l\ s^{-1}$ above the inlet compartment to create a droplet on the needle. Air bubble formation was further minimized by degassing the PBS buffer and the use of a bubble trap in the dilutor. Other problems, such as thickness of the needle, height of injection into the inlet compartment and diameter of the O-ring, had to be resolved to avoid leakage or flooding of the cells.

2.7. Analytical procedure

Atropine in the receptor solution was determined by means of a radioreceptor assay. Calibration curves were prepared from the atropine stock standard solution to provide concentrations ranging from 1×10^{-9} to 1×10^{-5} M. These calibration samples, together with the receptor solution samples, were analysed as follows. Aliquots of 50 μl of receptor solution samples or 50 μl of calibration samples (in duplicate) were placed in polyethylene tubes, then 50 μl of radiolabelled [^{3}H]NMS (4×10^{-9} M) and 400 μl of tissue suspension were added. The tubes were vortex mixed and incubated for 30 min at 37°C. After the addition of 4 ml of ice-cold 50 mM sodium phosphate buffer, the samples were immediately filtered through Whatman GF/B glass-fibre filters under vacuum using a filtration apparatus (48S, University Centre for Pharmacy, Groningen, Netherlands). The tubes were rinsed twice with 4 ml of ice-cold buffer, which was also filtered. The total filtration and rinsing process, taking place in ca. 15 s, was carried out on each tube in turn. The filters were transferred into mini-scintillation vials and dispersed in 3.5 ml of scintillation cocktail by shaking for 120 min. The vials were counted for 40 000 counts for 5 min in a liquid scintillation counter, whichever came first (Minaxi, Packard, Groningen, Netherlands). A 50 μl volume of the used [^{3}H]NMS solution was placed in two mini-scintillation vials and counted to measure the total activity added.

Calibration curves were filtered with the LIGAND curve-fitting program [11]. The binding values (*Bq*) obtained for the receptor solution samples were introduced in the calibration curves and the unknown concentrations of atropine were calculated. When the final concentration of the receptor solution samples exceeded the upper limit of quantitation of the calibration curve, the receptor solution samples were diluted (10–10 000 fold) and reanalysed.

3. Results and discussion

3.1. Experiments with Franz diffusion cells

Silastic membranes have been used in many drug permeation studies as a reasonable alternative to skin [3]. In the devolopment of new diffusion cells, synthetic membranes may be useful because they may minimize the variability in permeation between human epidermal membrane and Silastic membrane, some initial experiments were carried out in Franz diffusion cells.

The mean cumulative percentage of atropine permeating through the membranes, is shown in Fig. 5. After 5 h, the permeation of atropine through human skin was significantly higher compared with Silastic membranes (Mann–Whitney rank sum test, $p < 0.05$). The higher permeability found for human skin may be due to the fact that the material had been subjected to freezing and thawing [13–15]. Degeneration of the skin during the experiment may also have played a role.

To investigate the difference in variability between the two membranes, an F-test was performed on all measured time intervals (Table 1). The tested variances, corrected for the mean percentage of atropine permeation, were significantly higher for human skin at every time interval, except at $t = 1$ h ($p < 0.05$). Therefore, Silastic membrane was used in further experiments for the development of new cells.

3.2. Kelder cell design

The first design consisted of a small inlet compartment (Fig. 4A). However, air could be

Table 1
Variability in atropine permeation through human epidermal membranes ($n = 4$) and Silastic membranes ($n = 6$) using Franz diffusion cells.

Sampling time (h)	RSD[a] (%)	
	Human epidermis	Silastic
1	116.2	91.1
3	108.9[b]	16.8
5	160.2[b]	12.4
17	65.8[b]	21.3
19	83.7[b]	14.7
21	66.6[b]	25.1
23	68.0[b]	21.0
25	65.6[b]	19.5

[a] RSD = (SD/mean) × 100%.
[b] Significantly higher than for Silastic: F-test, $p < 0.05$.

trapped easily when the needle injected PBS buffer. The trapped air was pumped through the cell together with the buffer but then stayed behind in the receptor compartment. These air bubbles under the surface of the membrane could reduce the permeation by reducing the diffusion area [16]. Therefore, a second design was developed with an enlarged inlet compartment to prevent air bubbles (Fig. 4B). Although the problem with trapped air bubbles from the inlet compartment was solved, another problem became apparent. In some cases, the buffer was siphoned over to the outlet, resulting in empty cells. A third design was made with a large, but lowered inlet compartment to prevent emptying of cells (Fig. 4C). Also, the inlet channel, connecting the inlet and receptor compartment, was shifted from the centre of the bottom to the side corner. This circumvented the passage of air bubbles to the receptor compartment.

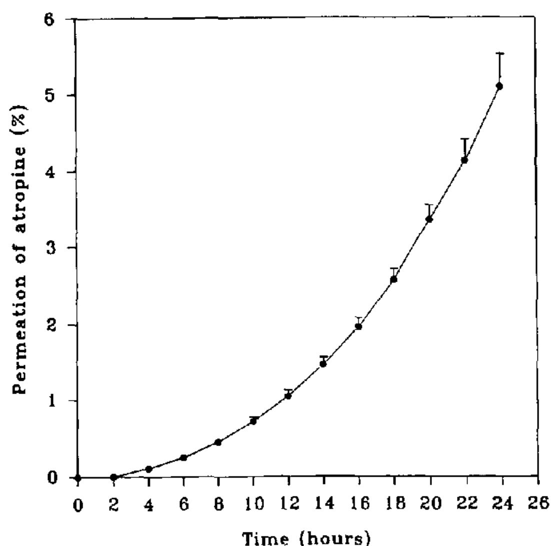


Fig. 6. Permeation of atropine through Silastic membranes ($n = 5$) with the third Kelder cell design.

3.3. Experiments with Kelder cells

With the first two designs, only one third of the cells could be analysed because the others contained air bubbles below the membrane. However, this problem was completely solved with the third design by changing the shape of the inlet compartment. In Fig. 6, the mean cumulative percentage of atropine permeating through Silastic membrane obtained using the third Kelder cell design, is shown. At room temperature (22°C), a mean (± SE) of 5.1% (± 0.4%) atropine permeated through the Silastic membrane in 24 h. With the Franz diffusion cells, the receptor solution was kept at 37°C and 8.3% (± 0.7%) of atropine permeated through the Silastic membrane in 24 h (Fig. 5). The higher percentage of permeated atropine using the Franz diffusion cells can be explained by increased permeation rates at higher temperatures [10].

3.4. Advantages and disadvantages

The objective in designing the Kelder cells was to develop an automated alternative for the Franz diffusion cells which will run unattended for at least 24 h. The manual sampling of the Franz cells requires constant attention and is often limited to normal laboratory hours, which means a less accurate fitting of the curve. Also, air bubbles are easily formed in the receptor compartment when withdrawing samples [12,17]. Initially, the entrapment of air bubbles was also a problem for the Kelder cells but could be avoided by improving the design of the cells.

The replacement of the receptor solution in the Kelder cell mimics the blood flow beneath the skin in vivo. This can be varied by changing the volume and flow rate through the receptor compartment. In order to maintain sink conditions, the volume of buffer pumped through a cell should be many times the volume of the receptor compartment [9,18]. Therefore, the total volume of the cell was minimized to 84 μl (receptor compartment plus dead volume in the outlet channel and tube). Magnetic mixing in the receptor compartment was not necessary because the flow rate of buffer through the Kelder cell was sufficient to remove the permeated drug completely [19]. However, with Franz cells magnetic stirring is crucial to provide a homogeneous receptor solution [1].

The temperature of an in vitro system should be controlled to maintain a target temperature and minimize variations in experimental conditions. With Franz diffusion cells, temperature control of receptor solution is maintained with a water-jacket. However, the temperature of the donor compartment is not thermostated and is therefore subject to variations in environmental temperature [2]. Our present experiments with Kelder cells were performed to focus on designs and were not temperature controlled. In future experiments, the ASPEC system will be placed in temperature-controlled room or box to provide a uniform temperature of the entire system.

Another advantage of Kelder cells is the reduced skin surface area, which allows smaller pieces of skin to be used. Also, the system can be linked to an HPLC system and automatically determine the amount of drug present in the receptor solution samples.

4. Conclusion

The third design of the Kelder cell is compact with a contact area of 0.51 cm^2 and a total volume of 84 μl (receptor compartment volume of 77 μl plus dead volume), and is air bubble free. With the liquid handling system ASPEC, permeation experiments can easily be automated. The volume and flow rate through the receptor compartment of the cell can be varied and the collection of receptor solution is reproducible and can be adapted to particular needs. The ASPEC system can be placed in a temperature-controlled room or box for adequate temperature control.

Acknowledgements

The authors thank Bert Schreuder and Chris Nienhuis for their technical assistance and Renger Jellema for preparing Fig. 4.

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