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An automated method for continuously monitoring diffusion cells in skin penetration studies

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Summary

A fully automated system for studying in vitro skin penetration has been developed. In the present system, frequent monitoring of drug transport through a membrane in a diffusion cell provides for the accurate measurement of steady-state flux and lag time, which is often difficult to monitor using conventional systems because of infrequent sampling. Each diffusion cell is connected to a micro flow cell in the spectrophotometer and the receptor cell fluid continuously flows through the micro flow cell back into the receptor cell due to the pressure caused by stirring inside the diffusion cell. This strirring also ensures mixing inside the receptor cell. The spectrophotometer is controlled by a personal computer so that drug penetration through the membrane can be monitored continuously because of frequent UV absorbance measurements. The usefulness of the system has been verified using shed snake skin as a model membrane.

Introduction

The transdermal route for administering drugs has become one of the major interests in pharmaceutical research. In vitro permeation of drug molecules through various model membranes, such as hairless mouse skin, has been studied using a number of different diffusion cell systems. One of the most popular diffusion cell systems is a Franz cell system for an infinite dose study as currently used by many researchers (Akhter et al., 1985; Coldmann et al., 1969; Franz, 1975; Keshary et al., 1984; Samitz et al., 1967; Southwell et al., 1984; Windheuser et al., 1982). Another type of diffusion cell system which is widely used for steady-state flux measurement is a side-by-side two-chamber diffusion cell system (Bellantone et al., 1986; Flynn et al., 1971; Tojo et al., 1987). The former cell design, a Franz cell system, is more versatile than the latter in that a wide variety of formulations, including ones which have volatile compounds, can be applied on a membrane. The former cell design also more closely simulates the in vivo situation because the membrane is exposed

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to ambient conditions and is not excessively hydrated as is usually the case in the side-by-side two chamber systems. In both systems, however, receptor fluid has to be sampled manually and the samples usually are analyzed by either liquid scintillation counting or HPLC. The sampling and subsequent analysis requires a lot of effort and time, often resulting in relatively infrequent sampling which gives some uncertainty in data analysis.

More recently flow-through diffusion cell systems have been developed (Akhter et al., 1984; Bronaugh et al., 1985; Cooper, 1984; Hawkins et al., 1986; Liron et al., 1984 and Okamoto et al. 1986). In those systems, the effluent can be collected in vials for subsequent liquid scintillation or HPLC analysis, or analyzed directly on a spectrophotometer. Such flow-through diffusion cells have an advantage over static cell systems mentioned above in that continuous supply of fresh receptor fluid assures sink condition throughout the diffusion measurement. The continuous supply of fresh receptor fluid, however, may lead to excessive dilution of the drug in the sampled solution, which makes it more difficult to analyze because the penetration through model skin membrane is usually slow for most drugs. Although slow flow rate coupled with receptor stirring can avoid the excessive dilution problem, this in turn may prevent frequent sampling because it takes a relatively long time to accumulate enough sample volume for analysis.

Shed snake skin is a non-living, pure stratum corneum which consists of 3 layers: the outermost, β-keratin rich β-layer, intermediate lipid-rich mesos layer, and the innermost, α-keratin rich α-layer. There has been reported to be some similarity between the mesos layer and mammalian stratum corneum in terms of structure, lipid content and composition (Landmann, 1979; Landmann et al., 1981; Roberts et al., 1980, 1983, Kligman, 1984; Dupuis et al., Ahern et al., 1973; Nicolaides, 1973; and Elias et al., 1977). It has also been reported that water permeability through snake skin was similar to that through human stratum corneum and that lipids play an important role in controlling water permeability both in shed snake skin and human skin (Roberts et al., 1980, 1983, Maderson et al., 1978; Blank, 1952; Berenson et al., 1951). Furthermore, since snake skin has no appendages (Maderson, 1972), the transfollicular route of penetration can be avoided, which considered to be of significant contribution in animal skins. Finally, the usefulness of shed snake skin as a model membrane in transdermal research has been reported by Ibuki (1985).

In the present study, a fully automated diffusion cell system has been developed, which requires no manual sampling of the receptor fluid. Since the receptor fluid flows through a spectrophotometer and back into a receptor compartment instead of being sampled for analysis, there is no excessive dilution of receptor fluid, which enables more reliable UV absorbance measurement. The system will be described in detail in this report with shed snake skin as a model membrane.

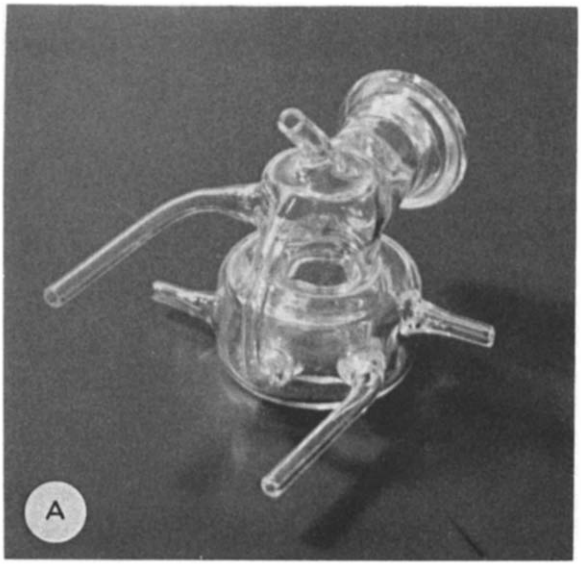
Materials and Methods

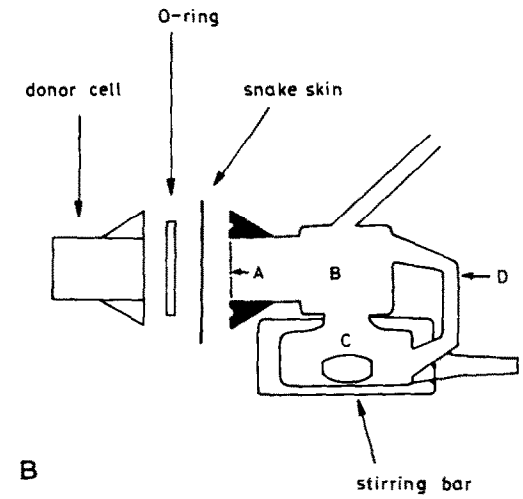
Minoxidil (Sigma), benzocaine (Sigma), lanolin-anhydrous (Aldrich), lauryl alcohol (Sigma), polyethylene glycol 400, ethanol, ethylacetate (all from Fisher Scientific), and Azone (Nelson Research Labs.) were used as received.

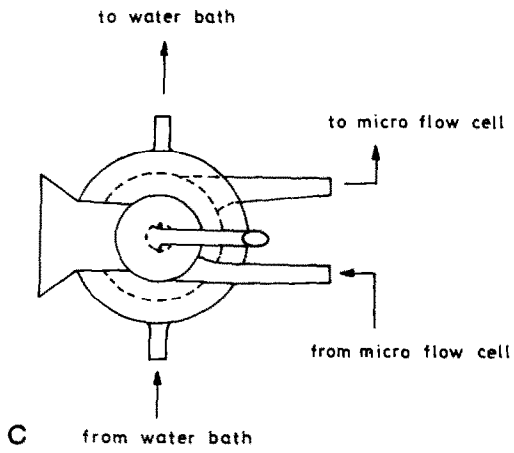
Minoxidil liquid formulation. Minoxidil (10 mg) was dissolved in a mixture of 50 mg lauryl alcohol and 200 mg polyethylene glycol 400. The weight was then adjusted to 1 g by adding ethanol to form a 1% w/w minoxidil liquid formulation. Of this solution 50 μl were applied on the shed snake skin for the penetration study.

Benzocaine liquid formulation. Benzocaine (10 mg) was dissolved in a mixture of 100 mg lauryl alcohol and 250 mg ethanol. The weight was then adjusted to 1 g with ethylacetate to form a 1% w/w benzocaine formulation. Of this solution 50 μl were applied for the penetration study.

Benzocaine ointment formulation. An ointment containing 1% w/w benzocaine, 5% w/w Azone, and 94% w/w lanolin was prepared by dissolving benzocaine in Azone in a vial over a 50 °C water bath, and then adding lanolin and a magnetic stirring bar. The vial was capped and placed in a 50 °C water batch over a magnetic stirrer and stirred for 2 h. The ointment was stored at room







temperature after preparation. Thirty mg of this ointment was applied for penetration study.

Diffusion cell system. A new diffusion cell developed in our laboratory is illustrated in Fig. 1 and the whole system is shown in Fig. 2. In order to avoid potential drug adsorption to materials to which drug molecules are exposed, the diffusion cell is made of glass and all the tubings are teflon. Each diffusion cell is connected to a micro flow cell in a spectrophotometer so that the fluid from the cell flows out of the lower chamber of the cell (C in Fig. 1), into a micro flow cell, and back to an upper chamber of the diffusion cell (B in Fig. 1). The model membrane, which has ca. 1.8 cm^2 of surface area, is placed in the cell at point A and the receptor phase (B and C) is filled with buffer solution. There is a magnetic stirring bar in the lower chamber (C), which spins at high speed (more than 500 r.p.m.). Because of this stirring action, the liquid in the lower chamber is pumped out toward a spectrophotometer at approximately 10 ml/min of flow rate. At the same time, there is a bypass tubing (D) such that there is a vigorous flow of solution from the lower (C) to upper chamber (D) and flows directly against the membrane. This vigorous flow of receptor solution ensures sufficient mixing in the receptor phase. Each of the diffusion cells has a magnetic stirrer under it to activate the pump and to maintain mixing in the cell. Also, there is a water jacket outside of the lower chamber in order to maintain the receptor fluid temperature. The UV or visible spectrum of the receptor solution is continuously monitored with the spectrophotometer.

The present arrangement includes 6 of these diffusion cells delivering their solutions to 6 micro flow cells in a single spectrophotometer, as shown in Fig. 2. The absorbance measurement with a spectrophotometer is controlled by a computer, which collects data and shows real time penetration results on the screen. The computer also stores data onto a diskette for later analysis of

Fig. 1. A newly developed diffusion cell. The surface area between the receptor and donor compartment is ca. 1.8 cm^2 and the total volume of the receptor compartment (B and C) is ca. 20 ml.

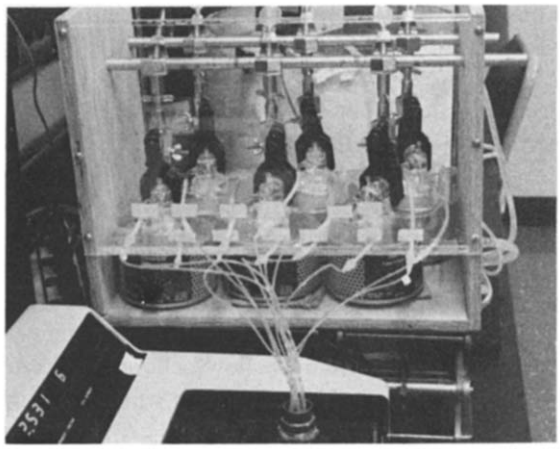


Fig. 2. The automated diffusion cell system which was used in the present study. Each of 6 diffusion cells is connected to a micro flow cell in the spectrophotometer with teflon tubing.

data. In the present system, we use an LKB spectrophotometer (Ultrospec II, Model No. 4050) and an AT&T personal computer (PC6300), which enables very frequent data collection - as often as every minute.

Model membrane. Snake skin molts of *Elaphe obsoleta* (black rat snake) were obtained from the Animal Care Unit of The University of Kansas and were stored at $-20\,^{\circ}C$ prior to use. Snake skin molts were brought to room temperature for 1 h before experiment. The ventral portion of the skin was removed, and the dorsal portion sectioned such that approximately a 5×5 cm central section of the dorsal portion remained. Each section of the dorsal skin was hydrated in $40\,^{\circ}C$ deionized water for 30 min. After hydration the skin was sonicated for 1 min., blotted dry, and then placed at point A of the diffusion cell (Fig. 1) with the natural outer side of the skin in the donor position of the cell. The skin was then fixed with an 0-ring between two sides and fastened tightly with a clamp. Ointment formulations were applied to the donor side of the skin before mounting the skin to the diffusion cell and liquid formulations were applied after mounting the skin. The receptor side was filled with approximately 20 ml of a buffer solution consisting of 0.15 M NaCl, 5.0×10^{-3} M NaH_2PO_4, and 2.0×10^{-3} M Na_2HPO_4 adjusted to pH 7.2 with NaOH. For the benzocaine ointment study 0.05% of gentamicin sulfate was added to the receptor fluid in order to inhibit microbial growth. The water jacket was then connected to a water bath in which the temperature was maintained at $32 \pm 0.5\,^{\circ}C$.

Conventional diffusion cell. Penetration of benzocaine in the liquid formulation through shed snake skin was measured with a Franz cell type diffusion cell, which is shown in Fig. 3, in order to compare the penetration rate of benzocaine between the present automated system and a conventional diffusion cell. This diffusion cell has ca. 1.8 cm^2 of the surface area between the receptor and donor cell with ca. 10 ml of receptor cell volume. The donor and receptor cells with an O-ring and shed snake skin were clamped tightly and immersed in a water bath at $32 \pm 0.5\,^{\circ}C$. Of the receptor solution 200 μl were sampled at appropriate time intervals with an equal amount of fresh buffer supplied to the receptor cell. The sampled receptor solution was analyzed by HPLC

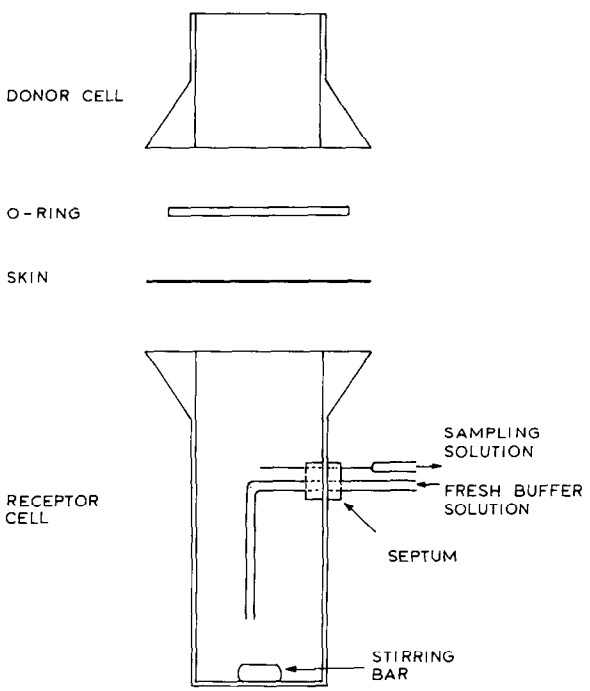


Fig. 3. A conventional Franz type diffusion cell as used in this study. The surface area between the receptor and donor compartment is ca. 1.8 cm^2 and the volume of the receptor compartment is ca. 10 ml.

with a C-18 column (5 μm of particle size) and 50% : 50% (v/v) of methanol : water as a mobile phase. Samples (15 μl) were injected and benzocaine was detected at 285 nm.

Results and Discussion

Penetration of minoxidil in the liquid formulation through shed snake skin was measured with the present automated system and the UV absorption of the receptor fluid at the wavelength of 230 nm was monitored at 30 min intervals. The absorbance change of the receptor solution as a function of time is shown in Fig. 4. Absorbance of the control solution (shed snake skin without minoxidil) was reasonably low, suggesting that the amount of UV absorbing substances leaking out of the shed snake skin was small under the present experimental conditions. The absorbance of control cell was then subtracted from that for other cells with minoxidil and the amount of minoxidil in the receptor fluid was calculated. The results for two different runs are shown in Fig. 5. Each set of data shows an apparent steady state flux (ca. 20 μg/h) up to ca. 18 h with a lag time of ca. 1 h. The permeability of minoxidil through shed snake skin was not calculated in this experiment because the minoxidil concentration could change with time due to evaporation of solvent in the formulation. The small lag time observed with the present system may not be detectable with conventional diffusion cell systems because of their incapability of frequent sampling, suggesting the advantage of the present system over conventional diffusion cell systems.

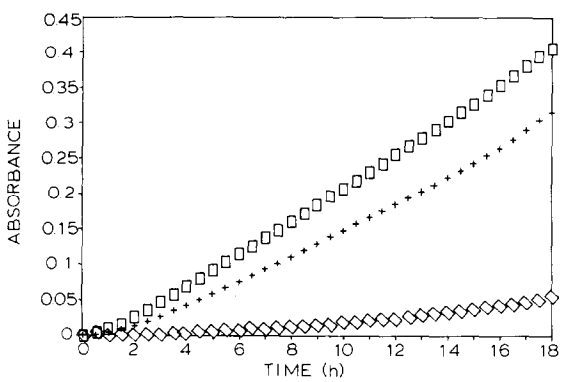


Fig. 4. The absorbance change of the receptor fluid with time in the minoxidil liquid formulation study. ◇, Control; □, +, minoxidil liquid formulation (two runs with the same formulation).

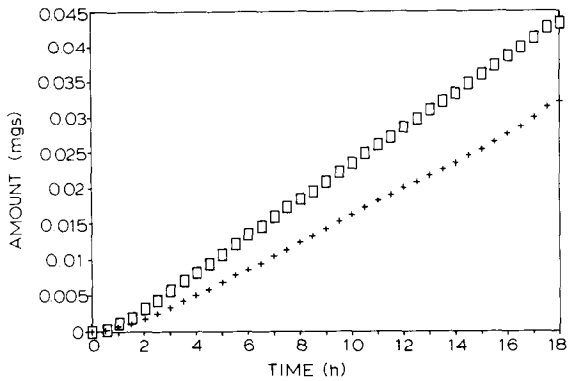


Fig. 5. The amount of minoxidil in the liquid formulation penetrated through shed snake skin measured with the present automated system. The results are shown for each of two identical runs.

The penetration of benzocaine in the liquid formulation through shed snake skin was measured every 30 min for 12 h at a wavelength of 285 nm. The result was compared to that obtained with a conventional diffusion cell system and both results are shown in Fig. 6. The apparent steady state flux obtained from each system was similar (12.9 μg/h for the present system and 10.2 μg/h for the conventional system). However, the lag time for the present system was very small compared to 0.4 h of the lag time observed with the conventional system. To examine the lag time of benzocaine in the liquid formulation, the same formulation was applied on the shed snake skin and the penetration rate was monitored at 5 min intervals. The result shown in Fig. 7 revealed a very small lag time (ca. 0.1 h) with an apparent steady state flux of ca. 14 μg/h. The difference in lag time between two systems was probably due to the longer set-up time of the present diffusion cell system. It takes approximately 0.5 h to set up and start measuring all 6 cells after applying the formulation on shed snake skin. If this set-up time is considered, the lag times between two systems appear to be no longer different. Although it has been reported by Nghiem and Higuchi (1988) that

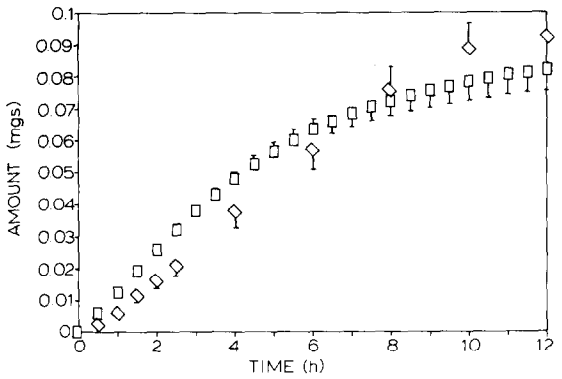


Fig. 6. Penetration of benzocaine from the liquid formulation through shed snake skin. □, Measured with the new automated system (mean ± S.E.M., $n = 4$); ◇, measured with the conventional Franz type diffusion cell with HPLC analysis (mean ± S.E.M., $n = 3$).

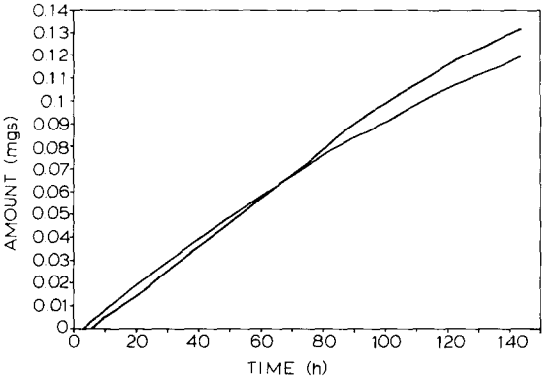


Fig. 8. Penetration of benzocaine from the ointment formulation through shed snake skin measured at 30 min intervals with the present automated system. The results are shown for each of two identical runs.

there exists esterase activity in shed snake skin, no *p*-aminobenzoic acid was observed in the receptor fluid with HPLC analysis, indicating that no benzocaine degradation occurred during penetration through the shed snake skin.

Fig. 8 shows the penetration rate of benzocaine in the ointment formulation through shed snake skin measured at 30 min intervals for 144 h with the present diffusion cell system. Absorbance of the receptor fluid was monitored at 285 nm. Apparent steady state flux of approximately 1 μg/h for up to 100 h with a lag time of 3–5 h is clearly shown in Fig. 8. This result suggests that more reliable steady state flux and lag time values can be obtained with less man hours using the present diffusion cell system. The penetration rate of benzocaine from the ointment formulation was approximately 10 times less than that from the liquid formulation, suggesting the potential poor bioavailability of benzocaine from ointment formulations. Again permeability was not calculated for the ointment formulation because the benzocaine solubility in the formulation could change with time due to relatively rapid penetration of Azone through shed snake skin (Ibuki et al.). Also penetration of Azone through the skin into the receptor fluid could decrease the penetration enhancing effect of this compound with time, which may be reflected in a slightly downward curvature of the penetration rate after 80 h in Fig. 8.

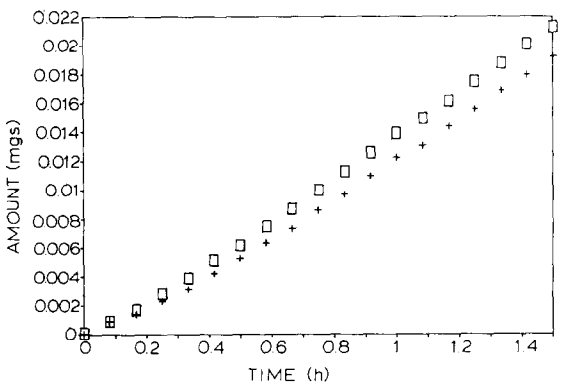


Fig. 7. Penetration of benzocaine from the liquid formulation through shed snake skin measured at 5 min intervals with the present automated system. The results are shown for each of two identical runs.

The potential usefulness of shed snake skin as a model membrane for transdermal research has been reported by Ibuki (1985) and shed snake skin from the same species (*Elaphe obsoleta* or black rat snake) was used in the present study. The types of the membrane which can be used as model membranes in the present automated system could be limited because of the possible release of some constituents of the membrane which would interfere with the spectrophotometric analysis. Shed snake skin, however, does not release a significant amount of absorbing species down to wavelengths as short as 210 nm, allowing its use as a model membrane in the present system. Syn-

thetic membranes are good candidates as a model membrane because of their rigid structure and pureness. In fact, synthetic membranes are being used in the present automated system in order to test the permeation of various compounds through those synthetic membranes and also to examine the drug release from various formulations using a porous membrane.

Since the receptor fluid in the present system is not continuously replaced as is the case in flow-through diffusion cells with subsequent sampling, the amount of compound used in this study was controlled so that the receptor concentration was maintained at less than 1% of the drug solubility even if all the drug molecules penetrate into the receptor fluid. This fact somewhat limits the amount of compound and the receptor fluid composition available in the present system. Furthermore, since the UV or visible absorption is monitored, the compound available in the present system must have UV or visible absorption. Although spectrophotometric analysis of the receptor fluid has a disadvantage compared to chromatographic methods with regard to sensitivity and differentiating capability between UV or visible absorbing compounds, frequent monitoring with the present system allows a large number of data collection, which may lead to more precise data analysis. If the conventional diffusion cell systems are used and subsequent HPLC analysis conducted, it will require a lot of personnel time to collect as much data as were generated in the present study. In this regard, the present automated system offers a great advantage over conventional diffusion cell systems.

In the present diffusion cell, the membrane is vertically attached to the receptor compartment in order to prevent air-bubble problems which are frequently observed with cells using the horizontal position. For Franz type diffusion cells with a membrane in a horizontal position, air bubbles tend to accumulate beneath the membrane which results in a reduced surface area available for diffusion.

In spite of some disadvantages, the present fully automated diffusion cell system should offer significant advantages over conventional systems, especially in terms of time and effort, and it should facilitate some of the normally time-consuming aspects of transdermal research.

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