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Comparison of Different Membranes with Cultures of Keratinocytes from Man for Percutaneous Absorption of Nitroglycerine

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Abstract

The permeability barrier function of cell-culture membranes to the permeation of nitroglycerine was evaluated to find an alternative to skin from man for ex-vivo skin-permeation tests.

The membranes were prepared, under submerged conditions, by inducing the growth of keratinocytes, from different donors, on a film of esterified jaluronic acid for different times (10, 20 and 30 days). Their permeability barrier functions were compared with those of some of the most widely used artificial membranes, silicone rubber (Silastic), cellulosic material (Cuprophan, Millipore HAWP), polysulphone membrane (Supor) and polytetrafluoroethylene membrane (TF-PTFE), and with those of biological membranes such as fresh and frozen skin, stratum corneum and epidermis from man, and hairless mouse skin. For each membrane the permeation profile was obtained and the flux was calculated. The permeation profiles for nitroglycerine were similar and linear in the first 2–3 h for all the synthetic membranes tested except TF-PTFE. For this membrane the profile was linear throughout the period considered and the amount permeating in 24 h (1603 $\mu g\,cm^{-2}$) was significantly lower than those obtained for the other artificial membranes (between 1926 and 2508 $\mu g\,cm^{-2}$). The amounts permeating through all the biological membranes in 24 h were in the range 520 to 781 $\mu g\,cm^{-2}$, except those for the keratinocyte–culture membranes, which were in the range 1730 to 2553 $\mu g\,cm^{-2}$. Prolonging the growth period of cultured keratinocytes did not affect nitroglycerine permeation.

The findings suggest that these keratinocyte–culture membranes have some advantages—good reproducibility if obtained from the same donor; many membranes can be obtained from the same donor; the preparation is simple; they can be handled more easily than traditional cell-culture membranes; and they afford constant penetration rates for a longer period than synthetic membranes. The membranes could be used for preliminary in-vitro permeation studies.

In-vitro evaluation of drug percutaneous absorption is needed in the design and development of transdermal delivery systems and can occasionally be used to predict the in-vivo drug uptake and associated plasma levels (Hadgraft et al 1993; Shah et al 1995). Standard regulatory requirements have not yet been established.

In-vitro permeation tests are generally performed using a diffusion cell system, with the donor and the receptor compartments separated by a suitable membrane. Excized skin from man is the membrane of choice but it is not readily available, there are ethical problems with its use, and there is great variability between specimens from different individuals, body sites and methods of collection (Wester & Maibach 1991).

Several materials have been investigated as an alternative to skin from man for ex-vivo evaluation of skin penetration. These materials have included synthetic membranes and a variety of excized animal skins (e.g. mouse, rat, pig, and rabbit). No synthetic membrane can really be used to predict percutaneous absorption, even if they have the advantages of giving reproducible data and meeting ethical standards. Animal skin is readily available,

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but its major disadvantages include distinct structural and chemical differences between these materials and skin from man (Tiemessen 1993)—animal skin is different in terms of lipid composition and physiological and morphological characteristics (e.g. thickness of the stratum corneum, number of pores and follicles) (Lin et al 1992).

Tissues from cultured keratinocytes have recently become available and have been tested as a skin substitute in percutaneous absorption studies (Ponec et al 1990; Regnier et al 1993; Godwin et al 1997).

The aim of this work was to evaluate the permeability barrier function to nitroglycerine permeation of cell cultures prepared by inducing the growth of keratinocytes on a film of esterified jaluronic acid.

The permeation studies were performed by use of a modified Franz-type diffusion cell. Minitran, a transdermal therapeutic system containing nitroglycerine, was chosen as a model system. The drug-release profile of the considered batch was obtained by dissolution tests performed according to the cell and disc-assembly methods of the European Pharmacopoeia (1997).

To compare the permeability barrier function of the cell cultures, permeation studies were conducted under the same experimental conditions, with some of the most widely used artificial and biological membranes—silicone rubber (Silastic), cellulosic material (Cuprophan, Millipore HAWP), polysulphone membrane (Supor), polytetrafluoroethylene (TF-PTFE). Experiments were also conducted with biological membranes—fresh and frozen skin from man, stratum corneum and epidermis (SCE) from man, hairless mouse skin, and cell cultures.

Materials and Methods

Materials

Minitran, batch no. 950160, was obtained from 3M Pharmaceuticals, MN.

The synthetic membranes used were: Millipore HAWP, a cellulose acetate filter, pore size 0·45 μm (Millipore, MA); Cuprophan 150 M 3·0, an inert, porous cellulosic material (Akzo Nobel Faser, Germany); Silastic 500–1, a medical grade silicone rubber, non-reinforced (Dow Corning Corporation, MI); Supor, a hydrophilic polysulphone membrane, pore size 0·45 μm (Gelman Sciences, MI); TF-PTFE, a polytetrafluoroethylene (PTFE) membrane with a polypropylene support, pore size 0·45 μm (Gelman Sciences, MI).

The biological membranes used were: fresh skin from the thigh of man; frozen (and stored at −80°C) skin from the thigh of man; stratum corneum and epidermis (SCE) from the thigh of man; hairless mouse skin from an animal aged 32 weeks; cell cultures from man obtained from four different donors (identified as A, B, C, D); cell cultures from man obtained from the same donor but after growth periods of 10, 20 and 30 days (identified, respectively, as A, A′ and A″).

Preparation of biological samples

Skin from man. Samples were removed from donors by means of a dermatome set at 200 μm. To minimize risk of infection, all biopsies were accompanied by a form recording all significant data about the donor. In addition, the biopsy was screened for the absence of hepatitis A and B, antibody anti-HCV, anti-HIV 1-2, RW, TPHA, CMV and herpes virus.

The skin biopsy, 2 cm × 3 cm, was placed in a sterile container containing phosphate-buffered saline (PBS, 30 mL), Dulbecco's modified Eagle medium (DMEM, 4·5 mg L^{-1} glucose), and antibiotics. Any adhering muscle or subcutaneous fat was surgically removed from the dermal side. The skin was used within 48 h of removal or after thawing.

Stratum corneum and epidermis (SCE) from man. The SCE was obtained by immersion of skin in distilled water at 60 ± 1°C for 1 min and peeling from the dermis. SCE membranes were dried in a desiccator at 25% RH (approx.) and then wrapped in aluminium foil and stored at 4 ± 1°C until use.

Hairless mouse skin. A hairless mouse was killed by cervical cleavage of the spinal cord and the full-thickness skin was removed from the back. The adherent fat and other visceral debris were removed from under the surface. The skin was used immediately.

Cell cultures. Biopsies from man were cut into smaller fragments (approx. 5 cm^2) which were subsequently digested with 2·5 mg mL^{-1} Dispase II (Boehringer Mannheim, Germany) in PBS. Incubation was performed for 4 h at 37°C or for 18 h at 4°C. The epidermal sheet was carefully peeled from the underlying dermis and trypsinized (0·05% trypsin–0·01% EDTA) to dissociate single cells further. Every 5–10 min pipetting was conducted to encourage detachment of basal keratinocytes and

enable complete cell recovery. Isolated cells were then collected, by centrifugation, after 20–30 min, after trypsin inhibition by addition of medium containing 10% serum.

Isolated keratinocytes were seeded for primary culture at a concentration of 30 000 viable cells cm^{-2} on a feeder layer of irradiated mouse fibroblasts in an 80 cm^2 plastic culture flask (Nunc, Denmark) with cell epidermal culture medium (CEC, 15 mL) without epithelium growth factor. The cultures were incubated at 37°C in 5% CO_2. CEC medium is a 3 : 1 mixture of DMEM and Ham's F12 (Irvine Scientific, CA) containing 10% foetal bovine serum (Hyclone, UT), 10^{-10} M cholera toxin, 0·4 $\mu g\,mL^{-1}$ hydrocortisone, 5 $\mu g\,mL^{-1}$ insulin, $1{\cdot}8 \times 10^{-4}$ M adenine, 5 $\mu g\,mL^{-1}$ transferrin, 2×10^{-9} M triiodothyronine (Sigma, St Louis, MO), 0·6 $mg\,mL^{-1}$ L-glutamine, 0·11 $mg\,mL^{-1}$ sodium pyruvate, 100 $mg\,mL^{-1}$ penicillin and 100 $\mu g\,mL^{-1}$ streptomycin (Irvine Scientific, CA). After 48 h culture 10 $ng\,mL^{-1}$ epithelium growth factor (Biosource, CA) was added to the cultures (complete CEC). Complete CEC medium was changed every two days until subconfluency. When primary keratinocytes achieved semiconfluence, trypsin–EDTA 1X (Trypsin 0·05%, EDTA 0·02% in phosphate buffer solution without calcium and magnesium) were added to the culture. After 10 min at 37°C the cells were centrifuged and seeded for second passage on an esterified jaluronic acid film (Laserskin, Fidia Advanced Biopolymers, Italy). Medium was completely substituted every 2 days until the cell cultures were tested.

Dissolution test

Dissolution was accomplished by use of an Erweka DT6 (Erweka, Germany) apparatus. The test was performed according to the European Pharmacopoeia, by either the disc-assembly method or a modified-cell method.

The extraction cell, made of polymethacrylate, was modified. The cavity of the central part was removed and the lower part was modified to minimize any dead volume. A sample cut from the patch was placed flat on the cell with the release surface uppermost and a Cuprophan 150 M 3·0 membrane on it. This membrane was rehydrated by immersion in distilled water for 1 h before use.

For both methods the vessels were filled with degassed distilled water (500 mL). The water bath temperature was maintained at 32 ± 0·5°C. The paddle speed was 50 rev min^{-1} and the diffusion area was 12·56 cm^2. Samples (10 mL) were collected after 0·25, 0·5, 1, 2, 3, 4, 5, 6, 7 and 8 h and analysed for nitroglycerine content by the HPLC method described below. The volume sampled was immediately replaced with fresh medium. Each value represents the average of six samples readings.

Permeation test

A sample (2·5 cm^2) was applied with slight pressure to the membrane. Before use each synthetic membrane and dried SCE sample was hydrated at room temperature by immersion in distilled water for 1 h and for 16 h, respectively. The biological membranes were placed on the diffusion cell with the dermal side in contact with the receptor solution and the stratum corneum side in contact with the patch. Each membrane was carefully mounted on the modified Franz-type diffusion cell, sealed with Parafilm and fastened with a rigid clamp.

These cells had a wider vertical column than the original Franz-type diffusion cell, and the bowl shape was removed. They had a diffusion area of 0·636 cm^2 and 5 mL (approx.) receiver capacity. The receiver volume of each cell was individually calibrated.

The receiver compartments were filled with freshly prepared degassed isosmotic pH 7·4 phosphate-buffered saline containing streptomycin (100 $\mu g\,mL^{-1}$, Sigma) (biological membranes) or degassed, HPLC-grade water (synthetic membranes).

Before use the solutions were sonicated under vacuum to remove dissolved air. The receiver solution was continuously stirred with a small magnetic bar. The receptor compartment was maintained at 32 ± 0·5°C. At predetermined times (0·25, 0·5, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h) 0·2 mL samples were withdrawn from the receiver compartment and immediately replaced with fresh receiver medium. Sink conditions were maintained throughout the experiment. Samples were analysed for nitroglycerine content by the HPLC method described below. Each value represents the average of five sample readings.

Drug assay

The concentrations of nitroglycerine in the receiver medium were determined by HPLC assay. HPLC was performed with a high-pressure pump (320 System; Kontron Instruments, Italy), an autosampler (Autosampler 460; Kontron Instruments), a variable wavelength UV detector (HP 1100 Series; Hewlett-Packard, CA) and an integrator-recorder (HP 3349, Hewlett-Packard).

Samples (10 μL) were injected at room temperature onto a C_{18} column (5 μm Spherisorb

ODS2, 20 cm, Shandon HPLC, UK). The mobile phase, methanol–water–tetrahydrofuran, 54 : 43 : 3 (v/v), was degassed before use; the flow rate was 0·9 mL min^{-1}. The UV detector was operated at 220 nm. The retention time of nitroglycerine was 7 min. The reference standards were from 1 to 100 μg nitroglycerine mL^{-1}. The nitroglycerine solutions were prepared using HPLC-grade methanol. The correlation coefficients of the standard curves were $0{\cdot}9999 \pm 3{\cdot}7 \times 10^{-5}$. The inter-assay coefficient of variation (n = 10) was 5·29 for a 5 μg mL^{-1} standard solution.

Data analysis

The amount (μg cm^{-2}) of nitroglycerine permeating through the membrane per unit area was calculated from the concentration in the receiving solution. The cumulative amount of nitroglycerine permeating into the receptor compartment was plotted against time to obtain the permeation profile. The steady-state flux (J, μg cm^{-2} h^{-1}) was estimated from the slope of the linear portion of the permeation profile. The estimated parameters are presented as means ± s.d. Tests for significant differences between means were performed by a one-way analysis of variance. Differences were considered significant at the $P < 0{\cdot}05$ level. Statistical analysis was conducted using SPSS 7.5 (SPSS Inc., IL).

Results and Discussion

Dissolution tests

The in-vitro release profiles of nitroglycerine from the Minitran were obtained by use of the using disc-assembly method and the modified-cell method. With the disc-assembly method 90% of the drug was detected in the dissolution media within 2 h whereas, as expected, release was slower for the cell method (90% in 6 h). The coefficient of variation was lower than 3% when the disc-assembly method was used; the value was 5% for the cell method. At the end of the experiments no relevant physical modification of the patches was apparent.

Permeation test

The amounts of nitroglycerine permeating through the synthetic membranes are reported in Figure 1, as are the amounts of nitroglycerine found in the receiver compartment using the cell without membrane. A permeability barrier function was shown for all the synthetic membranes as the amounts detected in a given time were always lower than those obtained without the membrane ($P < 0{\cdot}05$). The permeation profiles were similar and linear for 2–3 h for all the membranes tested except the TF-PTFE membrane.

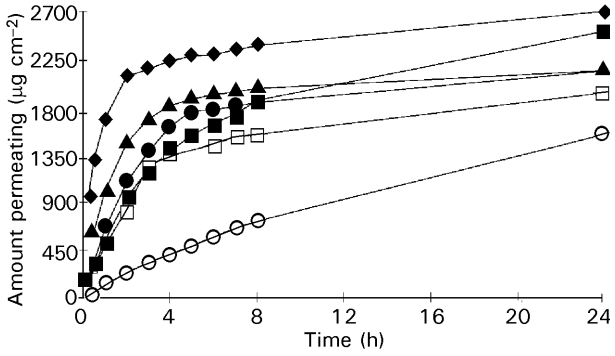

Figure 1. Permeation profiles of nitroglycerine through synthetic membranes. ■ Silastic, ▲ Supor, ● Cuprophan, □ Millipore HAWP, ○ TF-PTFE and comparison with a cell with no membrane (◆) (n = 5; s.d. values were < 10%).

The cumulative amount of nitroglycerine permeating through the TF-PTFE membrane had a linear profile for the period considered, without any marked lag time or burst effect phenomena, and the rate of permeation was significantly lower ($P < 0{\cdot}05$) than that obtained with the other artificial membranes (Wu et al 1992). With the Millipore mixed-cellulose ester membrane the rate of release of nitroglycerine was lower; again this has been reported previously (Wu et al 1992).

The amounts of nitroglycerine permeating through biological membranes in 24 h are shown in Figure 2. The permeation profiles obtained from fresh and frozen skin from man, removed from different donors, were different. Because other authors have shown that the permeability of skin from man was unaffected by freezing and thawing, even after prolonged freezing (Harrison et al 1984; Bronaugh et al 1986), this difference is probably

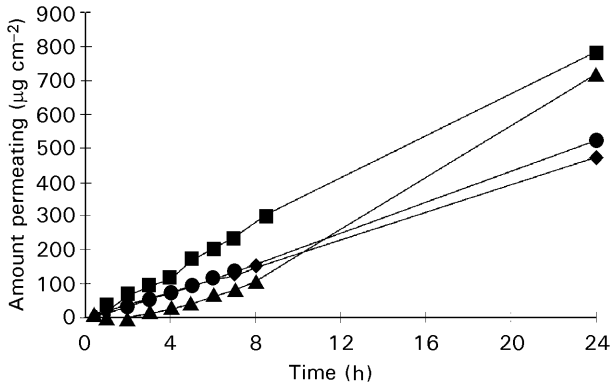

Figure 2. Permeation profiles of nitroglycerine through biological membranes. ■ Frozen skin from man, ▲ hairless mouse skin, fresh skin from man, ◆ SCE (n = 5; s.d. values were < 10%).

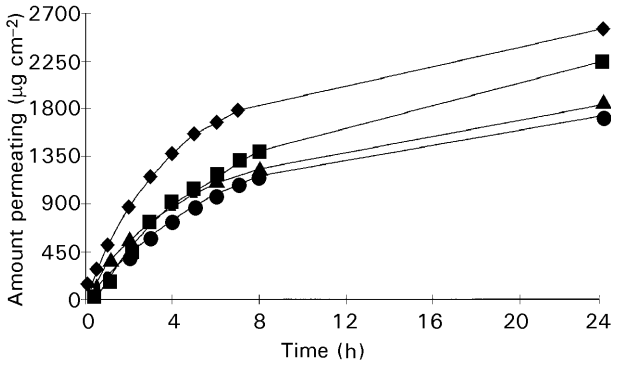

Figure 3. Permeation profiles of nitroglycerine through cell cultures obtained from different donors. ◆ A, ■ B, ▲ C, ● D (n = 5; s.d. values were < 10%).

because of inter-specimen variation and experimental error rather than freezing.

The observed flux through the SCE was very similar to that through the fresh full-thickness skin. The in-vitro permeation rates obtained with the samples of frozen skin from man are in agreement with the estimated in-vivo delivery rate claimed on the Minitran label (0·75 mg cm^{-2} for 24 h) (Hadgraft et al 1991).

Permeation through hairless mouse skin was characterized by a marked lag time that was not observed for skin from man.

The rates of penetration through all the keratinocyte cultures were constant for a longer period (5 h) than for those through synthetic membranes (Figure 3). For different donors the coefficient of variation of the nitroglycerine fluxes was 37·8%, less than those reported in the literature for nitroglycerine fluxes through SCE from different donors (42·9 and 47·7%; Langguth et al 1986a,b). The differences among donors was significant (F-test, $P < 0{\cdot}0001$).

Table 1. Flux through the membranes and total amount of nitroglycerine permeating in 24 h.

Membrane	Flux ($\mu g\,cm^{-2}\,h^{-1}$)	Amount of nitroglycerine permeating in 24 h ($\mu g\,cm^{-2}$)
Fresh skin from man	22·1 ± 2	520 ± 54
Hairless mouse skin	30·5 ± 6	716 ± 152
Frozen skin from man	33·2 ± 8	781 ± 185
SCE from man	23·4 ± 10	559 ± 263
†Cell cultures D	174·5 ± 8	1730 ± 157
†Cell cultures C	178·9 ± 19	1833 ± 159
†Cell cultures B	235·2 ± 39	2248 ± 288
†‡Cell cultures A	299·4 ± 28	2553 ± 167
‡Cell cultures A′	285·6 ± 18	2443 ± 225
‡Cell cultures A″	282·8 ± 21	2542 ± 143
TF-PTFE 0·45 μm	63·9 ± 7	1603 ± 166
Millipore	387·3 ± 22	1926 ± 130
Supor 0·45 μm	432·7 ± 37	2007 ± 253
Silastic	431·7 ± 95	2508 ± 98
Cuprophan	523·0 ± 69	2210 ± 73

Results are means ± s.d. †Cell cultures from man obtained from different donors. ‡Cell cultures from man obtained from the same donor but with different growth periods (A = 10 days; A′ = 20 days; A″ = 30 days).

Better reproducibility was obtained for the fluxes through culture samples from the same donor (CV = 7%, n = 15).

Prolonging the growth period of cultured keratinocytes from 10 to 20 or 30 days had no effect on nitroglycerine permeation (Table 1).

Nitroglycerine fluxes through synthetic membranes, except TF-PTFE, were much greater than those through excized skin from man; they were higher than those obtained from cell cultures grown on jaluronic acid, although of the same order of magnitude (Table 1).

The TF-PTFE membrane had a permeability barrier function to nitroglycerine similar to that of skin from man and animals (Juhasz et al 1996).

Conclusions

As expected the profiles of drug-release from Minitran obtained with the two dissolution methods tested (disc assembly and cell method) were different, although both methods are applicable.

The synthetic TF-PTFE membrane was highly resistant to permeation of nitroglycerine and its performance was very different from that of the other synthetic membranes.

The barrier functions of cell-culture membranes grown on jaluronic acid film were lower than those of skin from man and hairless mouse skin and higher than those of synthetic membranes. The period of steady-state flux was always longer for cell-culture membranes than for synthetic membranes. Prolonging the time of cell-culture growth from 10 to 30 days did not significantly change the permeability characteristics of the membranes. A 20-day period always ensured complete growth of the cells on the jaluronic acid film and can be regarded as the optimum period of growth.

Release rates for keratinocyte culture membranes obtained from the same donor were not significantly different. Although these membranes were more permeable than SCE from man, they have some advantages—good reproducibility if obtained from the same donor; less variability than observed for skin from animals and man; many membranes can be obtained from the same donor; the preparation is simple; they can be handled more easily than traditional cell-culture membranes; and they result in constant penetration rates for a longer period than do synthetic membranes. For these

reasons these membranes could be used for preliminary in-vitro permeation tests.

Further studies are in progress to increase the resistance of the cell cultures to the permeation of drugs by modifying the method of preparation.

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