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Quantification and localization of fentanyl and TRH delivered by iontophoresis in the skin

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Abstract

Autoradiography and the technique of stripping/slicing were used in order to investigate the pathways and to quantify drug penetration into skin after iontophoresis of two model compounds: fentanyl, a lipophilic molecule and TRH, a hydrophilic molecule. Iontophoresis was performed for 1, 4 and 6 h at a mean current density of 0.33 mA/cm^2 and was compared to passive diffusion. The quantification studies showed that iontophoresis increases the drug concentration in the part of the skin limiting molecule permeation: viable skin for fentanyl and stratum corneum for TRH. Even though, besides accumulation, autoradiography allows one to localize the route of passage, observations tend to confirm that transepidermal penetration can take place and that an important route of penetration is the transappendageal pathway.

Keywords: Iontophoresis; Penetration pathway; Fentanyl; TRH; Autoradiography; Stripping; Slicing

1. Introduction

Traditional transdermal drug-delivery systems are based on the transport of molecules into or through the skin by diffusion. In most cases, the transepidermal diffusion pathway is of a greater importance in the overall process of percutaneous penetration, although appendageal absorption may be the dominant mechanism during the initial transient stage of penetration (Cullander, 1992). However, the number of solutes which can be delivered by this route are limited due to the excellent barrier properties of the stratum corneum, the outermost layer of the epidermis. The stratum corneum has a very unique morphology, by which lipids form multi-lamellar arrays in the hydrophilic extracellular spaces surrounding the corneocytes. This morphology itself could account for the low permeability of the stratum corneum (Potts and Francoeur, 1991). As a consequence, most drugs delivered by the conventional transdermal systems for systemic use are small, potent and highly lipophilic molecules.

Different approaches were envisaged to enhance skin permeability and to extend the transdermal application field to new drugs (Chien, 1988).

Iontophoresis, the application of an external electrical field to promote drug penetration, has

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been used empirically for decades. This transdermal drug delivery system has the main advantage of enhancing skin penetration of ionizable or polar drugs with better transport control (Yoshida and Roberts, 1992). Several studies have recently been performed in order to resolve iontophoresis workings. However, the localization of the current flow paths remains poorly investigated. An understanding of these conductive routes is essential for the optimization of transdermal drug delivery and the treatment of dermatologic conditions.

When an electrical field is imposed across the skin, ions will move along the paths of lowest electrical resistance. Different studies tend to show that transdermal iontophoretic transport occurs primarily at discrete sites or 'pores'. Cullander and Guy (1991) used a vibrating probe electrode to identify and vectorize iontophoresis currents in mammalian skin preparation. They showed that iontophoretic currents were primarily appendageal with certain appendages appearing to carry most of the current. However, not all iontophoretic transport was localized. The vibrating probe located a small current going into (or coming out of) the skin at locations where no appendageal structures (or injuries) were apparent. Moreover, confocal microscopy showed that a paracellular path existed for polar and charged species. On the other hand, scanning electrochemical microscopy studies (SCEM) (Scott et al., 1993b) suggested that if hair follicles are the active pathways for iontophoretic transport, only a small fraction of them behave as such. Scott et al. (1993a) recently showed that activation of low resistance pores occurred during iontophoresis parallel to the increase in skin conductance.

The aim of our study was to quantify according to skin depth and to localize the pathways taken by two compounds during iontophoresis: a lipophilic (fentanyl) and a hydrophilic (TRH) molecule. The compounds studied were chosen because of their physicochemical properties, among which solubility is of great importance in percutaneous phenomena. Fentanyl has a molecular mass of 336 Da and presents an octanol/water partition coefficient of 717 at pH 7.4 (Roy and Flynn, 1988). TRH is a peptide of 362 Da and has a partition coefficient (octanol/water, pH 7.4) of 0.0376 (Dowty et al., 1992). Moreover, the efficiency of iontophoretic transport has already been demonstrated for both molecules. Fentanyl presented, under the same conditions as those discussed below, a transdermal flux of 1 ± 0 ng/cm^2 per h by passive diffusion which was increased up to 537 ± 31 ng/cm^2 per h when direct current was applied (over 6 h at a mean current density of 0.33 mA/cm^2) (Thysman et al., 1994). TRH diffused at a mean flux of 57.6 ± 16.2 ng/cm^2 per h. This flux increased up to 695.2 ± 115.1 ng/cm^2 per h under current application (6 h at 0.33 mA/cm^2) (unpublished data). Two different techniques were chosen because of their complementarity: autoradiography, which permits localization of the drugs and the technique of stripping and slicing (Schaefer and Lamaud, 1987; Green et al., 1992) for its quantification approach.

2. Materials and methods

2.1. Chemicals

Fentanyl citrate was purchased from Janssen Pharmaceutica and [H^3]fentanyl was a gift from Janssen Pharmaceutica (Beerse, Belgium). TRH was purchased from Sigma Chemical Co. (Sigma Aldrich, Bornem, Belgium) and [H^3]TRH from NEN Research (Du Pont de Nemours, Brussels, Belgium). Chemicals used to prepare buffers or to hydrolyse (analysis grade) were obtained from UCB (RPL, Leuven, Belgium) and Merck (Merck-Belgolabo, Overijse, Belgium). All solutions were prepared in ultrapure water (Sation 9000, Vel, Leuven, Belgium).

2.2. Apparatus and procedures

Iontophoresis was performed in vitro in horizontal cells. 3 cm^2 of freshly excised abdominal hairless rat skin (7–10 weeks old, Iffa Credo, St Germain-les-Arbresles, France) separated the donor and receptor compartments. The platinum electrodes (platinum pure, Johnson Matthey, Brussels, Belgium; 1 cm^2) were connected to a

direct current source (0.33 mA/cm^2). The cathode was introduced into the receptor compartment filled with 0.024 M phosphate buffer (pH 7.4) made isotonic with glucose (0.015 M). The donor compartment, facing the stratum corneum, was filled with fentanyl (40 μg/ml), [^{3}H]fentanyl (3 μCi/ml) introduced in a citrate buffer (0.01 M, pH 3.5) or with TRH (100 μg/ml), [^{3}H]TRH (3 μCi/ml) and citrate buffer (0.02 M, pH 4.2). The anode was immersed in the donor solution. Iontophoresis and diffusion were performed for 1, 4 and 6 h.

Quantifications were performed by horizontal slicing according to the model described by Schaefer and Lamaud (1987) and Green et al. (1992). At the end of the treatment, the electrodes were removed and the skin surface was gently wiped clean. The skin directly under the location of the donor compartment was excised, pinned flat and tape-stripped 10 times (Scotch Cristal, 602, Cergy Pontoise, France). Each strip was placed into a separate scintillation vial, digested over 24 h with 1 ml of 1 N NaOH, neutralized with 167 μl of 6 N HCl and mixed with liquid scintillation cocktail (Ready-Safe, Beckman, Belgium) acidified with acetic acid (7‰) to avoid chemoluminescence. Finally, each vial was counted for radioactivity (ß-counter; Wallac 1410, LKB, Pharmacia). The remaining underlying skin tissue was flattened on a glass slide laid over dry ice. Four to five biopsies (0.5 cm × 0.5 cm) were taken and mounted on a cryostat (2800 Frigocut, N Reichert-Jung). Ten 40-μm slices were cut at −22° C parallel to the skin surface and corresponding slices from the different biopsies were combined. The slices were digested (over 72 h) in NaOH (1 N, 0.5 ml), neutralized with HCl (6 N, 83 μl) and counted for radioactivity.

Localization was studied by autoradiography. At the end of the current application or diffusion, the skin was frozen in isopentane (Merck-Belgolabo, Overijse, Belgium) cooled in liquid nitrogen, and stored at −80° C. Sections perpendicular to skin surface (14 μm) were prepared in a cryostat (2800 Frigocut N Reichert-Jung) at −22° C and mounted on slides. Each treatment was repeated on three different rat skins. Slides were immersed for 10 min in 4% formaldehyde (Merck-Belgolabo, Overijse, Belgium) in two ultrapure water baths (5 min), coated with Kodak autoradiography emulsion (type NTB2, Tecnomara AG, Wullisellen, Switzerland) and exposed at 4° C in light-proof desiccator boxes. After time exposure (8 weeks), sections were developed in Kodak Dektol (Kodak, New Haven) for 2 min, rinsed twice in ultrapure water, fixed in Agefix for 5 min (Agfa Gevaert, Leverkusen, Germany) and rinsed in water. The slides were coloured using hematoxylin-eosin.

3. Results

3.1. Quantification

3.1.1. Fentanyl

As shown in Fig. 1, the quantity of fentanyl detected in stratum corneum did not vary with current application. Indeed, diffusion and iontophoresis induced the same distribution profile of fentanyl. Moreover, an increase in the duration of current application did not influence the quantity of fentanyl present in stratum corneum.

Fig. 2 demonstrates the epidermis/dermis distribution profile. In viable skin, iontophoresis enhanced fentanyl penetration (from 10- up to 40-fold) and a significant difference was observed between quantities detected after diffusion and iontophoresis. Moreover, the difference observed between iontophoresis and diffusion increased

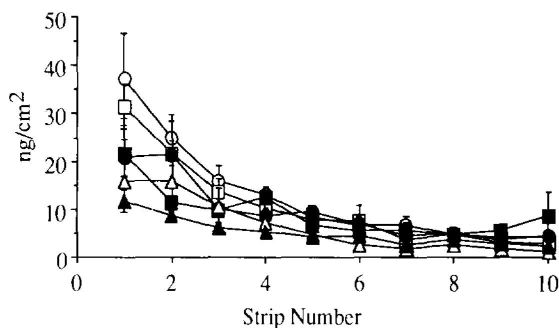

Fig. 1. Quantity of fentanyl (ng/cm^2) as a function of stratum corneum depth after iontophoresis applied at a current density of 0.33 mA/cm^2 for 1 h (▲), 4 h (●) and 6 h (■) or after diffusion for 1 h (△), 4 h (○) and 6 h (□).

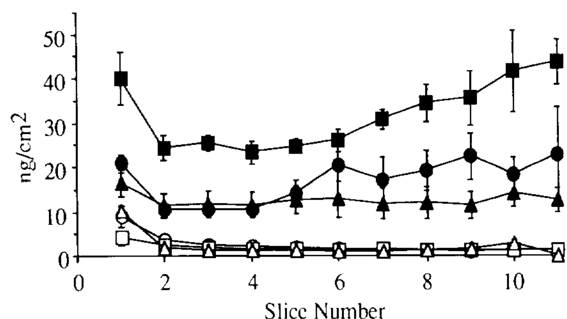

Fig. 2. Quantity of fentanyl (ng/cm^2) as a function of epidermis and dermis depth after iontophoresis applied at a current density of 0.33 mA/cm^2 for 1 h (▲), 4 h (●) and 6 h (■) or after diffusion for 1 h (△), 4 h (○) and 6 h (□).

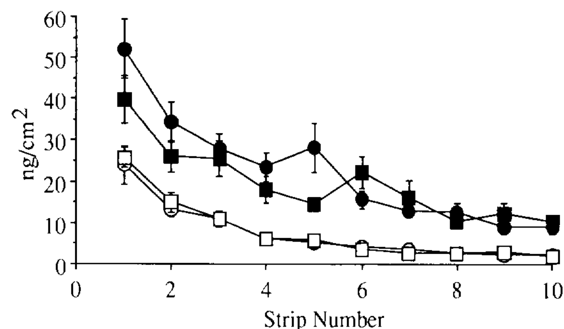

Fig. 3. Quantity of TRH (ng/cm^2) as a function of stratum corneum depth after iontophoresis applied at a current density of 0.33 mA/cm^2 for 4 h (●) and 6 h (■) or after diffusion for 4 h (○) and 6 h (□).

when the duration of iontophoresis was lengthened.

3.1.2. TRH

As shown in Fig. 3 and 4, iontophoresis significantly enhanced TRH quantities in both stratum corneum (by a factor of about 4) and dermis (by a factor of about 5–10) as compared to passive diffusion.

3.2. Localization

3.2.1. Fentanyl

During diffusion, the quantity of fentanyl in stratum corneum was greater than in underlying epidermis. Penetration of the compound into hair shafts and associated structures was progressively noted, with a slighter and delayed accumulation in sebaceous glands as compared to hair follicles.

The preponderance of fentanyl in stratum corneum (compared to underlying layers) was maintained in the case of current application. In addition, marked lines of silver grains were often observed between stratum corneum and the underlying epidermis, especially after 4 h iontophoresis (Fig. 5a). After 1 h iontophoresis, fentanyl was also localized at the edge of the hair shaft cells. Moreover, a well-defined marked accumulation of silver grains was detected in the sebaceous glands after 4 h and mainly 6 h iontophoresis (Fig. 5b).

Some of the autoradiograms clearly showed paracellular staining. No intracellular localization was observed.

3.2.2. TRH

After TRH diffusion, staining was equally divided in stratum corneum and underlying epidermis. Little TRH penetration was noted in rat skin appendages.

When TRH was delivered by iontophoresis, staining was more pronounced in stratum corneum than underlying layers. A high concentration of silver grains was observed in hair follicles although sebaceous glands were only slightly

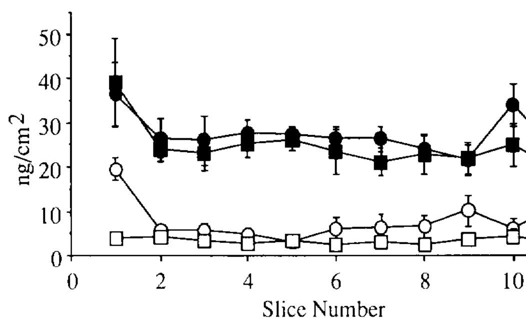

Fig. 4. Quantity of TRH (ng/cm^2) as a function of epidermis and dermis depth after iontophoresis applied at a current density of 0.33 mA/cm^2 for 4 h (●) and 6 h (■) or after diffusion for 4 h (○) and 6 h (□).

marked even after long-term current application (Fig. 5c).

4. Discussion

Passive skin absorption can be considered to be represented physicochemically by a series of consecutive partitioning and diffusional processes. In transversing the skin, the drug must first partition into the stratum corneum and diffuse through this impermeable barrier. The molecule may therefore interact with many potential binding sites, possibly forming a reservoir. Free drugs eventually reach the interface between the stratum corneum and the viable epidermis, where the drug has to partition once again between the lipophilic and the water-rich tissue. In the living skin, the drug may be confronted to a storehouse of enzymes. Moreover, additional receptors, metabolic and depot sites should intervene as the drug moves to a blood capillary, partitions into the walls and exits into the blood (Hadgraft, 1991).

In stratum corneum, the quantification study did not show any significant difference in fentanyl quantities detected after diffusion or iontophoresis. This could easily be explained by the fact that due to its lipophilicity, fentanyl or at least its unionized fraction diffuses easily through stratum corneum. Indeed, we can consider that, at its first level behavior, stratum corneum acts as a lipid membrane. Therefore, Fick's first law may be applied and shows the relation between partition coefficient (K_p) and the flux of penetrating drug (dQ/dt):

$$\frac{dQ}{dt} = \frac{K_p D_p}{l}(C_d - C_r)$$

where D_p is the diffusion coefficient, l membrane depth, C_d donor concentration and C_r receptor concentration

To a certain degree, for a very lipid soluble drug, the affinity for stratum corneum may be so high that the clearance from the horny layer replaces diffusion through the stratum corneum as the rate-limiting step. This is probably the case for the fentanyl molecule, and this should provide an explanation for its considerable lag time constant in passive transdermal studies (Roy and Flynn, 1990; Thysman et al., 1994). Indeed, as opposed to the intercellular multilamellar lipid matrix of the horny layer, it is well established that the cell mass of epidermis beneath stratum corneum contributes to an aqueous resistance to diffusion. In this hydrophilic environment, the fentanyl partition coefficient is not balanced in favor of viable skin and fentanyl tends to stay in stratum corneum. The preponderance of silver grains observed in stratum corneum compared to the underlying skin and, in some cases, the great accumulation at the entry of viable skin confirm the poor affinity of fentanyl for viable skin. It can be assumed that each strip removes one corneocyte layer (Peacock and Van Winkel, 1976). Moreover, rat stratum corneum is composed of eight layers and each one is 0.8–0.9 μm thick (Dupuis et al., 1984). Therefore, for stripping, the drug quantities were measured in slices approx. 0.85 μm thick. Consequently, we can note that the detected quantities measured in viable skin (in slices 40 μm thick) are dramatically less than in the upper layers. Iontophoresis tends to reduce this large difference: the fentanyl quantities detected in viable skin are significantly greater after iontophoresis than passive diffusion. Moreover, an enhancement in applied current duration tends to increase the quantities of fentanyl in the skin. This last fact is probably an indication that accumulation occurs in deep tissue.

Unfortunately, the technique of slicing does not permit us to distinguish between dermis matrix and appendageal accumulation. Besides the improvement in partitioning between the stratum corneum and viable tissue, the higher fentanyl quantities which were detected after iontophoresis and their enhancement with iontophoresis duration could also be due to the direct passage of fentanyl in hair follicles, sebaceous glands and accumulation in these appendices. It has been established that the routes of penetration differ between iontophoresis and diffusion: although appendageal routes play a minor role in passive permeation, they appear to become major pathways under the influence of an electrical driving force (Cullander, 1992). Nevertheless, observa-

tions of autoradiograms demonstrate considerable accumulation in the appendix and lead one to presume a definite role played by appendageal pathways in iontophoretic transport. However, the autoradiographic technique also has some disadvantages and extensive staining could also be due to a high affinity from the molecule to any binding site. Thus, fentanyl, because of its high partition coefficient, tends to accumulate in the lipophilic environment of the sebaceous glands which appear to serve as a site of retention and a reservoir for compound release.

Unlike fentanyl, the hydrophilic molecule penetrates poorly into the lipophilic environment of the horny layer (due to its low K_p), iontophoresis seeming to facilitate TRH penetration and to increase the permeability of the stratum corneum. Once the stratum corneum is crossed, TRH probably diffuses 'easily' through epidermis and dermis layers, even passively. The greater TRH

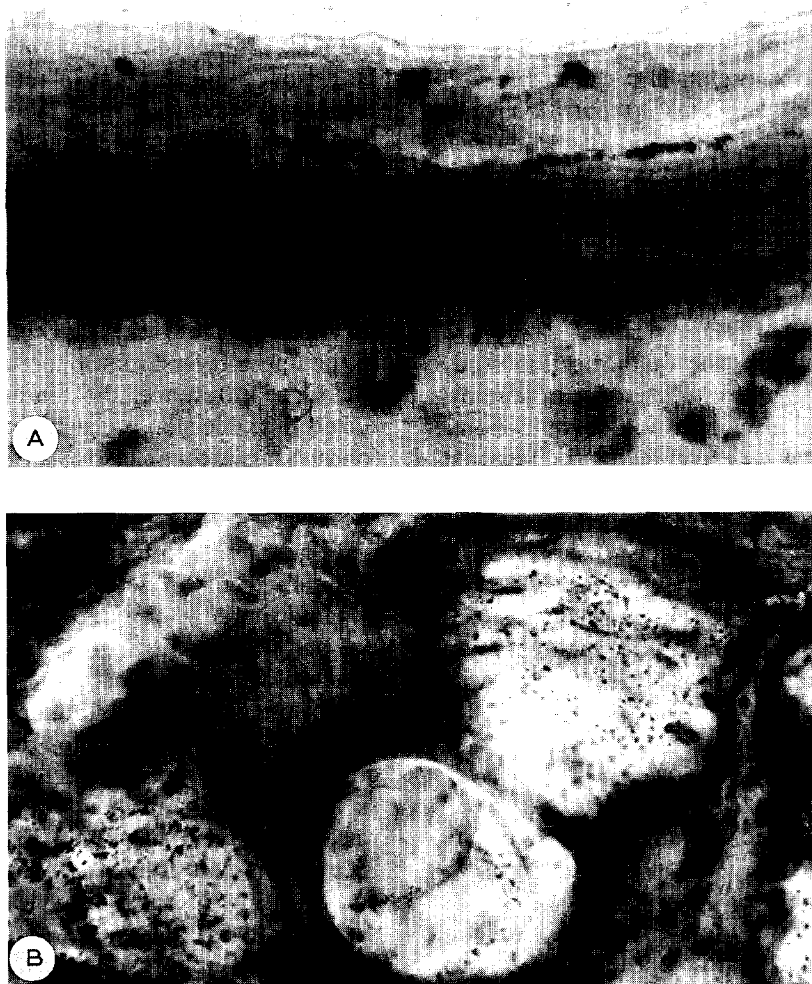

Fig. 5. (a) Accumulation of silver grains at the entry of viable epidermis. Fentanyl iontophoresis was performed for 4 h at a mean current density of 0.33 mA/cm^2 (magnifying power: ×400). (b) Accumulation of silver grains in sebaceous glands and hair shafts. Fentanyl iontophoresis was performed for 6 h at a mean current density of 0.33 mA/cm^2 (magnifying power: ×400). (c) Autoradiogram of rat skin after 1 h TRH diffusion. Presence of silver grains mainly in stratum corneum (magnifying power: ×250).

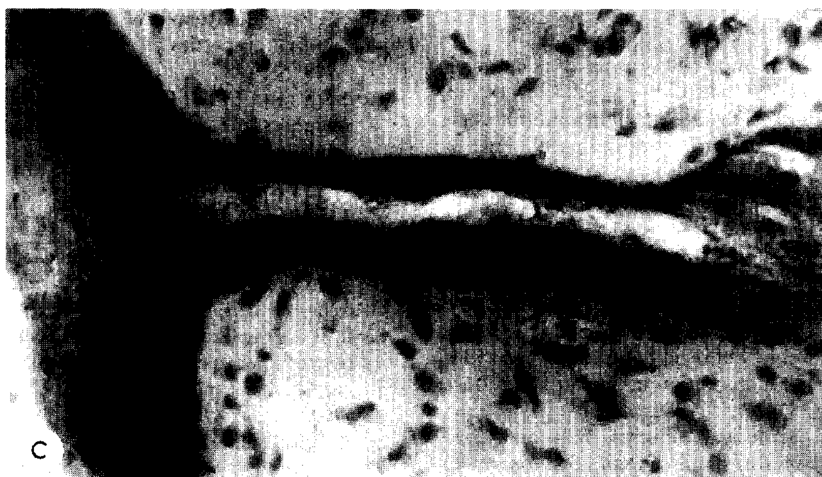

Fig. 5 (continued).

quantities and silver grains observed in stratum corneum compared to viable skin may be explained if we consider the reasoning given by Sage and Riviere (1992). It is believed that the highest concentration would be associated with the rate-limiting barrier. The barrier should consequently be the stratum corneum in this study. We could therefore imagine that the electric field propels the molecule down whatever aqueous pathways exist and, once there, the molecule tends to stay and diffuse slowly because of its relatively poor lipophilic properties. However, this theory has some limitations, since a high concentration may simply be associated with a high affinity.

The larger quantities detected in viable tissue after iontophoresis may be easily explained by Fick's first law: this equation shows a direct relation between penetration into a membrane (in this case epidermis) and donor concentration (in this case, stratum corneum concentration). Moreover, whereas by passive diffusion, fentanyl quantities decreased dramatically once in viable skin, the difference was less pronounced in TRH studies: diffusion occurred more spontaneously between stratum corneum and viable skin. In addition, the promoting effect due to electric current compared to passive diffusion was less distinct. This last fact correlates well with the lower enhancement factor observed in transdermal flux studies for TRH (57.6 ± 16.2 ng/cm^2 per h up to 695.2 ± 115.1 ng/cm^2 per h) as compared with fentanyl (from 1 ± 0 ng/cm^2 per h by passive diffusion up to 537 ± 31 ng/cm^2 per h).

5. Conclusion

In conclusion, iontophoresis increases drug concentration in the part of the skin limiting molecule permeation: viable skin for fentanyl and stratum corneum for TRH. This could at least partly explain why drug permeation is strongly enhanced after iontophoresis.

Although autoradiography allows one to localize accumulation and the route of passage, our observations tend to confirm that transepidermal penetration can take place and that an important route of penetration is the transappendageal pathway.

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