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Localization of lipophilic molecules penetrating rat skin in vivo by quantitative autoradiography

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Summary

A microcomputer-based image analysis autoradiographic method was used to characterize drug penetration profiles and drug localization in the various regions of the rat skin. By means of this quantitative autoradiographic method, it was possible to measure and visualize the effect of carriers and application time on the localization in the rat skin of two lipophilic compounds, tetrahydrocannabinol (THC) and oleic acid (OA). It was found that after 2 h, both compounds, THC and OA, show a similar profile of penetration: relatively low concentrations accumulated in the various layers of the skin. No significant difference ($p < 0.4$) was found between the concentration in the epidermis and in the appendages, either for THC or OA. The epidermis and the appendages contained twice the concentration measured in the dermis. After 24 h, a different behavior can be described for each compound. It appears that from the same carrier, Transcutol, THC has a much greater skin penetration, as well as a different localization, than OA. For THC, a concentration gradient can be observed from the epidermis through the appendages to the dermis. It appears that by increasing the time of application from 2 to 24 h, the preference of a molecule to 'choose' the penetration routes can be emphasized. It was also shown that a penetration enhancing agent such as oleic acid may affect the localization of THC in the various skin layers. The highest concentration of drug was found in the epidermis and appendages.

Introduction

In the process of drug delivery to the skin, the localization of the penetrating compounds in the skin layers and the identification of transport pathways is essential.

Recently, De Haan et al. (1989) published an elegant method for visualization of drug transport across the stratum corneum. The drug visualization is achieved by using electron microscope autoradiography. This qualitative method enables localization of the drug in the stratum corneum at a cellular level. Only a few tentative approaches to quantify the compound in the different skin layers and appendages have been reported. An important contribution to the quantitative evaluation was made by Schaefer and his colleagues (Schaefer et al., 1978; Schalla et al., 1989) which introduced the skin section method. With their

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technique, the authors obtained a concentration gradient in the skin with respect to layer depth. Since a distinction between the transepidermal and the transfollicular route of penetration is not possible from the skin sections parallel to the skin surface, the technique is supplemented by qualitative histoautoradiography. Thus, the combination of histoautoradiography and skin sections gives a quantitative determination of the drug in the different skin layers and may point towards privileged penetration routes.

Autoradiography is one of the techniques of choice for assessing localization of substances in tissues. The quantitative autoradiographic method is successfully used in brain research (Young and Kuhar, 1979; Unnerstall et al., 1981; Biegon et al., 1989).

However, most of the procedures used in skin autoradiography give only a qualitative localization and visualization of the drug in the skin layers.

The aim of the present investigation was to localize the compounds in their penetration pathways through the skin by means of a quantitative autoradiography technique using a microcomputer based image analysis. This is the first report on the use of a microcomputer-based image analysis autoradiogram in skin penetration studies. A further goal was to learn the effect of carriers on the drug distribution between the rat skin layers and appendages of two lipophilic compounds, tetrahydrocannabinol (THC) and oleic acid (OA), chosen as model molecules in the present work.

Materials and Methods

Materials

Pure Δ^8-tetrahydrocannabinol (Δ^8THC) was a gift from the laboratory of Professor R. Mechoulam (Hebrew University of Jerusalem). [^{3}H]Δ^8THC with a specific activity of 12.8 Ci/mol and a concentration of 1 mCi/ml was prepared by Kamag (Dimona, Israel). [^{3}H]Oleic acid with a specific activity of 15 Ci/mol and a concentration of 1 mCi/ml was purchased from ARC (U.S.A.). The other materials in this study: propylene glycol (PG) (BDH), polyethylene glycol 400 (PEG_{400}) (Merck), ethanol (EtOH) (Frutarom), diethylene glycol monoethyl ether (Transcutol) (Gattefosse), and oleic acid (OA) (Merck) were all analytical grade or conformed to B.P. requirements.

Animals

The animals used in the present investigation were male, Nu IOPS hairless rats (Iffa Credo, France), weighing 180–220 g.

Quantitative skin autoradiography method

The drug of interest was applied to a defined surface area (3.2 cm^2) of the rat skin for a chosen period of time and the skin was removed, sectioned, autoradiographed and stained. Quantitative measurements of the amount of drug localized in various regions of the skin were performed by a computerized image analysis system. The steps involved in this method are detailed below.

Skin samples for autoradiography

Formulations (0.8 g) containing 50 μCi of tritiated drug were applied to the dorsal region of hairless rats and covered with a Hill Top Chamber (Hill Co., U.S.A.). The duration of application varied between 2 and 24 h. The rats were then killed. The skin area of interest was removed by scalpel and scissors, cleaned with paper tissue in order to remove residual drug, immersed in Tissue Tek OCT 458 (Miles Lab.), and immediately frozen in isopentane cooled in liquid nitrogen (-130°C). The frozen blocks were sectioned transversely at -30°C in a cryomicrotome (Model TI, International Equipment Co.) into slices with a thickness of 6 μm. The sections were collected on glass slides (proper, premium microslides, pre-cleaned), and dried. The slides were introduced into cardboard X-ray film cassettes (Kodak) and covered by Hyperfilm ^{3}H 24/30 RPN-12 (Amersham, U.K.) at room temperature (23°C) under pressure. The exposure time varied from 4 to 7 weeks. The skin sections were kept together with the standards during the whole period of film exposure. Then the autoradiograms were developed, fixated, and the histological preparations were stained with haematoxylin and eosin (Baker et al., 1977).

Each autoradiogram was further photographed using a microscope camera, specially designed for photomicrography, with automatic exposure (MC63A, Zeiss). Autoradiograms and standards were photographed under the same light conditions. The corresponding skin slides of each autoradiogram were also photographed and used for the determination of the skin histology.

Standards: preparation and autoradiographic measurement

The standards were prepared by a modification of the method described by Rainbow et al. (1984) for brain tissue and autoradiography. Skin samples from the regions of the rat body, similar to those used for drug application were mashed in a blender several times until full homogenization. The mash was further homogenized (Heindolf Homogenizer RZR 2000) at 2000 rpm for 10 min. 1 g of the homogenate mass was mixed with various quantities (0.5–50 μCi) of radiolabeled material and was frozen in isopentane (-130°C) immediately after mixing. A block was obtained for each standard. Autoradiograms were prepared by the procedure which follows the steps described above for skin samples.

Several sections collected on glass slides were weighed for determination of the average wet weight of the tissue slice. A number of sections were collected into scintillation vials for dpm counting. Scintillation cocktail (Packard, U.S.A.) was added to the vials, which were then assayed using a Kontron Betamatic Scintillation Counter (Luminitron Scientific Instr.). The results are presented as mean of 5 replicates for each concentration. The remaining sections were collected on glass slides and exposed to the strip film together with the skin samples.

The drug concentration in the tissue standard was calculated from Eqn 1:

$$\frac{x \text{ mCi/g tissue}}{y \text{ mCi/mmol}} = z \text{ mmol/g} \qquad (1)$$

where x represents data from the scintillation counter in dpm translated to mCi; y is the specific activity of the tritiated compound; and z is the compound concentration per gram tissue weight.

The optical densities of the standard autoradiograms were plotted against the tritium concentration per gram tissue weight. Eqn 1 may be used for any tritiated compound with known specific activity. The calibration curve was plotted by a computerized image-analysis system. The characteristic of the system with applications for brain research are described by Isseroff and Lancet (1985) and Biegon et al. (1989). The image analysis system consists of a video camera (Model TC1005) with a 1 inch Newvidon Video Tube (RCA, Lancaster, PA), IBM-PC AT computer, television screen (Grundig), light-box designed to give equal illumination throughout the entire field, joystick (Model JS-1, Cromemco Inc.), and printer (Epson 80). The autoradiogram is placed on the light box. The video camera transfers the picture, and a software package translates the picture into the digital image, which appears on the television screen. The joystick is used to define the coordinates for densitometric measurement and to outline contours. Image acquisition, storage and display are performed by a CAT-800 System (Digital Graphic Systems, Palo Alto, CA). The system translates the grey level of each pic-

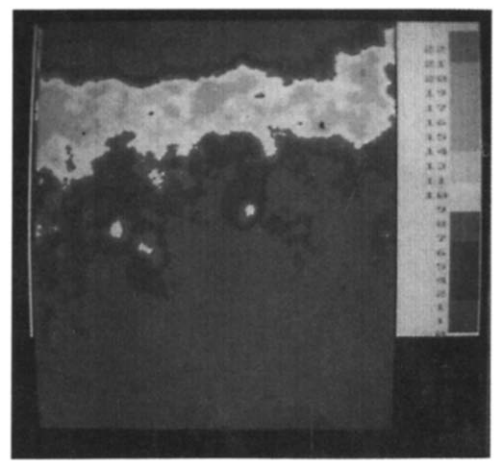

Fig. 1. Hairless rat skin pseudocolored computerized image autoradiogram after 24 h of application of 50 μCi tritiated THC in Transcutol. Color map-dark red 22 = 220 pmol/g; purple 1 = 10 pmol/g. (Color not shown here as reproduction of the figure is in black and white only.)

ture element, included in the area marked by the joystick, according to the standard curve. The biodata are averaged and printed.

By using a white/black method, it was possible to increase the contrast by weakening the bright parts of the picture and darkening the grey parts. This method of work enables recognition and better identification of specific regions in the whole picture. The use of color operation, which displays autoradiograms in pseudo-color representation of grey levels provides a more visually meaningful graduation of drug localization. The coding of grey level was selected through color mapping techniques. The map appears on the screen with each autoradiogram image.

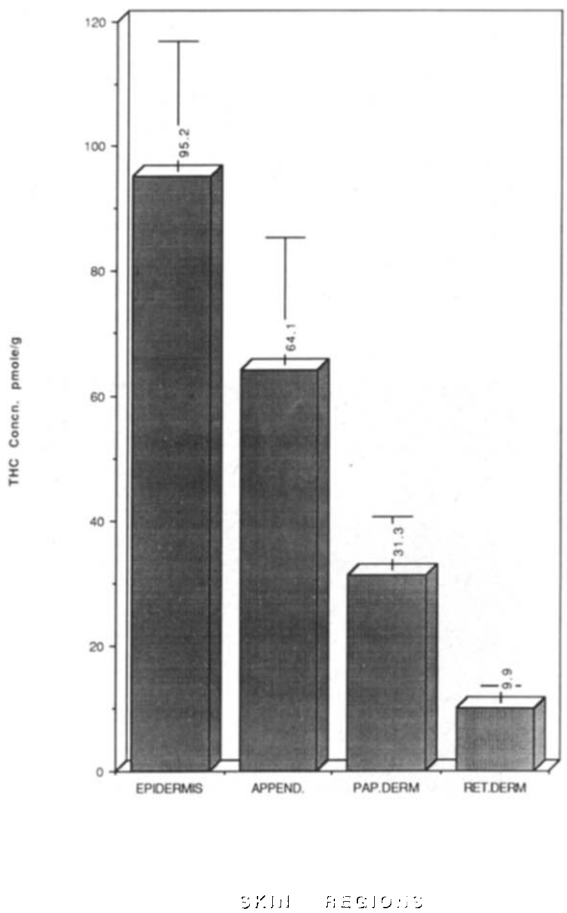

Fig. 2. Localization of THC in the various skin regions in autoradiogram presented in Fig. 1.

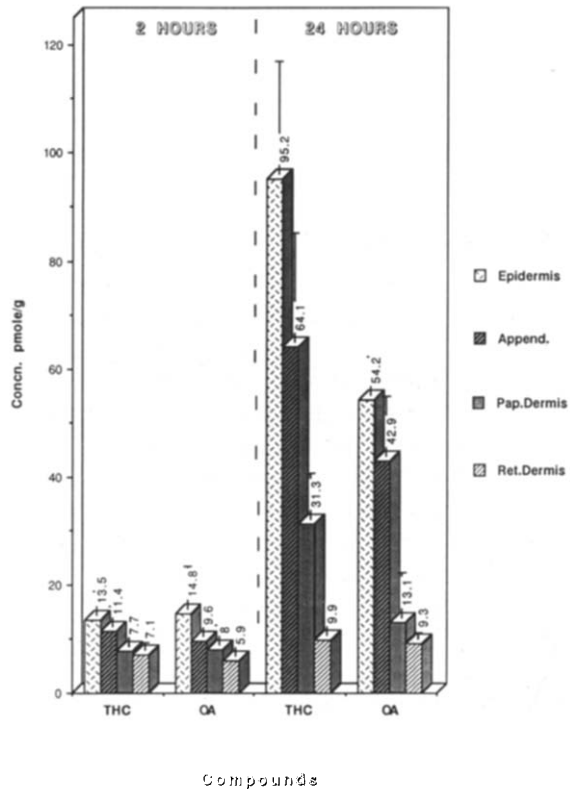

Fig. 3. Localization of THC and oleic acid (OA) in the epidermis, appendages and dermis of hairless rat skin after 2 and 24 h of application of 50 μCi tritiated compound in Transcutol.

Results and Discussion

Fig. 1 shows the pseudocolored computerized autoradiogram of the rat skin after 24 h application of 50 μCi THC in Transcutol. This figure illustrates the distribution of the radiolabeled drug among the various regions of the skin. In the pseudocolored autoradiogram, the colors represent the density of drug localization using the rainbow spectrum from purple to red for low to high drug concentrations. The computerized statistical densities in the epidermis, appendages, papillar dermis and reticular dermis are presented in the histogram in Fig. 2.

Using the computerized autoradiographic data, attempts were made to quantitatively localize penetrating compounds in their pathway through the skin from systems containing various carriers. The effect of the time of application was also studied.

The differing distributions of two lipophilic compounds, THC and OA, are given in Fig. 3 as a function of time. After 2 h, both compounds, THC and OA, show a similar profile of penetration: relatively low concentrations accumulated in the various layers of the skin. No significant differences ($p < 0.4$) were found between the concentration in the epidermis and in the appendages, either for THC or OA. The epidermis and the appendages contained twice the concentration measured in the dermis. After 24 h, a different behavior can be described for each compound. It appears that from the same carrier, Transcutol, THC has a much greater skin penetration, as well as a different localization, than OA. For THC, a concentration gradient can be observed from the epidermis through the appendages to the dermis, the values presented in Fig. 3 being statistically significant ($p < 0.005$). On the other hand, the concentration of OA localized in the epidermis is not significantly different from appendages ($p < 0.1$).

It is interesting to note that for both compounds the concentrations measured in the retic-

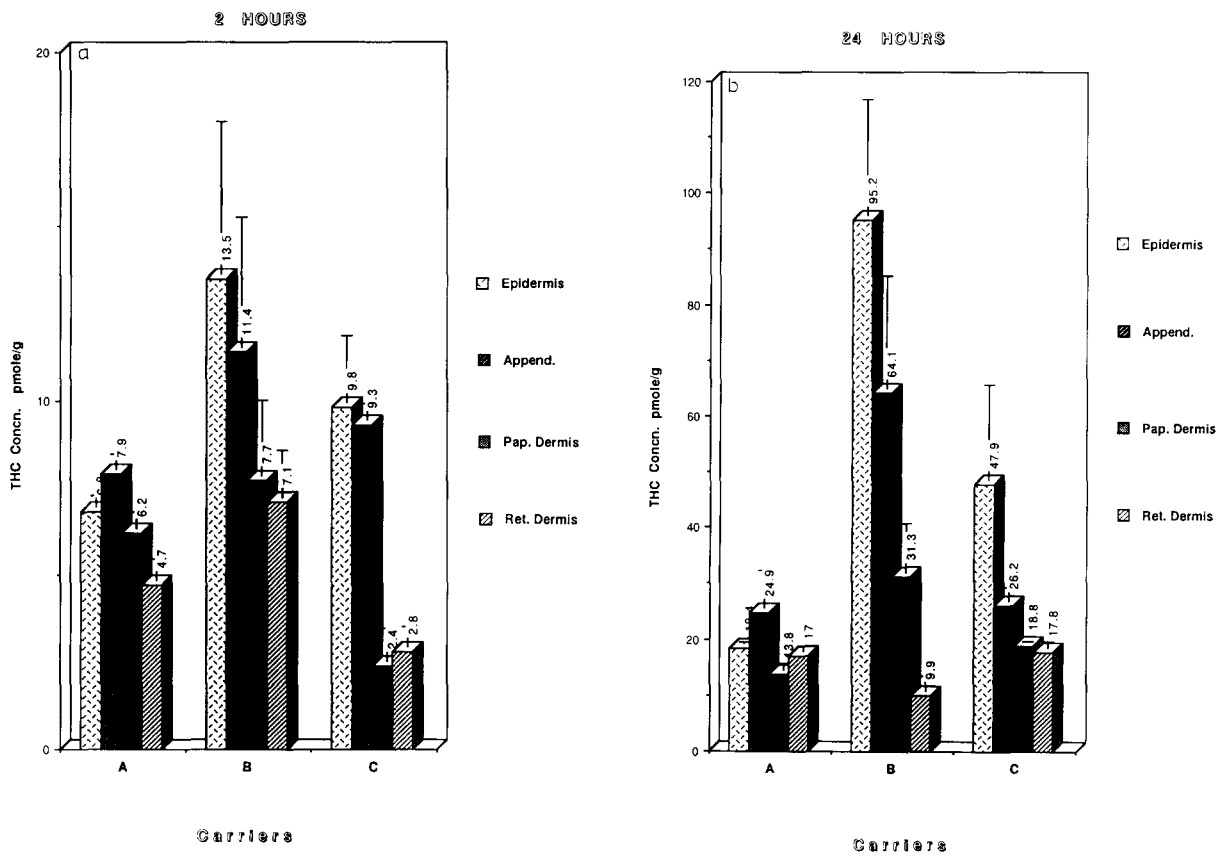

Fig. 4. a. Effect of carriers on the localization of THC in the epidermis, appendages and dermis of hairless rat skin after 2 h application. Carriers: A, PEG_{400}; B, Transcutol; C, PG:EtOH (7:3). b. Effect of carriers on the localization of THC in the epidermis, appendages and dermis of hairless rat skin after 24 h application. Carriers: A, PEG_{400}; B, Transcutol; C, PG:EtOH (7:3).

ular dermis were small, indicating that the lipophilic compounds could not easily reach this region of the skin.

From the above results, it appears that by increasing the time of application from 2 to 24 h, the preference of a molecule to 'choose' the penetration routes can be emphasized.

It was further interesting to learn whether the carrier could affect the drug pathway through the skin. For this purpose, THC was incorporated in: (1) PEG_{400}, (2) Transcutol, and (3) PG:EtOH (7:3), and applied for 2 and 24 h. The results are presented in Fig. 4a, b. After 2 h of application (Fig. 4a) the highest penetration was observed from Transcutol, and the lowest from PEG_{400}. Although the distribution among the routes of penetration is different for each carrier, there is no significant difference between the concentration of THC in epidermis and the concentration of THC in the appendages for all three systems tested. From the histogram in Fig. 4a it is clear that when applied in the PG:EtOH (7:3) mixture, the quantity of the THC which reaches the dermis (papillar and reticular) is very small, being the lowest among the three carriers.

After 24 h of application, the distribution profile of THC in the various skin layers changed for each of the three carrier systems. Fig. 4b shows that after 24 h of application, the Transcutol system had delivered the highest THC concentration to the various layers of the skin, as compared to the PG:EtOH (7:3) or PEG system; the lowest overall skin concentration was measured in the PEG system. For the PEG system, although relatively low concentrations of drug were measured in each of the regions tested (i.e., epidermis, appendages or dermis), no significant difference ($p < 0.4$) was found between them. This indicates that in 24 h the delivery of the drug to the epidermis was relatively low. Moreover, the drug was cleared from the epidermis, and a concentration was built up in the dermis. With the PG:EtOH (7:3) system, after 24 h, the epidermis held the highest THC concentration, and the dermis remained the skin region in which the drug concentration was the lowest, although the drug concentration even there increased 7-fold relative to that measured after 2 h.

For the Transcutol system (Fig. 4a, b), the increase in application time from 2 to 24 h did not change the general distribution profile; however, the concentration gradient was sharper. For example, the concentration in the reticular dermis was 10 times larger than that in the epidermis. The Transcutol system appears to deliver the highest concentration of THC to the epidermis, appendages, and papillar dermis.

In a previous investigation (Touitou et al., 1988), it was shown that in the presence of oleic acid, the skin permeability of hairless mouse skin to THC in vitro increased by a factor of 6. Fig. 5 illustrates the effect of OA (6% w/w) on the localization of THC in the various skin layers after 2 h of application on hairless rat in vivo and delivered from PG:EtOH (7:3). These results indicate a 6-fold increase in THC concentration in the epidermis and approximately a 3-fold in-

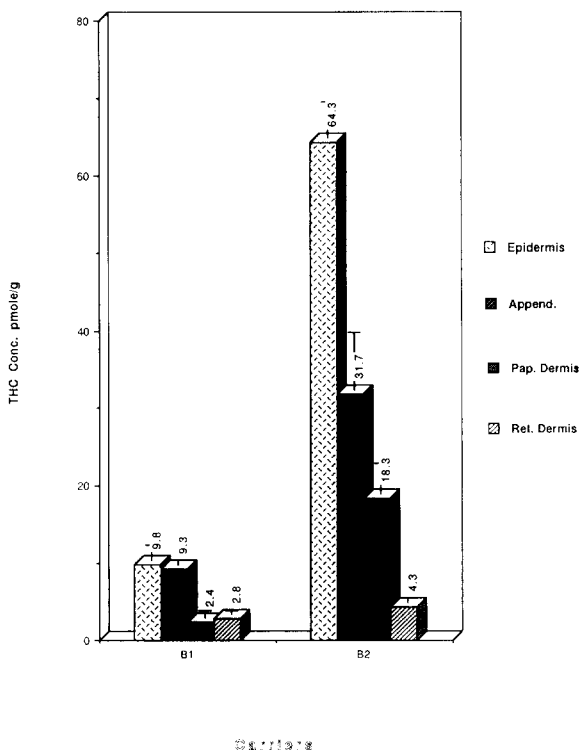

Fig. 5. Effect of oleic acid on the localization of THC in the epidermis, appendages and dermis after 2 h of application of 50 μCi tritiated drug. Carriers: B1, PG:EtOH (7:3); B2, 6% oleic acid in PG:EtOH (7:3).

crease in the appendages. These data conform well with the previously reported results. Moreover, by using the autoradiographic method, it can be seen that the presence of oleic acid in the delivery system applied to the skin, changed the distribution profile of the drug in the skin, as can be observed in histograms B1 and B2 in Fig. 5, where the concentration gradient between the epidermis and the reticular dermis increased sharply.

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