

Oxidative Degradation of a Drug during the Course of Diffusion Across the Skin

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Degradation of a compound with a hydroxyl group during the course of its diffusion across the skin was investigated. Based on the experimental findings of a shortened retention time of a degradant peak from post-diffusion samples and from the ability to evaporate radioactivity from such samples, it seems that during diffusion the parent compound degrades into a more hydrophilic product which is then oxidized. A tritium label at the carbon with a hydroxyl group was released as a tritiated water. When the post-diffusion samples were left open to the air allowing evaporation of water, there was a corresponding decrease in radioactivity of such samples. There was a linear relationship between the time left open and the fraction of radioactivity lost. When such samples were fractionated by HPLC, and then had their radioactivities measured by scintillation counting, two peaks were identified. The first peak, which may be attributable to tritiated water, was eluted at the same retention time as the solvent front. The second peak eluted at the retention time of the parent compound. When the evaporation/loss of radioactivity experiment was repeated using a ^{14}C -labeled compound there was no significant loss of radioactivity, indicating that the earlier loss with ^3H -labeled compound was related to the formation and loss of tritiated water.

Key words : Oxidative Degradation, Skin, Metabolism, Diffusion, Oxidation

INTRODUCTION

Over the past decade, transdermal drug delivery has been a subject of tremendous interest. Due to the inherently low permeability of the skin, many investigators have used radio-labeled compounds in percutaneous absorption studies, based on the ease of detecting such compounds and on the low detection limit achievable when using liquid scintillation counting as the method for analysis. However, scintillation counting can not differentiate metabolites and/or degradates from the parent compound, therefore, results from scintillation counting can sometimes be misleading. Using samples from a leucine-enkephalin percutaneous penetration study (Choi *et al.*, 1990), Choi *et al.* reported that there was a tremendous difference in detected penetrant when using scintillation counting versus HPLC assay as the method of analysis. Furthermore, it was asserted that this difference in the amount detected was due to enzymatic metabolism. Enzymatic metabolism of penetrants during the course of their diffusion across various skins has been studied

by many other investigators (Storm *et al.*, 1990; Guzek *et al.*, 1989; Potts *et al.*, 1989).

Since automating the diffusion apparatus with Franz-type flow-through diffusion cells and a fraction collector, it was observed that the radioactivity of certain samples was unusually low. All samples were collected in glass scintillation vials which were periodically capped in groups, as the fraction collector continuously advanced throughout the experimental period. It was found that the longer a sample was left open, the more loss of radioactivity. It was postulated that evaporation of a portion of the sample occurred and that this evaporation event may be related to the loss of radioactivity with time. It was theorized that oxidation of the permeant occurred as it diffused across the skin, wherein the tritium label was released to a water molecule. If the collected samples were left open and exposed to air then such tritiated water would have been free to evaporate. Thus any differences in radioactivity between collected samples where evaporation was free to occur and samples where evaporation was nearly completely hindered, would provide evidence to support that the drug degraded and lost tritium to the water. The present study is aimed at testing the above hypothesis and clarifying the mechanism of this degradation.

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MATERIALS AND METHODS

Materials

Glycerol formal was obtained from Sigma Chemical Co. (St. Louis, MO) and was used as received. All other chemicals were reagent grade and were used without further purification. The purity of radio-labeled compounds were checked by TLC and HPLC and was found to be greater than 99.0%.

Diffusion Experiments

An automatic flow-through diffusion cell system was used in this study, consisting of: a multi-channel peristaltic pump, a fraction collector, and vertical Franz-type diffusion cells. Samples were collected continuously at about 0.5 ml/hr. Each sample was collected for a 4 hr period. Full thickness cattle skin was obtained at the time the animal was sacrificed, which was immediately stored in a freezer at -20°C . This full thickness skin was thawed at room temperature and was used after removing subcutaneous fat with a dermatome and scalpel. The skin was mounted to each cell so that the epidermal side faced the donor cell and the dermal side faced the receiver cell. Constant temperature was maintained in each cell by circulating 37°C water through the outer jacket of each cell. A solution of 25% glycerol formal in water was used as the receiver cell medium. The selection of this medium was based on glycerol formal's ability to increase the solubility of the permeant in an aqueous solution, which maintained a sink condition. The contents of each receiver cell were stirred by externally driven, teflon-coated, magnetic bars to maintain continuously uniform samples. Thirty to sixty minutes after mounting skin sections on each diffusion cell, all remaining air bubbles were removed. A 100 μl volume of radioactive formulation was applied on the exposed outer surface of the skin. Depending on the study protocol, sample vials were either filled with scintillation cocktail or left empty prior to sample collection.

Assay Method

The permeant was analyzed on an Shimadzu HPLC system consisting of an automatic injector (SIL-6B), a UV detector (SPD-6AV), a high-performance pump (LC-600) and a data processor (C-R4A). A reversed phase column (Zorbax RX-C8) was used. Column temperature was maintained at 35°C using a thin foil temperature controller (CH1445, SYS-TEC Inc.). The mobile phase used consisted of acetonitrile/water (80/20) with 0.1% phosphoric acid and 0.1% triethylamine. All radioactive samples were analyzed with a liquid scintillation counter (Model 2500TR, Packard Instruments Co., Downers Grove, IL).

Data Reduction

The following equation was used to calculate the amount of the compound permeated (Choi *et al.*, 1994). When radio-labeled compound was used, the amount of permeant was calculated based on the fraction of the radio-labeled compound permeated.

$$M_n = C \times V + \frac{S_n}{2} + \sum_{i=1}^n S_i \quad (\text{when } n \geq 2)$$

$$M_n = C \times V + \frac{S_n}{2} \quad (\text{when } n=1)$$

where M_n : cumulative amount permeated
 C: concentration in the receiver cell
 V: volume of the receiver cell
 S_n : total amount in the nth sample

RESULTS AND DISCUSSION

Three groups were created for this study. To see the effect that evaporation had on the loss of radioactivity, the samples from the first group were collected in scintillation cocktail-filled vials, (evaporation protected). The sample composition (25% glycerol formal in water) was miscible with the scintillation cocktail (Ultima-Flo M, Packard Chemicals), however, the sample and the scintillation cocktail would not mix until the sample vial was capped and shaken. Instead, two distinct layers would form before shaking with the more dense aqueous sample resting on the bottom of the vial and the scintillation cocktail above it (creating an effective barrier between the sample and the air). A second group of samples were collected in vials without scintillation cocktail, (free to evaporate); these samples were left uncapped for a certain time period allowing for evaporation, following which they were mixed with scintillation cocktail and capped. Finally, the same formulation without any added radioactive compound was used in a third group in the experiment. These samples were collected in vials without cocktail and were analyzed by HPLC. Fig. 1 compares the cumulative amount (μg) of the compound permeated as a function of time. The group using scintillation cocktail-filled vials shows the highest cumulative amount permeated and the group assayed by HPLC shows the lowest cumulative amount permeated. The substantial difference in permeation, between the group where the scintillation cocktail was added before sampling and the group where the scintillation cocktail was added after sampling, clearly indicates that a large portion of the radioactivity in the latter samples was lost while they were left open to the air. It is likely that the loss of radioactivity, in the group without cocktail added initially, was related to evaporation of the sample, since evaporation of the other samples was minimized by the addition of scintillation cocktail pri-

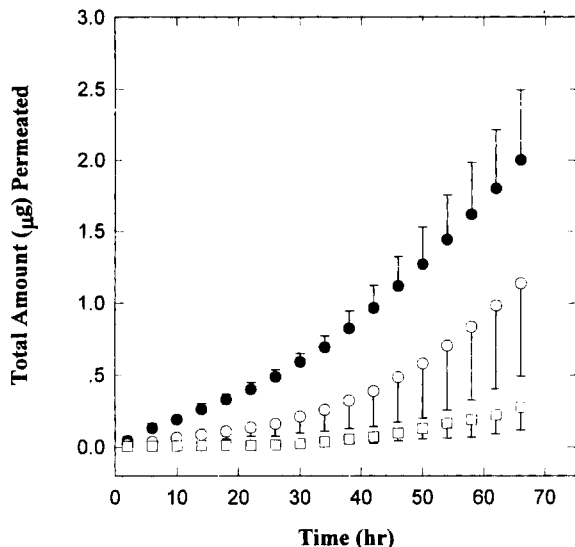


Fig. 1. Total amount (μg) of the permeant as a function of time when measured by different methods of analysis. Each point represents average of 6 measurements and the error bar shows standard deviation. Key: closed circle; samples collected in scintillation cocktail filled vial and analyzed by scintillation counting, open circle; samples collected in empty vial and analyzed by scintillation counting, open square; samples analyzed by HPLC.

or to their collection. As mentioned previously, the cocktail and the more dense medium formed two phases until mixing: an upper layer of cocktail and a lower layer of receiver cell medium. Thus, it was found that filling the vials with scintillation cocktail prior to the collection of samples resolved the problem with radioactivity loss due to evaporation, however, the extent of the degradation was still not known. The discrepancy between scintillation counting and HPLC assay indicates that the radioactivity measured did not solely result from parent compound, but instead from a combination of parent compound and one or more degradants. In the preliminary study, the parent compound was stable in the receiver cell medium for the time period used in the diffusion study, therefore, two possible degradation pathways are likely during the course of diffusion: chemical degradation and/or enzymatic degradation.

To further investigate the loss of radioactivity with time, the fraction of radioactivity lost was plotted vs. the time that each sample was left open to the air (Fig. 2). The fraction lost was obtained by dividing the difference between the average dpm/ml of samples with and without cocktail by the average dpm/ml of samples with cocktail at each time point. It is clear from the figure that the fraction lost is linear as a function of time with a correlation coefficient of 0.96. It is likely that the evaporation rate is also linear with time. This finding provides additional evidence

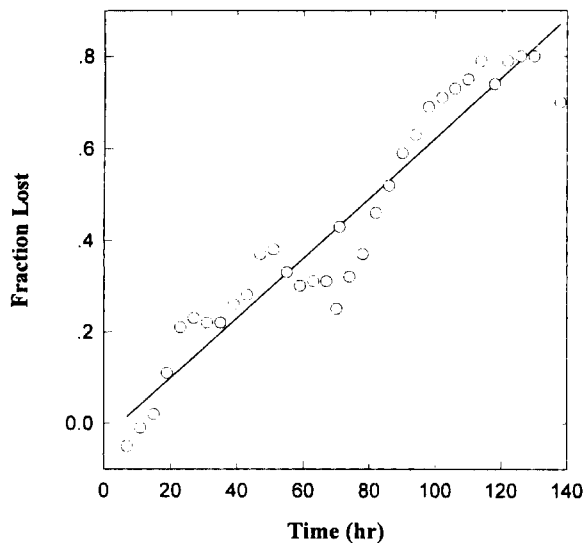


Fig. 2. Fraction of radioactivity lost as a function of time when each sample was left open to the air.

that the loss of radioactivity is directly related to the extent of evaporation of a sample, with water being the most likely component of the sample to undergo evaporation.

Fig. 3 compares the permeant concentrations of each group. Again, the concentration of the permeant projected from radioactivity may not represent the concentration of the parent compound alone. This plot shows trends that are similar to those found in Fig. 1.

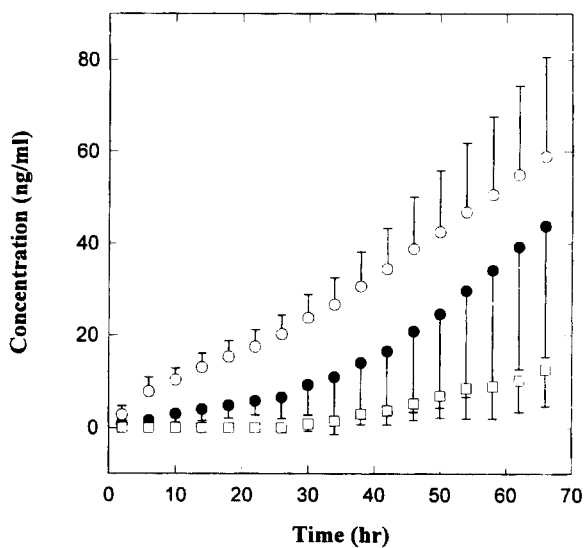


Fig. 3. Concentration of the parent compound when measured by different methods of analysis. Each point represents average of 6 measurements and the error bar shows standard deviation. Key: open circle; samples collected in scintillation cocktail filled vial and analyzed by scintillation counting, closed circle; samples collected in empty vial and analyzed by scintillation counting, open square; samples analyzed by HPLC.

The detection limit of the HPLC assay was less than 3 ng/ml. However, HPLC assay of the non-radioactive group did not show a detectable parent compound peak until the 30 hr diffusion sample was assayed, differing from the average calculated concentration (a value that was obtained from converting radioactivity measurements from the radio-labeled groups) which exceeded 3 ng/ml after only the 6 hr interval. It is notable that the ratio of the concentrations obtained from HPLC assay to those from radioactivity measurements increased from essentially zero in the earlier phase to a fraction of ca. 21% at the end of the experiment. The ratio of zero for the first half of the experiment implies that all permeant concentrations calculated from radioactive counts, from time 0 to time 30 hr, were due mostly to degradants. Furthermore, only from time 30 hr to the end of the experiment does one begin to see increasing detectable concentrations of parent compound from HPLC, meaning that the portion of permeant concentrations due to degradants is decreasing with time and the portion of permeant concentrations due to parent compound is only then beginning to increase. This implies that the larger fraction of the permeant is degraded in the earlier phase and/or the degradation process is saturable.

To identify the major degradate(s), the receiver cell medium remaining in a diffusion cell at the end of the experiment was injected into the HPLC and the eluted fractions were collected. In addition, a standard solution was also injected into the HPLC. This solution was made by diluting hot formulation with receiver cell medium. Injecting the standard solution allows for the comparison of chromatograms of drug that underwent cattle skin permeation to drug that did not undergo permeation. A total of 0.4 to 0.5 ml of the medium was injected and eluted fractions were collected for 30~60 sec intervals, depending on the time after injection. The samples were collected for up to 18 minutes. Each fraction was mixed with scintillation cocktail and counted. Each point in Fig. 4 represents the dpm of each fraction for both the receiver cell samples and that of the standard solution. Two separate samples were prepared from receiver cell medium and loaded onto HPLC separately. Two peaks were identified from the receiver cell samples. The first peak was collected from 2.5 to 3.5 min., which coincides with the retention time of the solvent front. The second peak was collected from 6 to 7 min., which coincides with the retention time of the parent compound. Less than 0.2 min. was elapsed from the detector response point to the collection of the eluent. The first peak accounted for about 70% of the total radioactivity and the second peak 30%. When water from the same sample was almost completely evaporated under nitrogen, about 50% of the total radioactivity was lost. This indicates that most of

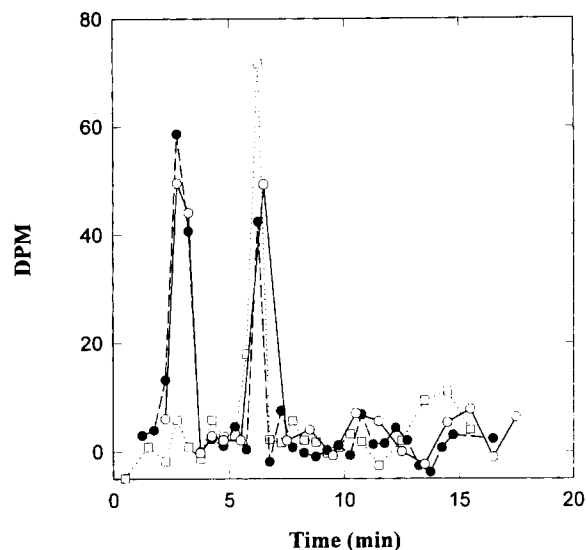


Fig. 4. Radioactivity of each sample fractionated by HPLC. Each fraction was collected every 30 to 60 sec interval depending on the time after injection. Standard solution was made by diluting the formulation with the receiver cell medium. Key: open square; standard solution, open and closed circle: receiver cell sample.

the radioactive eluent of the first peak was easily evaporable under nitrogen at about 40°C. These results are all consistent with the hypothesis that the compound was metabolized or degraded during the course of diffusion.

In order to further confirm that tritium was lost by way of evaporation, a diffusion study was conducted using a compound that had both a tritium label and a C-14 label. A sample was taken after ca. 48 hours of diffusion. The sample was divided in half and the radioactivity was measured before (first half) and after partial evaporation (second half) under nitrogen at room temperature. The evaporated sample was reconstituted with receiver cell medium to its original volume before evaporation. As before, the evaporated and the non-evaporated samples were injected into the HPLC and the eluents were collected into fractions which were counted on the liquid scintillation counter. When counting for tritium, the first peak showed an ca. 32% reduction in radioactivity after evaporation, while the second peak did not show any significant change (Fig. 5). When counting these eluent fractions for C-14 activity, both the first and the second peak did not show any significant change after evaporation (Fig. 6). This indicates that a portion of the first peak (when counting for tritium) was lost by evaporation, and also that the tritium label was cleaved from the unevaporable parent compound becoming an evaporable moiety that resided in water. At the same time, the C-14 label remained attached to the parent compound (or some portion of it) and accordingly remained unevaporable.

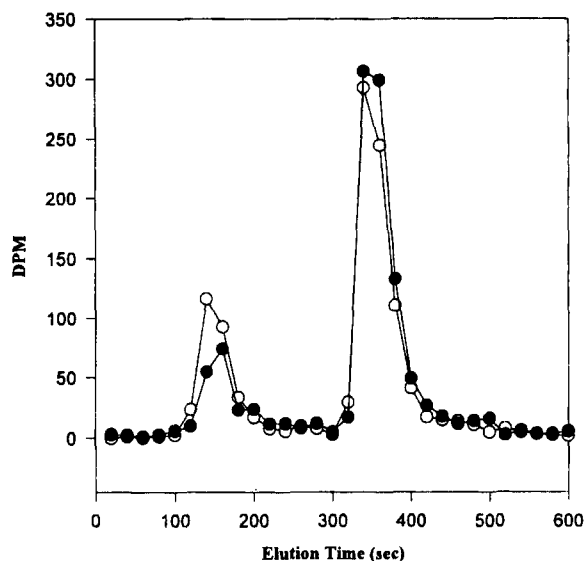


Fig. 5. Comparison of radioactivity (tritium) of HPLC fraction before and after partial evaporation. The evaporated sample was reconstituted with the receiver cell medium before the injection into HPLC. Key: open circle; fractions before the evaporation, closed circle; fractions reconstituted after evaporation.

If water accounts for a portion of the first peak (solvent front) then the evaporation of water should only effect tritium activity (Fig. 5), since the activity in water was not counted by the C-14 analysis (Fig. 6). Stated according to our theory: when the C-5 position where the hydroxyl group is attached was oxidized the tritium label was transferred to water with a corresponding decrease in measured radioactivity following evaporation, while, the C-14 label was retained with a corresponding constant level of radioactivity following evaporation. Therefore, the radioactivity of the first peak (when counting for tritium) was composed of tritiated water and one or more unknown degradates, while the first peak (when counting for C-14) consisted only of one or more unknown degradates and no compound with the C-14 label present in the water component. In addition, a change in the ratio of the first peak to the second peak before and after evaporation, respectively, indicated that tritiated water was lost by evaporation. For the tritium measurement this ratio changed from 40% to 24%, while the C-14 ratio changed from 26% to 22%; a significant reduction in the ratio when tritium was counted and no significant change in the ratio, as well as total radioactivity, when C-14 was counted. Thus, evaporation had no effect on C-14 counting.

On the basis of the fact that at least some of the degradates were easily evaporable and the retention time of the first radioactive peak coincided with the HPLC solvent front, it is likely that the tritium label at

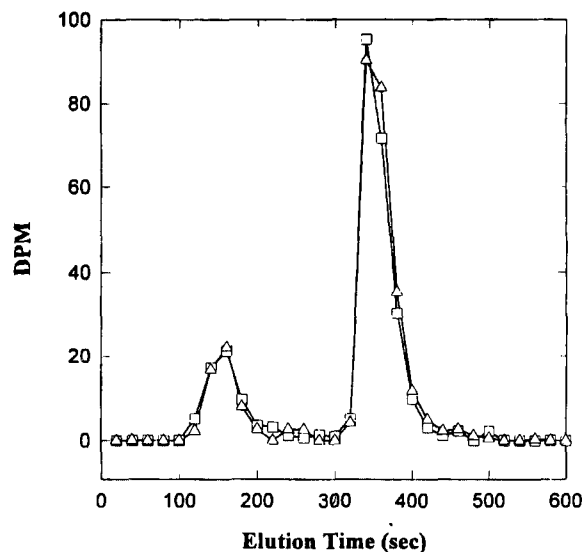


Fig. 6. Comparison of radioactivity (C-14) of HPLC fraction before and after partial evaporation. The evaporated sample was reconstituted with the receiver cell medium before the injection into HPLC. Key: open square; fractions before the evaporation, open triangle; fractions reconstituted after evaporation.

the C-5 was released as tritiated water when the C-5 was oxidized. However, the retention time of the degradate was shorter than the parent compound, indicating that the degradate was more polar. It can be speculated from the retention time that the compound was first degraded into more hydrophilic compound and then oxidized at the 5-position later. In the preliminary experiment, it was demonstrated that tritium exchange with water was very slow and the radio-label was stable in an ethanol stock solution. It was also demonstrated that the permeant had good chemical stability in the applied formulation. At this point, it is not clear whether it undergoes chemical degradation or enzymatic degradation. Further study is required to elucidate the mechanism of this degradation.

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