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# Cutaneous metabolism of nitroglycerin in viable rat skin in vitro

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**Summary**

Percutaneous penetration and metabolism of nitroglycerin (GTN) in vitro were compared in viable rat skin (perfused with minimal essential medium (MEM)) and nonviable rat skin (perfused with phosphate-buffered saline (PBSA)) in flow-through diffusion cells. The fraction of nitrates penetrating as dinitrate metabolites was significantly greater in MEM-perfused skin than in PBSA-perfused skin, while the fraction of nitrates penetrating as GTN was significantly less. Moreover, differences between MEM and PBSA in the fraction of absorbed nitrates present as GTN coincided in time with the loss of metabolizing capacity in PBSA-perfused skin, supporting the notion that it was the loss of metabolic capacity which led to an increase in GTN penetration. Over the 24 h experimental period, 29% of the GTN penetrating the MEM-perfused skin was metabolized and 71% of the absorbed dose was present as GTN, whereas only 14% of GTN penetrating PBSA-perfused skin was metabolized and 86% of the absorbed dose was present as GTN. These results demonstrate that metabolic viability of skin in vitro can affect not only the degree of cutaneous metabolism of penetrating compounds but also their rates of penetration.

The effectiveness of transdermal devices is due largely to their ability to deliver drugs into the general circulation at a controlled, steady rate and to avoid first-pass clearance by the liver. Transdermal delivery of drugs, however, does not completely avoid first-pass metabolic processes, since the skin, like the liver, has the ability to biotransform many compounds (Pannatier et al., 1978). Indeed, substantial cutaneous metabolism of transdermally delivered nitroglycerin (GTN), a drug which is very rapidly cleared from the bloodstream, has been demonstrated in Rhesus monkeys in vivo (Wester et al., 1983) and in mouse and human skin up to 6 h after application in vitro (Santus et al., 1987).

As suggested in the theoretical works of others (Ando et al., 1977a,b; Fox et al., 1979; Hadgraft, 1980; Guy and Hadgraft, 1982), it seems possible that a marked degree of cutaneous metabolism, such as that of GTN, may have a separate and significant impact on the amount of topically applied parent compound penetrating the skin. Consequently, when percutaneous penetration is as-

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sessed in vitro, it may be critical to maintain not only the barrier properties of the skin but also its metabolic viability. This has not ordinarily been done in in vitro penetration studies.

One of the most commonly used receptor fluids in in vitro percutaneous penetration studies has been phosphate-buffered saline (PBSA). It has recently been demonstrated, however, that both glycolytic and xenobiotic metabolic activity of skin perfused with PBSA shows a steady decrease over 12 h and finally a complete loss of activity. In contrast, minimal essential medium (MEM, Earle's salts) maintains glycolytic and xenobiotic metabolic activity for 24 h or more (Collier et al., 1989). In this experiment, penetration and metabolism of GTN were determined in skin perfused with MEM and compared to penetration and metabolism of GTN in skin perfused with PBSA in order to assess the effect of maintaining metabolic viability of skin in vitro on the quantification of GTN penetration.

Intact skin specimens were prepared and flow-through diffusion cells were assembled as previously described (Bronaugh and Stewart, 1985, 1986). One female fuzzy rat (obtained from the Skin and Cancer Hospital, Temple University, Philadelphia, PA), 3–5 months of age, was killed by $CO_2$ asphyxiation. Back skin was removed, a dermatome section of 200 $\mu$m was prepared, and eight circular skin specimens were punched out and assembled into diffusion cells, allowing for 0.32 $cm^2$ of exposed surface area. MEM containing 10% fetal calf serum (FCS) (Flow Labs, McLean, VA) and gentamycin sulfate (Sigma, St. Louis, MO) at 50 mg/l and continuously gassed with 95% $O_2$ : 5% $CO_2$ was pumped (Manostat peristaltic pump, New York, NY) at a rate of 1.5 ml/h beneath the skin in four of the diffusion cells. PBSA (pH 7.4) was pumped beneath the skin in the remaining four diffusion cells, also at a rate of 1.5 ml/h. Skin surface temperature was maintained at 32°C with a circulating water bath. Excess 2% nitroglycerin ointment (Nitrostat, Parke-Davis, Ann Arbor, MI; mean dose $\pm$ S.E. of $15.5 \pm 0.8$ mg (equivalent to $971.1 \pm 51.3$ $\mu$g GTN/$cm^2$)) was applied to the surface of each specimen and the diffusion cell was covered with Parafilm (American Can Co., Neenah, WI). Receptor fluid was collected at 3-h intervals over 24 h. When either receptor medium, freshly prepared and spiked with GTN, remained at room temperature for periods of 24 h or longer, significant amounts of both 1,2- and 1,3-dinitrate (10–15%) formed in the media. When the media were frozen immediately after addition of GTN, no dinitrates formed. Therefore, fractions were frozen and stored at 0°C as soon as possible after collection.

Receptor medium fractions were thawed and extracted with 3.0 ml of *tert*-butyl methyl ether (t-BME) containing internal standard (*o*-chloronitrobenzene). The extracts were then diluted 1 : 5 with t-BME, and 1.0 ml of each was dried with approx. 500 mg of anhydrous sodium carbonate. GTN and its metabolites, 1,2-glyceryl dinitrate and 1,3-glyceryl dinitrate, were quantified by the gas chromatographic method of Carlin et al. (1988). Recoveries of small amounts of nitrates, equivalent to experimentally observed amounts (about 5.0 $\mu$g of GTN/ml and 0.2 $\mu$g of glyceryl dinitrates (GDN)/ml), were 92–96% from MEM and 100–105% from PBSA.

To examine whether partitioning of GTN between skin and MEM differed from that between skin and PBSA, concentrations of GTN in PBSA and MEM (2.5–250.0 nmol/ml; 0.6–60.0 $\mu$g/ml) were compared after 24 h equilibration with an equal volume of *n*-hexane. No differences were observed, suggesting that GTN has an equal tendency to partition from the skin into either PBSA or MEM.

To examine whether glutathione-*S*-transferase, the enzyme responsible for GTN metabolism (Habig et al., 1975), would leach into receptor fluid and contribute to the formation of dinitrate metabolites, a separate skin specimen was perfused with MEM for 7 h. Next, 4.0 $\mu$g (17.6 nmol) of GTN was added to the 7-h collection fraction, which was then incubated for 1 h at 37°C and analyzed for the presence of dinitrates. Very little dinitrate formation was observed (0.04 $\mu$g or approx. 1% of the added amount), suggesting that dinitrates present in the receptor fluids perfusing the GTN-treated skin specimens were present as a result of GTN metabolism during its penetration and not as a result of the leaching of glutathione-S-transferase from skin into receptor media.

Steady-state rates of nitrate penetration were achieved by the 6 h collection period for skin perfused with both PBSA and MEM. No significant difference existed in the total amount of nitrates (GTN and metabolites) present in the MEM and PBSA fractions. However, as illustrated in Fig. 1, the percent of total nitrates present as GTN was significantly less in MEM than in PBSA (two-way ANOVA, repeated measures, $p = 0.01$). Analysis of differences between means revealed that significantly smaller fractions of total nitrates were present as GTN in MEM than in PBSA during each collection period after 9 h. Moreover, as illustrated in Fig. 2, the percent of total nitrates present as either 1,2-GDN or 1,3-GDN was significantly greater in MEM than in PBSA (two-way ANOVA, repeated measures, 1,2-GDN, $p < 0.01$; 1,3-GDN, $p = 0.01$). Mean values for both metabolites in MEM were significantly greater than mean values in PBSA at every collection period after 9 h.

Thus, penetration of GTN was significantly lower and the metabolite formation was significantly greater in skin perfused with MEM, which maintained metabolic viability over the entire 24 h experimental period, compared to skin perfused with PBSA, which did not. Additionally, there was a coincidence in time of the significant differences between MEM and PBSA in the fraction of absorbed nitrates present as GTN and metabolites with the decrease in viability of PBSA-perfused skin previously observed (Collier et al., 1989). Only when metabolism was sufficiently compromised by perfusion with PBSA (by the 12 h collection period) did significant differences between MEM-perfused and PBSA-perfused skin emerge. The metabolic status of the skin thus has an effect not only on the amount of dinitrates formed but also on the amount of GTN penetrating skin in vitro.

Over the total 24 h period, 71% of the nitrates penetrating the MEM-perfused skin were present as GTN and 29% were present as dinitrate metabolites, whereas 86% of the nitrates penetrating the PBSA-perfused skin were present as GTN and 14% were present as dinitrate metabolites. The total fraction of absorbed GTN metabolized in MEM-perfused skin is similar to previous

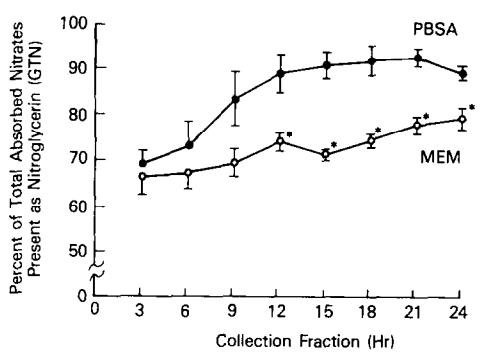


Fig. 1. Percent of total absorbed glyceryl nitrates present as nitroglycerin (GTN) in either MEM- or PBSA-perfused fuzzy rat skin in flow-through diffusion cells treated with 2% nitroglycerin ointment (Nitrostat, Parke-Davis) (mean ± S.E.). * Significantly different from corresponding PBSA mean, $p < 0.05$.

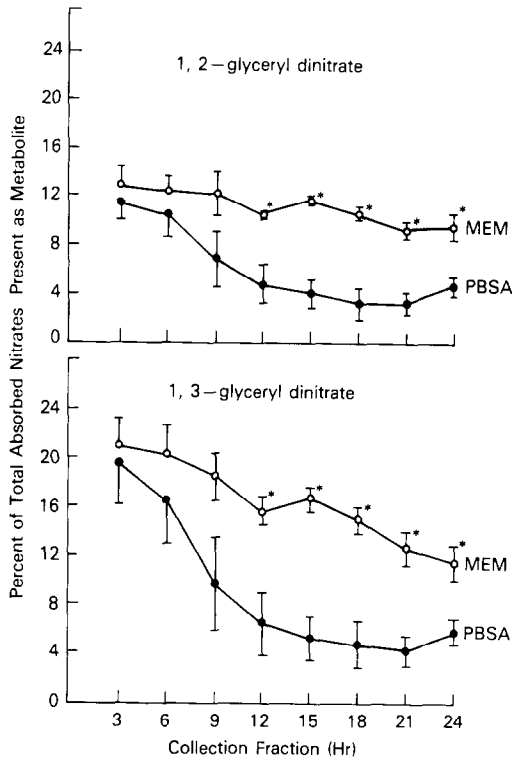


Fig. 2. Percent of total absorbed glyceryl nitrates present as nitroglycerin dinitrate metabolites in either MEM- or PBSA-perfused fuzzy rat skin in flow-through diffusion cells treated with 2% nitroglycerin ointment (Nitrostat, Parke-Davis) (mean ± S.E.). (top) 1,2-Glyceryl dinitrate (1,2-GDN); (bottom) 1,3-glyceryl dinitrate (1,3-GDN). * Significantly different from corresponding PBSA mean, $p < 0.05$.

TABLE 1

*Estimates of cutaneous metabolism of transdermal nitroglycerin*

| Experimental subject | Percentage of transdermal nitroglycerin metabolized by skin |
|---|---|
| Fuzzy rat, PBSA-perfused [a] | 14 ± 4 |
| Fuzzy rat, MEM-perfused [a] | 29 ± 4 |
| Rhesus monkey [b] | 16, 21 |
| Human [c] | 24, 32 |

[a] Values are means ± S.E. from four skin specimens.

[b] Wester et al. (1983); values are means and were derived from bioavailability determinations based on measurements of plasma (21%) or urine (16%) radioactivity after topical and intravenous administration of [$^{14}$C]nitroglycerin in three different monkeys (S.E. values not reported).

[c] Nakashima et al. (1987); values were derived from clearance rates of dinitrate metabolites from plasma after topical and intravenous administration of nitroglycerin in two different human subjects.

estimates based on in vivo observations in monkeys and humans as shown in Table 1.

These results emphasize the importance of using a receptor medium which maintains metabolic viability of skin over the entire experimental period when assessing percutaneous penetration and/or metabolism in vitro. Not only metabolism but also penetration estimates may be compromised if viability is not maintained.

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