Cutaneous Metabolism of Nitroglycerin *in Vitro*. I. Homogenized Versus Intact Skin

Naruhito Higo,[1,2] Robert S. Hinz,[1] David T. W. Lau,[1] Leslie Z. Benet,[1] and Richard H. Guy[1,3]

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The metabolism of nitroglycerin (GTN) to 1,2- and 1,3-glyceryl dinitrate (GDN) by hairless mouse skin *in vitro* has been measured. In the first set of experiments, GTN was incubated with the 9000*g* supernatant of fresh, homogenized tissue in the presence and absence of glutathione (GSH), a cofactor for glutathione-*S*-transferase. After 2 hr of incubation with GSH, 30% of the initially present GTN had been converted to 1,2- and 1,3-GDN; without GSH, less than 5% of the GTN was metabolized. The ratio of 1,2-GDN to 1,3-GDN produced by the homogenate was 1.8–2.1. In the second series of studies, GTN was administered topically to freshly excised, intact hairless mouse skin in conventional *in vitro* diffusion cells. The concurrent transport and metabolism of GTN was then monitored by sequential analysis of the receptor phase perfusing the dermal side of the tissue. Three topical formulations were used: a low concentration (1 mg/ml) aqueous solution, a 2% ointment, and a transdermal delivery system. Delivery of total nitrates (GTN + 1,2-GDN + 1,3-GDN) into the receptor phase was similar for ointment and patch formulations and much greater than that from the solution. The percentage metabolites formed, however, was greatest for the solution (61% and 2 hr, compared to 49% for the patch and 35% for the ointment). As has been noted before, therefore, the *relative* level of skin metabolism is likely to be greatest when the transepidermal flux is small. Distinct from the homogenate experiments, the 1,2/1,3-GDN ratios in the penetration studies were in the range 0.7–0.9. It would appear that homogenization of the skin permits GTN to be exposed to a different distribution of enzymes than that encountered during passive skin permeation.

KEY WORDS: transdermal delivery; cutaneous metabolism; nitroglycerin; percutaneous absorption.

INTRODUCTION

The potential of transdermal drug delivery (TDD) has been realized in recent years with the approval of several dosage forms designed to produce pharmacological effect resulting from systemic drug availability. However, despite the fact that the skin is known to be metabolically active, few investigations have addressed the reduction of topical drug bioavailability via a "cutaneous first-pass effect." The diverse biotransformation reactions of which the skin is capable have been summarized in a number of reviews (1–5). Nevertheless, a frequently quoted advantage of TDD is that presystemic metabolism is avoided. In this, and a subsequent (6) paper, we have examined how a number of variables may impact upon the degree to which a transdermally delivered drug may be metabolized within the skin.

Specifically, the cutaneous transformations of nitroglycerin (GTN) to its 1,2- and 1,3-glyceryldinitrate (GDN) metabolites have been studied *in vitro*. GTN is widely used in the treatment of angina pectoris. It is the most successful transdermally delivered drug, with many formulations currently on the market. Previous research has established that GTN is susceptible to epidermal metabolism. Wester *et al.* (7), for example, estimated that the cutaneous first-pass effect *in vivo* in rhesus monkeys reduced GTN bioavailability by 16–21%. *In vitro*, using both human and mouse skin, Santus *et al.* (8) demonstrated significant GTN metabolism during percutaneous penetration and indicated that the percentage of metabolites formed was very sensitive to skin permeability. Elsewhere, the possible inactivation of GTN by microorganisms resident on the skin surface has been reported (9,10), and the incorporation of skin metabolism into a physiologically based pharmacokinetic model of transdermal GTN delivery has significantly improved the interpretation (11).

Here, we again consider GTN metabolism by skin *in vitro*. Using freshly excised tissue, the biotransformation of the drug by a homogenate has been compared to that observed during the percutaneous penetration process through the intact membrane. Different drug delivery systems (i.e., different GTN delivery rates to the epidermal surface) have been employed in the intact membrane studies, as have skin membranes taken from different anatomic sites.

MATERIALS AND METHODS

Nitroglycerin Formulations. Three dosage forms were employed in the experiments described below: (i) an aqueous nitroglycerin solution (Tridil, American Critical Care Co., Agunadilla, PR) diluted with pH 7.4 phosphate-buffered saline (PBS) to a concentration of either 1 μg/ml or 0.1 mg/ml; (ii) a 2% GTN ointment (Nitrocor, Recordati Co., Milan); and (iii) the Nitro-Dur II (Key-Schering, Miami, FL) transdermal delivery system (20 mg, 5 cm^2).

Assay of GTN and 1,2- and 1,3-GDN. All samples were assayed by a common gas chromatographic procedure using an electron capture detector (12). Prior to injection, the samples were extracted three times with 10 ml of a mixture of *n*-pentane (Burdick & Jackson Laboratories, Muskegon, MI) and methyl t-butyl ether (EM Science, Gibbstown, NJ). The internal standard, 2-iodobenzyl alcohol (Aldrich Chemical Co., Milwaukee, WI), was added to the samples (at a concentration of 80 ng/ml) before extraction. The gas chromatograph (Varian 6500, Varian Associates, Walnut Creek, CA) employed an HP-1 fused silica capillary column (0.32-mm internal diameter, 25-m length, 1-mm film thickness; Hewlett-Packard Co., Palo Alto, CA).

Skin Source. Full-thickness skin was removed immediately post-sacrifice from 10- to 15-week-old hairless mice (SKH: hr-1, Skin Cancer Hospital, Philadelphia). Dorsal skin was used in all experiments except for a flux compari-

[1] Departments of Pharmacy and Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, California 94143-0446.

[2] Permanent address: Hisamitsu Pharmaceutical Co., Inc., 408 Tashiro, Tosu, Saga 841, Japan.

[3] To whom correspondence should be addressed at School of Pharmacy, University of California, San Francisco, San Francisco 94143-0446.

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son between dorsal and ventral skin, as explicitly stated below.

Homogenate Experiments. The skin was homogenized in 0.05 *M* Tris buffer (0.15 *M* KCl, pH 7.4) at 4°C using a tissue homogenizer (Sorvall Omni-mixer, Sorvall, Inc., Newton, CT). The skin–buffer mixture was 1:5 (w/v). Subsequently, the homogenate was centrifuged at 9000*g* (L8-60M Ultracentrifuge, Beckman Instruments, Fullerton, CA) and the supernatant obtained. To one-half of the supernatant, glutathione (GSH; Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 2 m*M*. Both supernatant solutions (+GSH and −GSH) were then warmed to 37°C and GTN (1 μg/ml) was introduced to produce an initial concentration of 25 ng/ml. Samples of the solutions were withdrawn at 0.5, 1, and 2 hr post-GTN addition for assay. Further reaction was stopped by placing the samples into a mixture of dry ice and methanol. As negative controls, GTN was introduced into buffer, without homogenized skin, in the presence and absence of GSH. The experiments were performed six times.

Percutaneous Transport Studies. The concomitant penetration and metabolism of GTN were measured in glass, flow-through diffusion cells (Laboratory Glass Apparatus, Berkeley, CA) (13) as previously described (8). The fresh skin was clamped between the upper and the lower halves of the diffusion cell. The area of skin exposed to the donor phase was 0.95 cm^2. The receptor chamber (volume, approx. 3 ml) was perfused with pH 7.4 PBS at 5 ml/hr, and (hourly) fractions were automatically collected (Gilson FC 220 Fraction Collector, Gilson Medical Electronics, Inc., Middleton, WI) and then frozen for later analysis. GTN was administered to the skin surface in one of three formulations: as an aqueous solution (1 ml at a concentration of 0.1 mg/ml), in an excess of 2% ointment (0.5 g spread evenly with a glass rod), and via the transdermal system (cut to the size of skin and attached through the patch adhesive). With the solution, penetration and metabolism were also measured using ventral skin. In the transdermal delivery system experiments, an individual patch was used for each piece of skin (i.e., patches were not subdivided). All transport experiments were performed at least six times.

RESULTS

Metabolism of GTN was observed in the supernatants from skin homogenates. Significantly more metabolism ($P < 0.01$) occurred in the presence of the cofactor GSH, and the GTN concentration dropped from an initial value of 25 to 14 (±2) ng/ml after 2 hr of incubation. With no GSH, the GTN concentration decreased to only 22 (±2) ng/ml in the same time period. In the presence or absence of GSH, metabolism to 1,2-GDN was preferred over that to 1,3-GDN by a factor of 1.8 to 2.1. The 2-hr levels of 1,2-GDN and 1,3-GDN were 4.4 (±0.6) ng/ml and 1.7 (±0.6) ng/ml, respectively, with GSH present; the corresponding values with no GSH were 0.9 (±0.4) ng/ml (1,2-GDN) and 0.4 (±0.2) ng/ml (1,3-GDN). In control experiments (incubation of GTN in buffer alone or in buffer + GSH), no GDN metabolites were detected.

The fluxes of nitrates (GTN, 1,2-GDN, and 1,3-GDN) into the receptor phase, as a function of time, following application of GTN in three topical formulations, are presented in Table I. The overall delivery of nitrates from the ointment and from the transdermal system were similar and significantly greater than that from the aqueous solution. It should be noted, however, that the initial GTN concentration in the solution was relatively small compared to that in the ointment and in the patch. When GTN was exposed to intact skin, irrespective of the delivery system, there was slightly preferential formation of 1,3-GDN compared to 1,2-GDN (Table I). The ratio (1,2/1,3) at all time points was less than unity and typically in the range 0.7–0.9. This finding agrees with the previous report of Santus *et al.* (8). After 2 hr, following GTN administration as an aqueous solution, almost 61% of the nitrates which had penetrated the skin were

Table 1. Nitrates (Mean ± SD) Penetrating to the Receptor Phase, as a Function of Time, Following Topical Application of GTN in Three Formulations

Time (hr)	GTN (nmol/hr)	1,2-GDN (nmol/hr)	1,3-GDN (nmol/hr)	Ratio[a]
Aqueous solution (n = 6)				
2	1.9 ± 1.0	1.6 ± 0.7	2.0 ± 1.0	0.84 ± 0.07
3	3.7 ± 1.7	2.1 ± 0.5	2.5 ± 1.2	0.86 ± 0.10
4	5.0 ± 2.4	2.3 ± 0.7	2.5 ± 1.2	0.91 ± 0.10
5	6.0 ± 3.2	2.0 ± 0.5	2.3 ± 0.5	0.90 ± 0.07
6	6.5 ± 3.2	1.7 ± 1.7	1.9 ± 0.7	0.92 ± 0.10
Ointment (n = 7)				
2	25.7 ± 10.1	5.3 ± 1.3	6.9 ± 1.3	0.77 ± 0.08
3	50.9 ± 21.4	7.1 ± 1.9	8.5 ± 1.9	0.84 ± 0.05
4	67.5 ± 24.1	7.5 ± 2.1	8.5 ± 2.1	0.87 ± 0.05
5	81.0 ± 35.2	7.5 ± 2.4	8.5 ± 2.1	0.87 ± 0.05
6	90.0 ± 32.5	7.2 ± 1.9	7.9 ± 1.9	0.92 ± 0.05
Transdermal delivery system (n = 6)				
2	18.3 ± 4.7	7.1 ± 1.5	10.0 ± 1.5	0.72 ± 0.05
3	39.2 ± 17.1	10.6 ± 1.7	14.5 ± 2.7	0.74 ± 0.07
4	58.3 ± 24.5	12.8 ± 2.0	17.1 ± 3.2	0.75 ± 0.05
5	69.0 ± 27.2	12.6 ± 1.5	16.6 ± 2.0	0.76 ± 0.05
6	86.5 ± 27.7	12.8 ± 1.2	16.4 ± 1.2	0.78 ± 0.05

[a] Ratio of 1,2-GDN to 1,3-GDN.

metabolites. The corresponding values for the ointment and patch were approximately 35 and 49%, respectively. Beyond 2 hr, the percentage of metabolites in each sample gradually decreased, such that 6 hr postapplication the values stated above had been essentially halved.

Finally, the fractions of metabolites formed, following GTN dosing as an aqueous solution to ventral and dorsal skin samples, were compared. While slightly higher metabolism by dorsal skin was apparent, the difference was not significant (data not shown). Similarly, total nitrate flux through ventral skin was indistinguishable from that across the dorsal membrane. These results contrast distinctly with those of Santus *et al.* (8) using *human* abdominal and breast skin. In that case, GTN flux across abdominal skin was much slower, and the percentage metabolism (as a consequence) considerably greater.

DISCUSSION

Cutaneous metabolism is of considerable fundamental and applied interest. The epithelial cells of the viable epidermis demonstrate a high level of metabolic activity during the elaboration of skin barrier function. The biotransformation potential of the skin has been viewed both positively, from the standpoint of prodrug delivery and release (2,14), and negatively with respect to the activation of xenobiotics to toxic products (15,16). In this work, where we have focused upon the cutaneous metabolism of GTN, the relevant issues are (i) To what extent is the systemic bioavailability of GTN compromised by a "local" first-pass effect? and, concomitantly, (ii) To what degree are potentially active metabolites being introduced into the general circulation as a result of this biotransformation?

The metabolism of GTN in numerous systems has been widely studied and reviewed (17,18). The involvement of glutathione-*S*-transferase in the reduction of GTN to its dinitrate metabolites has been inferred by the enhanced metabolism observed in the presence of the cofactor GSH (19). This hypothesis is supported by the homogenate experiments. The ratio of 1,2-GDN to 1,3-GDN, formed from the metabolism of GTN by the 9000*g* supernatant of hairless mouse skin homogenate, is approximately 2, a value which would be predicted on purely statistical grounds (i.e., if the metabolic process showed no specificity whatsoever, and the nitrate groups on GTN were reduced randomly, then there is a 2-to-1 probability of forming 1,2-GDN over 1,3-GDN). However, this metabolic pattern is not common to all tissue homogenate systems, some of which produce 1,2-GDN/1,3-GDN ratios quite different from 2 (20,21). Furthermore, *in vivo,* administration of GTN by different routes results in a variety of metabolite ratios in the systemic circulation (22).

The relative production of 1,2- and 1,3-GDNs during transdermal passage is quite distinct from that of the homogenate (see Table I). As previously reported by our laboratories (8) a slightly preferential formation of 1,3-GDN (ratio 1,2/1,3 = 0.8) is measured. What explanations can account for the discrepancy? First, it is quite possible that tissue homogenization causes the release of enzymes to which permeating GTN molecules are not usually accessible. Alternatively, if separate metabolic pathways to 1,2-GDN and to 1,3-GDN exist, it may be that saturation (of either or both) occurs in the homogenate experiment (where GTN "delivery" to the viable skin regions is not controlled by the formulation or by the intact stratum corneum); such an alteration in the kinetics of substrate presentation to the enzyme(s) may lead to a different distribution of metabolite products. Another possibility might be differential participation of skin surface microorganisms to the GTN degradation process. In other words, homogenization of the skin tissue could alter the metabolic contribution from these microbes. One might also anticipate that the destruction of the natural pH gradient, which exists across intact skin (23), can also contribute to the altered metabolism produced by the homogenate (particularly when it is recognized that GTN stability is less in alkaline conditions than in acid).

The delivery of total nitrate species across the skin *in vitro* is dependent upon the GTN formulation applied (Table I). The relatively low concentration aqueous solution results in a considerably lower flux than the ointment and patch, which contain much higher levels of GTN. The ointment and the transdermal delivery system lead to essentially identical nitrate delivery rates, suggesting that the skin may be rate-controlling under the conditions of these experiments (the alternative explanation is that these two very different formulations release GTN at the same rate, which is slower than the rate of stratum corneum permeation).

Although the flux of substrate from the ointment and patch formulations appears to be reaching a steady state, there is a continually increasing tendency over the 6-hr duration of the experiment (Table I). If the skin is acting as a rate-controlling membrane, then this trend may reflect a long diffusional lag time to steady-state transport. Alternatively, the observation may be the result of a time-dependent experimentally induced change in hairless mouse skin barrier function. The flux of the dinitrates appears to have plateaued at 6 hr, suggesting that the loss of tissue viability is beginning to cause observable effects (see below).

Table I shows that the lower GTN delivery from the aqueous solution significantly affects the level of cutaneous metabolism. With a smaller flux of the parent compound, the epidermal enzymes are able to transform a larger percentage of GTN into 1,2- and 1,3-GDN [a phenomenon which has been reported previously (8)]. A practical consequence of this observation is that the use of skin penetration enhancers may reduce the potential problems associated with cutaneous metabolism. By increasing the parent drug flux, and reducing the residence time of diffusing molecules within the skin, these adjuvants may lower the relative degree of "cutaneous first-pass effect."

It should also be noted that the results in Table I demonstrate a consistent decrease in enzyme activity with time. Diminished activity, rather than saturation, is implicated because identical temporal patterns are observed for all three delivery systems (one of which, the solution, delivers GTN at a much lower rate). Conceivably, the reduced metabolism could be due to cofactor depletion (as opposed, for example, to enzyme denaturation). No effort was made to maintain epidermal viability for long periods in these studies [as others (24) have successfully reported]. The objective here was to gather qualitative information about the cutaneous metabolism process and to initiate investigation of the interplay

between biological and pharmaceutical variables. Quantitative data obtained from hairless mouse skin are not easily converted into values pertinent to man at this time. However, one can expect that the general principles established by these *in vitro* experiments will be relevant to the clinical application, and the pharmaceutical development, of transdermal drug delivery systems. The interrelationships among GTN delivery rate, skin condition, and cutaneous metabolism are examined further in the subsequent paper (6).

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REFERENCES

1. P. K. Noonan and R. C. Wester. Cutaneous metabolism of xenobiotics. In R. L. Bronaugh and H. I. Maibach (eds.), *Percutaneous Absorption,* Dekker, New York, 1985, pp. 65–85.
2. D. A. W. Bucks. Skin structure and metabolism: Relevance to the design of cutaneous therapeutics. *Pharm. Res.* **1**:148–153 (1984).
3. R. H. Guy, J. Hadgraft, and D. A. W. Bucks. Transdermal drug delivery and cutaneous metabolism. *Xenobiotica* **17**:323–343 (1987).
4. R. J. Martin, S. P. Denyer, and J. Hadgraft. Skin metabolism of topically applied compounds. *Int. J. Pharm.* **39**:23–32 (1987).
5. D. R. Bickers. The skin as a site of drug and chemical metabolism. In V. A. Drill and P. Lazar (eds.), *Current Concepts in Cutaneous Toxicity,* Academic Press, New York, 1980, pp. 95–125.
6. N. Higo, R. S. Hinz, D. T. W. Lau, L. Z. Benet, and R. H. Guy. Cutaneous metabolism of nitroglycerin *in vitro*. II. Effects of skin condition and penetration enhancement (accepted for publication) (1992).
7. R. C. Wester, P. K. Noonan, S. Smeach, and L. Kosobud. Pharmacokinetics and bioavailability of intravenous and topical nitroglycerin in the rhesus monkey. *J. Pharm. Sci.* **72**:745–748 (1983).
8. G. C. Santus, N. Watari, R. S. Hinz, L. Z. Benet, and R. H. Guy. Cutaneous metabolism of transdermally delivered nitroglycerin *in vitro*. In B. Shroot and H. Schaefer (eds.), *Skin Pharmacokinetics,* Karger, Basel, 1987, pp. 240–244.
9. S. P. Denyer, W. B. Hugo, and M. O'Brien. Metabolism of glyceryl trinitrate by skin staphylococci. *J. Pharm. Pharmacol.* **36**:61P (1984).
10. S. P. Denyer and C. McNabb. Microbial metabolism of topically applied drugs. In J. Hadgraft and R. H. Guy (eds.), *Transdermal Drug Delivery,* Dekker, New York, 1989, pp. 113–134.
11. E. Nakashima, P. K. Noonan, and L. Z. Benet. Transdermal bioavailability and first-pass skin metabolism: A preliminary evaluation with nitroglycerin. *J. Pharmacokin. Biopharm.* **15**: 423–437 (1987).
12. F. W. Lee, N. Watari, J. Rigod, and L. Z. Benet. Simultaneous determination of nitroglycerin and its metabolites by capillary gas chromatography with electron-capture detection. *J. Chromatogr.* **426**:259–266 (1981).
13. C. L. Gummer, R. S. Hinz, and H. I. Maibach. The skin cell: A design update. *Int. J. Pharm.* **40**:101–104 (1987).
14. N. Bodor, J. Zupan, and S. Selk. Improved delivery through biological membranes VII: Dermal delivery of cromoglycic acid (cromolyn) via its prodrug. *Int. J. Pharm.* 7:63–75 (1982).
15. E. Boyland. The biological significance of metabolism of polycyclic compounds. *Biochem. Soc. Symp.* **5**:40–54 (1960).
16. H. Marquardt, T. Kuroki, E. Humberman, J. K. Selkirk, C. Heidelberger, P. L. Grover, and P. Slims. Malignant transformation of cells derived from mouse prostate by epoxides and other derivatives of polycyclic hydrocarbons. *Cancer Res.* **32**:716–720 (1972).
17. F. J. Di Carlo. Nitroglycerin revisited: chemistry, biochemistry, interactions. *Drug Metab. Rev.* **4**:1–38 (1975).
18. S. H. Curry and S. M. Aburawi. Analysis, disposition and pharmacokinetics of nitroglycerin. *Biopharm. Drug Dispos.* **6**:235–280 (1985).
19. P. Needleman and F. E. Hunter, Jr. The transformation of glyceryl trinitrite and other nitrites by glutathione-organic nitrate reductase. *Mol. Pharmacol.* **1**:77–86 (1965).
20. P. A. Cossum and M. S. Roberts. Metabolite inhibition of nitroglycerin metabolism in sheep tissue homogenates. *J. Pharm. Pharmacol.* **37**:807–809 (1985).
21. H. L. Fung, S. C. Sutton, and A. Kamiya. Blood vessel uptake and metabolism of organic nitrates in the rat. *J. Pharmacol. Exp. Ther.* **233**:334–341 (1984).
22. P. K. Noonan and L. Z. Benet. Variable glyceryl dinitrate formation as a function of route of nitroglycerin administration. *Clin. Pharmacol. Ther.* **237**:629–635 (1986).
23. M. Katz and B. J. Poulsen. Absorption of drugs through the skin. In B. B. Brodie and J. R. Gillette (eds.), *Handbook of Experimental Pharmacology,* Springer-Verlag, New York, 1971, pp. 103–173.
24. S. W. Collier, N. M. Sheikh, A. Sakr, J. L. Lichtin, R. F. Stewart, and R. L. Bronaugh. Maintenance of skin viability during *in vitro* percutaneous absorption/metabolism studies. *Toxicol. Appl. Pharmacol.* **99**:522–533 (1989).