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The in-vitro percutaneous absorption of glycerol trioleate through hairless mouse skin

STEPHEN J. MOLONEY, *Department of Toxicology, Avon Products, Division Street, Suffern, NY 10901, USA*

Abstract—The chemical composition of the aqueous receptor fluid in one-chambered diffusion cells used for in-vitro percutaneous absorption studies has been shown to significantly affect the apparent extent of absorption of the triglyceride, glycerol trioleate. Using murine skin samples it was found that the addition of albumin to the receptor fluid resulted in an increase in the apparent extent of absorption, while the presence of the bacteriostatic agent thiomersal resulted in a decrease. The viability of the skin samples had no effect on absorption. It was determined that the chemical species in the receptor fluid was the free fatty acid. Albumin presumably bound the fatty acid, therey creating a sink which enabled the fatty acid to partition from the skin into the receptor fluid.

The systemic and local availability of topically applied compounds is dependent on the rate of permeation through the stratum corneum. Quantitation of this process is of importance to both delivery system design and risk assessment. Measurement of percutaneous absorption in-vitro using the finite dose technique has been proposed as a relevant model for estimating the in-vivo rates of delivery of chemicals (Franz 1975, 1978; Bronaugh et al 1982). This method consists of a diffusion cell where the dermis is bathed by isotonic saline (receptor fluid) and the epidermis is exposed to the atmosphere under ambient conditions. However, some limitations of this system have been described. Compounds that are hydrophobic will not readily partition from the skin into the aqueous receptor fluid (Bronaugh & Stewart 1984; Hawkins & Reifenrath 1986). This can result in an underestimation of the rate of percutaneous absorption. Addition of agents such as non-ionic surfactants and protein to the receptor fluid have been used in an attempt to obviate this problem (Bronaugh & Stewart 1984). Secondly, the skin is usually considered to be a passive, inert barrier. Viability of the excised skin, however, has been reported to affect the absorption of certain chemicals such as benzo[a]pyrene (Kao et al 1984). Elucidation of the appropriate conditions is of importance for the general application of this in-vitro technique. This report details the effects of viability of the excised skin and the addition of protein, surfactants, and antibiotics to the receptor fluid on the in-vitro percutaneous absorption of the hydrophobic lipid, glycerol trioleate.

Materials and methods

Carbon-14 labelled glycerol trioleate, oleic acid, ascorbic acid and tritiated water were purchased from Amersham, Arlington Heights, IL. Glycerol trioleate, oleic acid, 1,2-dioleylglycerol, thiomersal, and bovine serum albumin fraction V (BSA) were purchased from Sigma, St. Louis, MO. Gentamicin sulfate was purchased from Gibco, Chagrin Falls, OH. Polyethylene glycol 20 oleyl ether (PEG-20 oleyl ether) was obtained from Croda Inc, New York, NY. All other chemicals were of laboratory grade or analytical grade when available.

Percutaneous absorption studies were carried out using one-chambered static diffusion cells (Crown Glass, Sommerville, NJ). Full thickness Skh-1 hairless mouse skin (Temple University, Philadelphia, PA) pieces from the mid-dorsal region were mounted on the cells with the dermal side in contact with the receptor fluid and the epidermis open (0·78 cm^2 surface area) to the ambient atmosphere. Glycerol trioleate and oleic acid were applied as 10 μL aliquots of 40 $\mu g\ mL^{-1}$ solutions in ethanol and ascorbic acid as 10 μL aliquots of a 1 $mg\ mL^{-1}$ solution in ethanol. The label was spiked so as to apply 2–4 μCi of activity per diffusion cell. Water was spiked so as to achieve 5 $\mu Ci\ mL^{-1}$ and 200 μL aliquots were applied to the diffusion cells, which were then occluded with Parafilm. This latter step was to avoid evaporation of tritiated water into the laboratory. The recovery of radiolabel in the receptor fluid was quantitated with a Packard Tri-Carb 4530 liquid scintillation counter.

The receptor fluid was modified by making additions to the 10 mM phosphate buffered saline, pH 7 (PBS). Gentamicin sulphate and thiomersal were added at 0·01% w/v; BSA was added at 3·0% w/v; PEG-20 oleyl ether was added at 6·0% w/v; glucose was added at 0·1% w/v; potassium cyanide was added at 0·8% w/v. The receptor fluid was maintained at 37°C and was stirred continuously.

For experiments that used frozen and thawed skin, excised skin was kept at −20°C for 24 h before thawing at room temperature (20°C) and then mounted in diffusion cells.

For determination of the disposition of the parent compound and metabolites of glycerol trioleate in the epidermis, dermis, and receptor fluid, diffusion cells were set up as previously described. After 24 h the epidermal surface was washed with ethanol to remove any triglyceride that had remained unabsorbed prior to removal of the skin samples. The epidermis was separated from the dermis by scraping. The triglyceride and metabolites were extracted from the epidermis, dermis and receptor fluid by the method of Bligh & Dyer (1959). Portions of the extracts were applied with lipid standards onto silica gel TLC plates and developed with hexane–diethyl ether–acetic acid (66:33:1 v/v). Lipids were visualized by iodine vapour and quantitated by scraping the appropriate areas into scintillation vials for quantitating the radioactivity.

Results

The bacteriostatic agents thiomersal and gentamicin were added to the receptor fluid to control bacterial growth in this compartment. When PBS containing gentamicin was used, the extent of absorption of glycerol trioleate as detected by radioactivity in the receptor fluid was very small (Table 1). Addition of 6% PEG-20 oleyl ether to PBS and gentamicin had no apparent effect

Table 1. Effect of the receptor fluid composition on the in-vitro percutaneous absorption of glycerol trioleate

Receptor fluid composition	Apparent percutaneous absorption*
PBS + gentamicin	0·3 ± 0·2
PBS + gentamicin + PEG-20 oleyl ether	0·2 ± 0·1
PBS + gentamicin + BSA	4·7 ± 0·8
PBS + gentamicin + BSA**	4·5 ± 1·2
PBS + gentamicin + BSA + glucose	4·3 ± 0·8
PBS + gentamicin + BSA + KCN	4·5 ± 1·0
PBS + thiomersal + BSA	1·7 ± 0·3

* Apparent extent of percutaneous absorption expressed as the mean and standard deviation (n = 6) of the percentage of the applied radioactivity recovered in the receptor fluid after 24 h.
** Skin samples had been frozen and thawed.

while addition of 3% BSA had a significant effect with a 16-fold increase in recovery of radioactivity in the receptor fluid as compared to PBS with gentamicin alone. When gentamicin was replaced with thiomersal, there was a greater than 50% reduction in the apparent extent of absorption compared with that observed with PBS with BSA and gentamicin. Addition of glucose or potassium cyanide to PBS and BSA or the use of skin samples that had been frozen and thawed had no effect on the apparent extent of absorption of glycerol trioleate. Use of frozen skin and the addition of potassium cyanide to the receptor fluid have been used previously to decrease skin viability in percutaneous absorption studies (Holland et al 1984, Kao et al 1985).

To ascertain if the thiomersal was acting by being absorbed into the skin from the receptor fluid, skin samples were preincubated for 6 h in diffusion cells that had either PBS with BSA and gentamicin or PBS with BSA and thiomersal. After 6 h the receptor fluid in both groups was removed and replaced with PBS with BSA and gentamicin. Glycerol trioleate was then applied to the epidermal surface as described previously. The apparent extent of absorption per 24 h after preincubation with thiomersal, $3{\cdot}6 \pm 0{\cdot}4$ percent of the applied dose ($n=8$), was significantly less ($P<0{\cdot}01$) than the rate observed after preincubation with gentamicin, $4{\cdot}8 \pm 0{\cdot}8$ ($n=8$). The extent of absorption after the 6 h preincubation with thiomersal was not as low as that observed when skin samples were kept in contact with thiomersal-containing receptor fluid for 24 h.

The addition of thiomersal to the receptor fluid had no effect on the percutaneous absorption of oleic acid, ascorbic acid or water when compared with the rate observed in the presence of gentamicin (Table 2). The absorption of oleic acid was of interest as it is a metabolite of the triglyceride, while ascorbic acid and water were used as model compounds to estimate general effects on permeability.

The effect of the presence of BSA on the percutaneous absorption of oleic acid was determined. Addition of 3% BSA to PBS and gentamicin increased the apparent extent of percutaneous absorption from $0{\cdot}7 \pm 0{\cdot}1$% ($n=4$) of the applied dose per 24 h to $66{\cdot}1 \pm 7{\cdot}2$% ($n=4$), a 94-fold increase.

Table 2. Comparative effects of addition of thiomersal and gentamicin to a phosphate buffered saline receptor fluid on the in-vitro percutaneous absorption of various compounds

	Percutaneous absorption*	
	PBS + thiomersal	PBS + gentamicin
Oleic acid**	$64{\cdot}1 \pm 15{\cdot}8$	$68{\cdot}2 \pm 12{\cdot}4$
Ascorbic acid	$1{\cdot}4 \pm 0{\cdot}7$	$1{\cdot}8 \pm 0{\cdot}7$
Water	$18{\cdot}2 \pm 3{\cdot}7$	$20{\cdot}4 \pm 3{\cdot}8$

* Percutaneous absorption expressed as the mean and standard deviation ($n=4$) of the percentage of the applied radioactivity recovered in the receptor fluid after 24 h.
** Receptor fluid also contained 3% BSA.

Table 3. Metabolite profile in epidermis, dermis, and receptor fluid 24 h after the application of glycerol trioleate to diffusion cells.

		Relative distribution of radioactivity**		
	Recovery*	Trigyceride	Diglyceride	Fatty Acid
Epidermis	14·6	96·2	1·0	0·6
Dermis	8·6	94·8	0·7	2·5
Receptor fluid***	3·6	2·3	0·9	93·8

* Percentage of the applied radioactivity applied to the diffusion cells recovered in the fractions after 24 h. Results are expressed as the mean of 2 experiments.
** Relative distribution expressed as the percentage of the total radioactivity recovered in each fraction associated with the triglyceride, diglyceride or free fatty acid.
*** Receptor fluid consisted of PBS with BSA and gentamicin.

The analysis of the epidermis, dermis, and receptor fluid (PBS with gentamicin and BSA) for metabolites 24 h after the application of glycerol trioleate is summarized in Table 3. The percentage of recovery of the applied dose diminished in the order epidermis > dermis > receptor fluid. The major component in the epidermis and the dermis was the parent compound, glycerol trioleate. The major component in the receptor fluid was oleic acid, with only traces of the tri- and diglyceride being detected.

Discussion

PEG-20 oleyl ether has previously been reported to be more effective than BSA at improving the recovery in the receptor fluid of the hydrophobic compounds cinnamyl anthranilate and acetyl ethyl tetramethyl tetralin during in-vitro absorption experiments (Bronaugh & Stewart 1984). That the reverse was observed in this case with glycerol trioleate was due to the fact that the chemical entity that was recovered in the receptor fluid was the free fatty acid. It is well-known that fatty acids bind with high affinity to serum albumin, the major transport system for unesterified fatty acids in the bloodstream (Brown & Shockley 1982). Addition of the BSA enabled the receptor fluid to act as a "sink" for the free fatty acid as shown by the dramatic increase in the apparent extent of percutaneous absorption when oleic acid was applied directly to the skin. Likewise, oleic acid formed by hydrolysis of the triglyceride during absorption could, under these conditions, partition into the receptor fluid where it was predominately located 24 h after application of glyceryl trioleate. None of the conditions tested apparently enabled the di- or triglycerides to partition readily into the receptor fluid.

Thiomersal, an organomercurial agent, has previously been used as an antibacterial agent in the receptor fluid of diffusion cells (Bronaugh et al 1982). Of the compounds tested, the observed decrease in recovery of radioactivity when using thimerosal in the receptor fluid as compared to gentamicin was specific for glycerol trioleate. The reason for this decrease is uncertain although the reduction in the apparent extent of absorption after preincubation with a thiomersal-containing receptor fluid indicates that it may be a direct effect on the skin rather than an effect on partitioning from the skin into the receptor fluid. That no effect was observed on the percutaneous absorption of oleic acid itself indicates that the decrease in the apparent extent of absorption of glycerol trioleate may be due to inhibition of lipase activity with a concomitant reduction in oleic acid formation. Irrespective of the mechanism, this phenomenon highlights the importance of using appropriate receptor fluid constituents and the need to modify the composition as necessary when measuring the absorption of diverse chemicals.

Viability and the extent of metabolism of the permeating chemical have been reported to affect the in-vitro absorption of benzo[a]pyrene across mouse skin (Kao et al 1984). In this study, viability did not appear to be of major importance as the presence of potassium cyanide or freezing of the skin samples did not decrease the extent of apparent absorption. In addition, incorporation of glucose in the receptor fluid as an energy source did not enhance absorption. The oxidative metabolism of benzo[a]pyrene is dependent on the reduced nucleotide NADPH, the level of which is dependent on the viability of the tissue. In contrast, the hydrolysis of glycerol trioleate is not dependent on cofactors. Differentiation between hydrolysis by the microflora on the skin surface or by cutaneous enzyme activity is not possible with the experimental protocol employed in this study. Cutaneous lipase activity has been shown to be located in intercellular domains in mouse and human epidermis (Menon et

al 1986) and as such the activity may appear to be independent of cutaneous viability. Metabolism by the cutaneous microflora may also appear to be independent of viability. However, as with benzo[a]pyrene, the apparent extent of absorption of glycerol trioleate will be dependent on the extent of metabolism.

The data from this experiment imply that the use of a standard set of conditions developed for measuring the in-vitro percutaneous absorption of one compound cannot necessarily be used for other compounds with different physicochemical properties. Use of inappropriate conditions will lead to erroneous assumptions as to the ability of chemicals to permeate the skin.

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Action of a chronic arecoline administration on mouse motility and on acetylcholine concentrations in the CNS

L. MOLINENGO, A. M. FUNDARÓ, M. C. CASSONE, *Institute of Pharmacology and Pharmacognosy, University of Turin, C.so Raffaello 31, Torino, Italy*

Abstract—The modifications of mouse motility and of the levels of acetylcholine (ACh) in two sections of the CNS caused by a chronic administration of 4·5; 9·5; 28·5 and 60 mg kg^{-1} day^{-1} of arecoline for 20 days have been studied. At low doses (4·5 and 9·5 mg kg^{-1} day^{-1}), arecoline caused no modification of the ACh levels and of the motility. The higher doses (28·5 and 60 mg kg^{-1} day^{-1}) caused a reduction of the mouse motility and an increase of the ACh levels in the subcortical structures of the CNS of the mouse.

There is evidence that the administration of arecoline may improve memory (Bartus et al 1980; Flood et al 1984). Pharmacological alleviation of memory disorders necessarily involves the prolonged use of drugs and it is important to verify the incidence of collateral effects in a chronic drug administration. With these premises we studied in the mouse the modifications of motor activity and of the levels of acetylcholine (ACh) in the CNS after a chronic arecoline administration.

Methods

Female albino "Swiss Morini" mice, 20–25 g, were housed in groups of 20. Because of the short duration of the arecoline effect (Pradham & Dutta 1970), frequent drug administration was needed, therefore, arecoline was dissolved in the drinking water. The concentrations used were: 0·05, 0·1, 0·4 and 0·7 gL^{-1}. The four experimental groups (of 20 mice each) drank the various arecoline solutions and the fifth group drank water (controls).

The pharmacological treatment lasted 20 days. In this period, the mean liquid consumption for each mouse was 2·02 mL^{-1} in the controls and 2·00 mL day^{-1} at 0·05 gL^{-1}, 2·10 mL day^{-1} at 0·1 gL^{-1}, 1·78 mL day^{-1} at 0·4 gL^{-1} and 1·95 mL day^{-1} at 0·7 gL^{-1} of arecoline.

Correspondence to: L. Molinengo, Institute of Pharmacology and Pharmacognosy, University of Turin, C.so Raffaello 31, Torino, Italy.

A modification of the open field for rats (Brimblecombe 1963) was used to evaluate the spontaneous mouse activity. The floor of a square box (side 35 cm, peripheral wall 25 cm high) was divided in 25 squares (side 7 cm). The floor was covered with a transparent plastic sheet to facilitate its cleaning. A lamp (80 W) at a distance of 150 cm gave a uniform illumination of the box floor. The mice were tested individually. To reduce or to eliminate the initial exploratory activity which may interfere with the evaluation of the simple motor activity, the mouse was put in the centre of the box for 10 min; in the subsequent 5 min period the number of squares crossed was determined. A square was considered crossed only when the animal entered the square with all four paws. All experiments were performed in the morning at the end of the chronic pharmacological treatment. The mice still had the drug concentrations at the time of testing.

The same day in which the test of motility was performed, at least five mice of the various experimental groups were killed by microwave irradiation of the head. The skull was opened and the brain frozen (−30°C). The brain was cut through the crus cerebri; cerebellum and pons were discarded. The cortex was collected and weighed. The remaining part of the brain (subcortex) was also weighed. ACh was extracted by the method given by Beani and Bianchi (1964). The tissue after homogenization in 2 mL of McIlvaine's citric disodium phosphate buffer (0·014 M; pH 4), was kept for 30 s in boiling water, then transferred to ice cold water and diluted with an equal volume of frog Ringer solution containing eserine salycilate (2×10^{-5} gL^{-1}) and a double salt concentration to obtain an isotonic medium. The extracts were centrifuged (3000 rev min^{-1}) for 30 min. The supernatant was collected for the bioassay of ACh on the rectus abdominis of the frog. The procedure given by the Staff of the