Characterization of Esterase and Alcohol Dehydrogenase Activity in Skin. Metabolism of Retinyl Palmitate to Retinol (Vitamin A) During Percutaneous Absorption

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Retinyl palmitate, a widely used ingredient in cosmetic products, is promoted for its beneficial effects on the appearance of skin. Previous studies suggest that enzymes are available in skin to metabolize this ingredient during skin absorption. Esterase activity hydrolyzes retinyl palmitate to retinol (vitamin A), which is oxidized in many tissues to retinoic acid primarily by alcohol dehydrogenase. The activities of esterase and alcohol dehydrogenase were characterized in hairless guinea pig skin by using flow-through diffusion cells and radiolabeled model compounds (methyl salicylate and benzyl alcohol) previously shown to be metabolized by these enzymes. Methyl salicylate was hydrolyzed by esterase to a greater extent in viable skin than in nonviable skin. Glycine conjugation of salicylic acid and benzoic acid occurred only in viable skin. The metabolism of methyl salicylate and benzyl alcohol occurred to a greater extent in male guinea pig skin than in female guinea pig skin. The percutaneous absorption of both radiolabeled compounds was similar in viable and nonviable skin. About 30 and 18% of topically applied retinyl palmitate were absorbed from an acetone vehicle by hairless guinea pig skin and human skin, respectively. Less than 1% of the applied dose of this lipophilic compound diffused from skin into the receptor fluid. Retinol was the only detectable metabolite of retinyl palmitate in both hairless guinea pig and human skin. In human skin, 44% of the absorbed retinyl palmitate was hydrolyzed to retinol. The use of retinyl palmitate in cosmetic formulations may result in significant delivery of retinol into the skin.

KEY WORDS: percutaneous absorption; metabolism; retinyl palmitate; hairless guinea pig.

INTRODUCTION

The skin can serve as a portal of entry for chemicals applied in topical formulations. Both local and systemic effects may result from the absorption of a chemical applied to the skin's surface. Because all major metabolic biotransformation reactions occur in skin (1,2), skin metabolism as well as skin absorption data must be obtained to fully assess the effects of topically applied drugs and cosmetics.

Retinyl palmitate is a widely used ingredient in cosmetic formulations. Because it is the most stable of available vitamin A esters, it can be directly incorporated into an anhydrous base or the oil phase of a cosmetic cream or lotion (3). Often these products are advertised as having beneficial effects on the appearance of skin. However, at least a portion of retinyl palmitate could be hydrolyzed by esterase enzymes in skin to retinol (vitamin A), a compound with known pharmacological activity (4). Histochemical studies have shown that rodent epidermis and subdermal muscle layer are sites of intense esterase activity (5). Previous *in vitro* diffusion cell studies have demonstrated esterase activity during percutaneous absorption in viable (human and hairless guinea pig) (6) and nonviable skin (6–8).

Because use of topically applied all-*trans*-retinoic acid (Retin-A) has been claimed to produce younger-looking skin (9), whether retinol (formed from retinyl palmitate) was further metabolized to retinoic acid during percutaneous absorption was also investigated. Evidence suggests that skin might contain enzymes capable of oxidizing alcohols to their corresponding acids (1,10). A small quantity of retinoic acid was reported to be formed in hairless mouse skin after topical application of retinol (11). Benzoic acid was formed from benzyl alcohol after oral and topical application of benzyl acetate to rats (12,13). However, metabolism in skin could not be quantitated in the *in vivo* studies.

Recent advances have made it possible to maintain the viability of skin in an *in vitro* flow-through diffusion cell system so that skin absorption and metabolism can be measured simultaneously (14). In initial studies, enzyme activity in skin required for the biotransformation of retinyl palmitate to retinol and retinoic acid was characterized using readily absorbed model compounds known to be substrates for the enzymes (8,12). The hydrolysis of methyl salicylate by esterases in hairless guinea pig skin was measured after application to viable and nonviable skin from male and female animals. The oxidation of benzyl alcohol by alcohol dehydrogenase was similarly characterized with excised hairless guinea pig skin. The importance of maintaining skin viability and the importance of sex-related differences in metabolism could therefore be assessed. The absorption and metabolism of retinyl palmitate were then determined in female human skin and in hairless guinea pig skin from animals of both sexes.

MATERIALS AND METHODS

Radiolabeled test compounds were used to increase sensitivity of the method and to facilitate determination of metabolites. [*Carboxy*-^{14}C]salicylic acid (specific activity, 11.7 mCi/mmol; 98% purity) was purchased from Sigma Chemical Co. (St. Louis, MO). Radiolabeled methyl salicylate was synthesized from the salicylic acid by an esterification reaction (15). Briefly, the reaction consisted of refluxing methanol and [^{14}C]salicylic acid acidified with sulfuric acid and then separating the [^{14}C]methyl salicylate by solid-phase extraction on a Sep-Pak C_{18} cartridge (Waters Corp., Milford, MA). The purity of the product was determined to be 99% by high pressure liquid chromatography (HPLC).

[*Carbinol*-^{14}C]benzyl alcohol was obtained at a specific

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activity of 14.8 mCi/mmol and 96% purity (Amersham Corp., Arlington Heights, IL). Research Triangle Institute (Research Triangle Park, NC) synthesized [^{14}C]retinyl palmitate at a specific activity of 37 mCi/mmol and 95% purity. The purities of benzyl alcohol and retinyl palmitate were verified by HPLC.

Absorption and metabolism experiments were conducted *in vitro* using flow-through diffusion cells (16). The system was sterilized by using 70% ethanol. Skin was obtained from 3- to 6-month-old male and female hairless guinea pigs (Charles River Laboratories, Wilmington, MA). For the retinyl palmitate studies, viable human skin was obtained immediately after surgery on two donors (abdominoplasty and breast reduction). The integrity of the barrier of human skin was verified by determining [^{3}H]water absorption (17). Split-thickness preparations of skin approximately 250-μm thick were prepared from all skin tissues by using a Padgett dermatome (Padgett Instruments, Kansas City, MO).

Skin viability was maintained in the diffusion cells by using Hepes buffered Hanks' balanced salt solution (HHBSS) as the receptor fluid (14). For the retinyl palmitate studies, HHBSS containing 4% bovine serum albumin (BSA) was used. Mixing with a stirring bar in the diffusion cell facilitated partitioning of the water-insoluble compound into the receptor fluid. Nonviable skin controls were produced by perfusing the cells with distilled water instead of HHBSS. Under these conditions, glucose utilization of skin is terminated within 1 hr, and the structural integrity of skin is compromised (6).

Retinyl palmitate studies were conducted under dark room conditions to prevent photodegradation of metabolites. The diffusion cell apparatus was housed in a large chamber protected from light. Preparation of receptor fluid and tissues for HPLC analysis was conducted in a hood with yellow lights.

Compounds were applied to skin in an acetone vehicle at a chemical dose of approximately 5, 3, and 20 μg/cm^2 for methyl salicylate, benzyl alcohol, and retinyl palmitate, respectively. The benzyl alcohol cells were occluded with a Teflon plug to prevent evaporation of the volatile compound (18). After 24 hr, the surface of each skin was washed three times with 1% soap:99% water to remove any unabsorbed test material.

Tissues from methyl salicylate and benzyl alcohol experiments were homogenized in HHBSS by a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY). Radioactivity in aliquots of the homogenates was measured in a Beckman LS-9000 scintillation counter (Beckman Instruments, Inc., Irvine, CA). Because only small amounts of the applied radioactivity (<5%) were found in skin homogenates from the methyl salicylate and benzyl alcohol studies, metabolism was not measured in these tissues. Skin treated with retinyl palmitate was homogenized in 8 mL of acetone to solubilize the parent compound and metabolites. The homogenate was then centrifuged at 3000 rpm for 10 min, and the supernatant was concentrated to 0.5 mL under a stream of nitrogen for further analysis by HPLC.

Aliquots of all receptor fluid fractions were analyzed by liquid scintillation counting to determine total radioactivity of absorbed compounds. Aliquots of receptor fluid from the methyl salicylate and benzyl alcohol studies were directly injected into the liquid chromatograph for metabolism measurements. Preliminary studies showed that both compounds incubated in HHBSS were stable for at least 24 hr. The HHBSS had previously been in contact with skin in a diffusion cell for 24 hr. These findings show that methyl salicylate and benzyl alcohol do not spontaneously degrade in the receptor fluid and that the metabolism that occurs takes place during absorption through the skin and not as a result of contact with the receptor fluid.

Receptor fluid fractions from retinyl palmitate studies were treated to remove protein before HPLC analysis. BSA in the fractions was precipitated with 2 volumes of ethanol. The radiolabeled compounds were extracted into 2 volumes of ethyl acetate, and the extract was evaporated to dryness under a stream of nitrogen. The residue was dissolved in methanol for HPLC analysis. In preliminary studies, >92% of the radiolabeled compounds were extracted by this procedure.

The HPLC system consisted of two Altex Model 110 pumps, an Altex Model 420 gradient controller, and a 5-μm Econosphere C_{18} reversed-phase column (Alltech Associates, Inc., Deerfield, IL). Radioactivity was quantitated by using a Radiomatic radioisotope detector (Packard Instruments, Downers Grove, IL). Nonradiolabeled standards were measured by using a Spectroflow UV detector at 340 nm (Kratos Analytical Instruments, Ramsey, NJ). A gradient elution of compounds was obtained starting with 50% methanol (MeOH):50% 0.01 *M* ammonium acetate (v/v). For the next 40 min, the solvent system was increased in MeOH by 0.25%/min to 60% MeOH, then by 2%/min for the next 5 min. From 45 to 75 min, the solvent system remained constant at 70% MeOH:30% 0.01 *M* ammonium acetate. Then MeOH was increased by 6%/min to 100% MeOH, which was required to elute the highly nonpolar retinyl palmitate. The flow rate throughout the entire gradient remained constant at 1 mL/min.

Absorption and metabolism data were analyzed for significance by using the Student's *t* test (Instat, Graphpad Software, San Diego, CA).

RESULTS

Percutaneous absorption values for methyl salicylate through viable and nonviable hairless guinea pig skin were not significantly different (Table I). Skin permeation of meth-

Table I. Percutaneous Absorption of Methyl Salicylate and Benzyl Alcohol in Hairless Guinea Pig Skin[a]

Compound	Sex	Viable skin[b,c]	Nonviable skin[b,c]
Methyl salicylate	Male	55 ± 6	47 ± 2
	Female	56 ± 16	50 ± 20
Benzyl alcohol	Male	61 ± 16	58 ± 16
	Female	63 ± 10	65 ± 3

[a] Percentage of applied dose absorbed in 24 hr.
[b] Mean ± SE of receptor fluid measurement using skin from 3 animals (3–4 repetitions per animal).
[c] The values obtained for viable and nonviable male and female guinea pig skin for each compound were not significantly different when compared by the two-tailed *t* test ($P < 0.05$).

yl salicylate was similar through back skin of animals of either sex (Table I). Absorption was rapid with over 75% of the absorbed compound found in the initial 6-hr receptor fluid collection interval (data not shown).

Differences in the metabolism of methyl salicylate depended on skin viability and whether the skin of males or females was used in the experiments (Table II). Only esterase activity was observed in nonviable skin: 38% of the absorbed parent compound was metabolized to salicylic acid. In viable skin, esterase activity was significantly increased. Salicylic acid was further metabolized by glycine conjugation to salicyluric acid with a total of 57% of the absorbed dose hydrolyzed. A marked difference in metabolism was observed between male and female hairless guinea pig skin (Table II). Similar amounts of radiolabeled methyl salicylate were absorbed through male and female guinea pig skin, but the total metabolism in male guinea pig skin occurred at more than twice the extent of that in female guinea pig skin. At the end of the 24-hr study, the level of salicylic acid formed by male guinea pig skin (35.6% of absorbed dose) was significantly greater than that formed by female guinea pig skin (12.3% of absorbed dose).

The time course of metabolism of methyl salicylate is shown in Fig. 1. Only at the 6-hr interval was the extent of metabolism in male and female guinea pig skin significantly different. (A similar effect was observed in benzyl alcohol metabolism.) The formation of salicylic acid in male guinea pig skin represents 34% of the absorbed radioisotope compared with 5% in female guinea pig skin.

The percutaneous absorption of benzyl alcohol was similar in viable and nonviable hairless guinea pig skin from animals of the same sex (Table I). The absorbed radioactivity was also found predominantly in the initial 6-hr collection (data not shown).

Oxidation of benzyl alcohol to benzoic acid was observed in viable and nonviable skin from animals of either sex (Table III). However, the percentage of metabolism of the total absorbed compound was approximately 3-fold greater for viable skin from males than for viable skin from females (marginally significant difference, $P > 0.07$). Only with viable skin was benzoic acid further metabolized to its glycine conjugate, hippuric acid.

The percutaneous absorption of the lipophilic compound retinyl palmitate was determined by summation of the material absorbed in the skin and partitioned in the receptor fluid at the end of the 24-hr study (Table IV). Even when the receptor fluid contained 4% BSA, <1% of the absorbed material partitioned into it from the skin. Substantial metabolism of retinyl palmitate to retinol was measured, but no additional metabolites were observed. The percentage of the applied dose absorbed was lower for human skin than for hairless guinea pig skin. About 44% of the absorbed dose was metabolized to retinol in human skin. In human or hairless guinea pig skin experiments, <1% of the radioactivity in the receptor fluid was determined to be from the metabolite retinol.

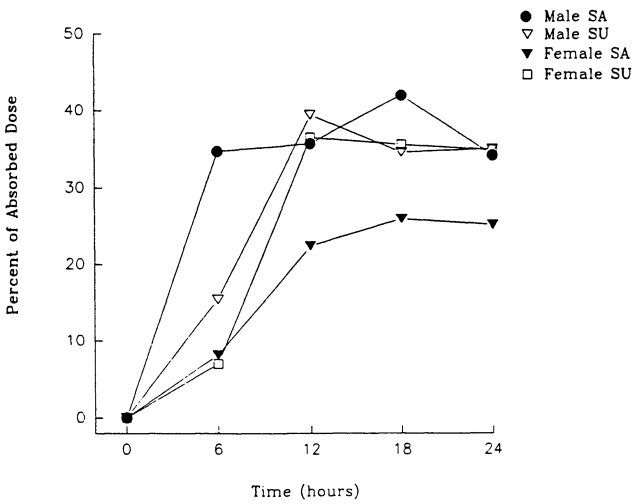

Fig. 1. Metabolism of methyl salicylate in viable guinea pig skin. Values are means of measurements from 3 animals (3–4 repetitions per animal). Standard error bars have been omitted for clarity. SA = salicylic acid; SU = salicyluric acid. Male SA and female SA are significantly different at 6 hr ($P < 0.01$).

DISCUSSION

The absorption and metabolism studies of the model compounds, methyl salicylate and benzyl alcohol, seem to confirm that enzyme activity in hairless guinea pig skin is capable of hydrolyzing esters to their corresponding alcohols and oxidizing alcohols to their acid metabolites. We observed that ester hydrolysis occurs to a greater extent in viable skin. Also, the conjugation reactions with glycine to

Table II. Metabolism of Methyl Salicylate in Hairless Guinea Pig Skin[a]

	Viable skin[b]			Nonviable skin[b]
Sex	Salicyluric acid	Salicylic acid	Total	Salicylic acid
Male	21 ± 5	36 ± 6[c]	56 ± 5[c,d]	38 ± 5[c,d]
Female	12 ± 4	12 ± 2[c]	25 ± 3[c,d]	13 ± 3[c,d]

[a] Percentage of absorbed dose metabolized.
[b] Mean ± SE of determinations in 3 animals (3–4 repetitions per animal).
[c] Significant male vs female difference by the two-tailed t test ($P < 0.01$).
[d] Significant viable vs nonviable skin difference, same sex ($P < 0.05$).

Table III. Metabolism of Benzyl Alcohol in Hairless Guinea Pig Skin[a]

	Viable[b]			Nonviable[b]
Sex	Hippuric acid	Benzoic acid	Total	Benzoic acid
Male	8 ± 2	44 ± 8[c]	53 ± 10[c]	30 ± 16
Female	4 ± 2	16 ± 8[c]	20 ± 10[c]	12 ± 6

[a] Percentage of absorbed dose metabolized.
[b] Mean ± SE of determinations in 3 animals (3–4 repetitions per animal).
[c] Marginally significant male vs female difference by the two-tailed t test ($P < 0.07$).

Table IV. Percutaneous Absorption and Metabolism of Retinyl Palmitate

Skin type	Skin[a]		Receptor fluid[a]	
	Radioactivity absorbed (%)[b]	Metabolized (%)[c]	Radioactivity absorbed (%)[b,d]	Metabolized (%)[c]
Guinea pig				
Male	30 ± 4	38 ± 13	0.5 ± 0.2	100
Female	33 ± 2	30 ± 16	0.6 ± 0.3	100
Human				
Female	18 ± 1	44 ± 5	0.2 ± 0.01	100

[a] Mean ± SE of determinations from 2 human donors (3–4 repetitions per donor) and 3 animals (3 repetitions per animal).
[b] Absorption is expressed as % of applied dose in skin and receptor fluid.
[c] Metabolism is expressed as % of the absorbed retinyl palmitate hydrolyzed to retinol.
[d] 0–24 hr fractions were combined.

form either salicyluric acid or hippuric acid proceeded only with viable skin. Therefore, *in vitro* diffusion cell studies cannot accurate measure skin metabolism unless viability of the skin is maintained, and previously reported values for methyl salicylate metabolism (8) are questionable.

Interestingly, the biotransformation of benzyl alcohol to benzoic acid (presumably by alcohol dehydrogenase) occurred in both viable and nonviable skin. Because alcohol dehydrogenase requires a pteridine cofactor for activity, one would expect less enzymatic oxidation in nonviable skin than in viable skin in diffusion cell studies. The sex-related differences in benzyl alcohol oxidation also suggest that an enzyme-mediated reaction occurred. Mouse epidermis has previously been shown to contain enzymes that oxidize retinol to retinoic acid (19).

An important observation in this study was the large difference in the extent of metabolism of methyl salicylate in male and female hairless guinea pig skin. As in other tissues of the body, sex-related differences in metabolism in skin should be considered in the evaluation of topical products from either a pharmacologic or a toxicologic standpoint. The formation of salicylic acid was only significantly greater in male skin at the 6-hr interval (Fig. 1), which is also the interval where the majority of radioactivity was absorbed. It appears that the hydrolytic capacity of female skin is saturated at this early time-point.

Although ester hydrolysis of methyl salicylate was greater for male hairless guinea pig skin than for female guinea pig skin (Table II), no differences were observed for the hydrolysis of retinyl palmitate (Table IV). This inconsistency may be caused by the esterase activity of different groups of enzymes, which have been classified according to the chemical structure of the substrates they prefer (20). Although some substrate specificities overlap, arylesterases may be primarily responsible for the metabolism of methyl salicylate, and other enzymes, carboxyesterases, may metabolize retinyl palmitate in the skin.

Interestingly, in spite of the effects of skin viability and the sex of the animal supplying the skin on metabolism during absorption of chemicals through skin, no significant effects on percutaneous absorption of radioactivity were caused by these different types of hairless guinea pig skin for either methyl salicylate or benzyl alcohol (Table I). The rate of skin permeation for these compounds seems to be determined by diffusion through the stratum corneum, and the primary metabolism seems to occur in the viable tissues beneath the stratum corneum after the rate of permeation has been determined. Metabolism can affect skin permeation when the viable epidermis contributes to barrier function (21).

Retinyl palmitate, a lipophilic ester of retinol, is photostable and can be incorporated into cosmetic products. It was absorbed into the upper layers of human and hairless guinea pig skin, but because it is insoluble in water, it did not readily partition from skin into the diffusion cell receptor fluid (Table IV). A substantial amount of retinol was formed in skin by hydrolysis of retinyl palmitate. Retinoic acid or its hydroxy metabolites were not detected in skin or receptor fluid. The limit of detection of retinoic acid is approximately 10 pmol, as determined by the minimum detectable radioactivity in our HPLC system and the specific activity of the isotope. Thus, amounts of this compound formed but undetected under conditions of this study probably would be of no pharmacological significance. For example, when 0.05% Retin-A cream was applied to the face of human volunteers for 10 hr (2 mg cream/cm^2 skin), 5.2% of the applied dose of retinoic acid was absorbed (22). Therefore, under clinical conditions of use, 0.052 μg retinoic acid (170 pmol)/cm^2 skin was absorbed. This level is 17-fold greater than the level of sensitivity of our method and would be easily detected in our system.

Connor and Smit (11) observed the formation of all-*trans*-retinoic acid after topical application of all-*trans*-retinol *in vivo* in hairless mouse skin. They applied retinol to a large area of skin on each animal (15 cm^2, which is presumably the skin of the whole animal) and subsequently measured very small quantities of retinoic acid in skin extracts (3 pmol/mg epidermis 2 hr post application). Mouse skin is frequently more metabolically active than other rodent and human skin (23,24). This minimal conversion of retinol to retinoic acid would not have been detected in our studies and may not be relevant to the application of formulations containing retinyl palmitate to human skin. In cultured dermal fibroblasts, retinol was metabolized to retinyl fatty acid esters, but retinoic acid formation was not observed (25).

Both retinol and retinoic acid can have pharmacological effects on human skin. Retinol is about 50% as potent as retinoic acid at inducing epidermal hyperplasia in the hairless mouse (26). The induction of ornithine decarboxylase activity in phorbol ester-treated mouse epidermis was inhibited 57% by 0.17 nmol of all-*trans*-retinoic acid and about 50% by 1.7 nmol of retinol (27). In cellular assays, comparisons of retinol and retinoic acid from tissue other than skin have shown larger differences in activity. Retinol was approximately 1000-fold less potent than retinoic acid in the induction of differentiation in a leukemia cell line (28) and a stem cell line (29).

Our studies demonstrate that retinol is formed in hairless guinea pig and human skin after topical application of retinyl palmitate and that if further metabolism to retinoic acid occurs, it is too small to detect. Any biological response of skin treated with retinyl palmitate formulations may be due to ester hydrolysis of the parent compound to retinol.

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