Toxicology and Applied Pharmacology 231 (2008) 117–121

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

In vitro and *in vivo* percutaneous absorption of retinol from cosmetic formulations: Significance of the skin reservoir and prediction of systemic absorption ☆

Jeffrey J. Yourick [a,*], Connie T. Jung [b], Robert L. Bronaugh [a]

[a] U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD 20740, USA
[b] U.S. Food and Drug Administration, Office of the Commissioner, Office of Policy, Rockville, MD 20857, USA

ARTICLE INFO

Article history:
Received 7 January 2008
Revised 8 April 2008
Accepted 10 April 2008
Available online 20 April 2008

Keywords:
Skin
Dermal
Absorption
Retinol
Lipophilic

ABSTRACT

The percutaneous absorption of retinol (Vitamin A) from cosmetic formulations was studied to predict systemic absorption and to understand the significance of the skin reservoir in *in vitro* absorption studies. Viable skin from fuzzy rat or human subjects was assembled in flow-through diffusion cells for *in vitro* absorption studies. *In vivo* absorption studies using fuzzy rats were performed in glass metabolism cages for collection of urine, feces, and body content. Retinol (0.3%) formulations (hydroalcoholic gel and oil-in-water emulsion) containing ^{3}H-retinol were applied and absorption was measured at 24 or 72 h. All percentages reported are % of applied dose. *In vitro* studies using human skin and the gel and emulsion vehicles found 0.3 and 1.3% retinol, respectively, in receptor fluid at 24 h. Levels of absorption in the receptor fluid increased over 72 h with the gel and emulsion vehicles. Using the gel vehicle, *in vitro* rat skin studies found 23% in skin and 6% in receptor fluid at 24 h, while 72-h studies found 18% in skin and 13% in receptor fluid. Thus, significant amounts of retinol remained in rat skin at 24 h and decreased over 72 h, with proportional increases in receptor fluid. *In vivo* rat studies with the gel found 4% systemic absorption of retinol after 24 h and systemic absorption did not increase at 72 h. Retinol remaining in rat skin after *in vivo* application was 18% and 13% of the applied dermal dose after 24 and 72 h, respectively. Similar observations were made with the oil-in water emulsion vehicle in the rat. Retinol formed a reservoir in rat skin both *in vivo* and *in vitro*. Little additional retinol was bioavailable after 24 h. Comparison of these *in vitro* and *in vivo* results for absorption through rat skin indicates that the 24-h *in vitro* receptor fluid value accurately estimated 24-h *in vivo* systemic absorption. Therefore, the best single estimate of retinol systemic absorption from *in vitro* human skin studies is the 24-h receptor fluid value. However, the receptor fluid value from the 72-h extended study may be used in a worst-case exposure estimate. In conclusion, *in vivo* skin absorption studies can be useful in determining whether to include material in the *in vitro* skin reservoir as absorbable material in estimates of systemic absorption.

Published by Elsevier Inc.

Introduction

Retinol (vitamin A) and its esters such as retinyl palmitate are widely used ingredients in cosmetic products. These ingredients are used to improve the appearance of skin by reducing fine lines and wrinkles and to in general improve the appearance of skin. Retinol can be converted in skin by oxidative metabolism to retinal followed by further oxidation to retinoic acid (Bailly et al., 1998; Marill et al., 2003). The topical drug, retinoic acid (tretinoin, Retin-A®), appears to show some effectiveness in treating the appearance of photoaging (Kang et al., 2001). The mechanisms responsible for this action may include the proliferation of keratinocytes resulting in increased shedding of corneocytes (Baumann et al., 2005) and may also be associated with the formation of new collagen in the upper dermis (Gilchrest, 1997). Application of retinol to skin has been reported to induce expression of cellular binding proteins and result in other molecular changes that are similar to those seen after treatment with retinoic acid (Kang et al., 1995). The effects of retinol on skin are thought to be substantially weaker than those seen with retinoic acid and much higher concentrations of retinol are required to produce epidermal thickening and enhanced expression of genes for cellular retinoic acid binding proteins (CRABP) (Kang et al., 1995).

Animal studies have found teratogenicity when high doses of retinol were administered (Geelan, 1979). High vitamin A intake may cause a teratogenic risk associated with increased levels of retinoic acid generated from the metabolism of retinol (Collins et al., 1992).

Only limited skin absorption studies for retinol have been conducted and the systemic absorption of the compound from cosmetic

☆ The opinions and conclusions expressed in this article are solely the views of the authors and do not necessarily reflect those of the Food and Drug Administration. This study was not conducted under the Guidelines of Good Laboratory Practice.

* Corresponding author. Office of Regulatory Science, U.S. Food and Drug Administration, Harvey Wiley Building, HFS-717, room 4E-019, 5100 Paint Branch Parkway, College Park, MD 20740, USA. Fax: +1 301 436 2694.
E-mail address: jeffrey.yourick@fda.hhs.gov (J.J. Yourick).

0041-008X/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.taap.2008.04.006

products is not well understood. The percutaneous absorption of retinyl palmitate was determined in human skin by *in vitro* techniques after application in an acetone vehicle (Boehnlein et al., 1994). At the end of their 24-h study, only 0.2% of the applied dose was absorbed into the receptor fluid beneath the skin and 18% had penetrated the skin but still remained there at the end of the study. The small amount of material in the receptor fluid was completely metabolized to retinol. The *in vivo* and *in vitro* systemic absorption of retinoic acid was determined in human skin after application in a cream (Franz and Lehman, 1990). Systemic absorption of 7.1% of the applied dose was determined *in vivo* after facial application and collection of radioactivity excreted in the urine.

In vitro skin absorption studies have been conducted with excised human skin in flow-through diffusion cells. Retinol was applied to skin in either an oil-in-water (o/w) emulsion or a gel vehicle. Because of the substantial skin reservoir for retinol found at the end of 24-h human skin studies, additional studies were conducted to compare the *in vitro* and *in vivo* skin absorption of retinol in the fuzzy rat. Results from these additional rat studies were used to help interpret the significance of the *in vitro* retinol human skin reservoir in the determination of systemic absorption.

Methods

Chemicals. ^{3}H-Retinol (specific activity; 47 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA) and had a radiochemical and chemical purity of >99%. Retinol, retinoic acid, and retinal were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were reagent grade and obtained from either Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical. HPLC-grade solvents were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). HEPES-buffered Hanks' balanced salt solution (HHBSS) (dry power packets, prepared by Gibco BRL, Life Technologies, Grand Island, NY) was freshly prepared before each study.

Animals. Female fuzzy (Hsd:Fuzzy-fz) rats (Harlan Sprague Dawley, Inc., Indianapolis, IN), 3-14 months of age, were used in these experiments. Rats were acclimatized on arrival for at least three days and screened for evidence of disease. Rats were housed singly in polycarbonate shoe box cages on hardwood chip contact bedding (Sani-Chip®). Animals were provided pelleted rodent chow (Purina® certified #5002C) and water (reverse osmosis-deionized) *ad libitum*. Animal holding rooms were maintained at 21±1 °C with 50±10% relative humidity. At least 10 complete changes per hour of 100% conditioned fresh air were made. The rats were on a twelve-hour light/dark full spectrum lighting cycle with no twilight.

Fresh viable human skin was obtained as a result of abdominoplasty procedures from a local cosmetic surgeon. The skin was placed on ice in HHBSS and transported to the laboratory.

Retinol (0.3%) dosing formulations. ^{3}H-Retinol was added to 0.3% (w/w %) retinol (cold) gel or oil-in-water emulsion for a radioactive dose of approximately 0.7 μCi/cell. The hydroalcoholic gel was 2% hydroxypropylcellulose dissolved in 90% ethanol with 0.1% butylhydroxytoluene (BHT). The oil-in-water emulsion was formulated as 3% polyglyceryl-3-distearate, 3% cetyl stearyl alcohol, 5% propylene glycol, 10% light mineral oil, 78% distilled water, 0.5% methyl-*p*-hydroxybenzoate, 0.5% propyl-*p*-hydroxybenzoate, and 0.1% BHT.

In vitro skin absorption studies. Percutaneous absorption studies were performed in a manner similar to those previously described for flow-through diffusion cells (Kraeling and Bronaugh, 1997; Bronaugh and Collier, 1991). Fuzzy rats were euthanized with carbon dioxide and the fine hair was cut with electric clippers. The dorsal skin was removed and the subcutaneous fat was trimmed away. Human skin sections were removed from the transport buffer (HHBSS) and the subcutaneous fat was removed. The skin was then gently cleaned with a 10% soap solution and thoroughly rinsed with distilled water. A split-thickness layer of rat or human skin (200–320 μm) was prepared with a dermatome (Padgett Instruments, Kansas City Assemblage Co., Kansas City, MO). Discs of dermatomed skin were obtained using a brass punch (13/16 in. diameter). Each skin disc was mounted with the epidermal side up in the diffusion cell (exposed surface area, 0.64 cm^2). The flow-through diffusion cell system was disinfected with 70% ethanol and then flushed with receptor fluid before beginning each study. The receptor fluid used in this study was HHBSS+4% bovine serum albumin+0.001% BHT, pH 7.4 (Collier et al., 1989). The flow rate of the receptor fluid was approximately 1.5 ml/h. The skin surface temperature was maintained at 32 °C by circulating 35 °C water through the diffusion cell holding block. Skin-loaded diffusion cells were allowed to equilibrate for at least 30 min before dosing. The barrier integrity of human skin discs was verified by using the 20-min ^{3}H-water test (Bronaugh et al., 1986). Human skin diffusion cells that exhibited a percent of the applied dose of ^{3}H-water absorbed greater than 0.35% (historical limit) were discarded. The retinol dose (2 mg/cm^2 application amount) was applied to each diffusion cell for 24 h, and then washed off to remove any unabsorbed material. Amounts remaining in the skin and absorbed into the receptor fluid were determined. A fraction collector was used to collect receptor fluid as 6-h fractions for a total of 24 or 72 h. At 24 h after dosing, the retinol remaining on top of the skin as unabsorbed material was removed by washing three times with 0.1 ml of a 10% (v/v) liquid detergent solution that was pipetted onto the skin surface. The skin surface was gently rubbed with cotton-tipped swabs to remove the detergent solution. The skin was rinsed two times with 0.2 ml of distilled water. All cotton swab tips were collected in a scintillation vial as a measure of the amount of material remaining on the skin surface. At the end of the study (24 or 72 h), the skin was removed from the diffusion cell and the amount of retinol remaining in the skin was determined. Skin discs were tape stripped ten times to remove the stratum corneum, thus producing a viable epidermal/dermal disc. Each tape strip was placed into a scintillation vial. Skin discs containing the viable epidermis/dermis were then frozen for skin content analysis later. The procedure for using 10 tape strips to remove the stratum corneum has been extensively used in our laboratory. The number of tape strips necessary to remove the stratum corneum varies by technique and the type of tape used. In 2005, Zhai and others reviewed the subject of tape stripping to remove the stratum corneum and revealed that a range of 7–20 tape strips were cited for the use of Scotch tape (Zhai et al., 2005). In addition, FDA suggests using 12 tape strips to recover topically applied drugs in the stratum corneum when conducting *in vivo* bioavailability and bioequivalence studies for drug products (FDA, 1998).

The viable skin content was determined from the amount of radioactivity in the skin homogenate. Skin discs were thawed and homogenized on ice in HHBSS with a hand-held homogenizer (Omni® Micro Homogenizer, Omni International, Inc., Gainesville, VA) by three–four 15 s bursts at full speed, with each burst followed by approximately 45 s of cooling. The total volume of homogenate was 4 ml. A 1 ml aliquot was removed and mixed with 3 ml of tissue solubilizer (Scintigest®, Fisher Scientific, Fair Lawn, NJ). The digest was incubated in an oven (approximately 60 °C) overnight or until the tissue was dissolved. After the skin was dissolved, the amount of radioactivity was determined by liquid scintillation counting.

In vivo skin absorption studies. An area of application of 3.0×3.0 cm was delineated on the mid-scapular region of the rat. The dosing area was enclosed and protected with a Stomahesive® patch glued directly to the animal's skin. Rats were dosed, the patch covered with a screen, and the animals were placed in metabolism cages for 24 or 72 h to collect urine and feces. The rats were then euthanized. The treated skin site was washed 24 h after application (similar to *in vitro* studies) with soap and water to remove unabsorbed material. The washes were collected to determine the amount of unabsorbed material. The skin dosing site and remaining carcass were each dissolved in concentrated (5 M) potassium hydroxide. Aliquots of the urine, feces, and dissolved carcass were analyzed for radioactivity by liquid scintillation counting.

The distribution of radioactivity was determined in the area of the dosing site. The skin with the patch still intact was cleaned of fat from the underlying tissue. The patch was then removed and the remaining glue on the skin was removed with hexane-soaked cotton swabs. A dermatome was used to create a split-thickness skin section (180–330 μm). The area containing the dosing site was punched into one or two skin

Table 1
In vitro percutaneous absorption of retinol in human skin using gel and oil-in-water emulsion vehicles

Recovery site	24-h gel	72-h gel	24-h emulsion	72-h emulsion
Receptor fluid	0.3±0.1[a]	0.5±0.01[b]	1.3±0.1	2.2±0.2[b]
Stratum corneum	3.5±0.4	2.8±0.8	5.9±1.4	4.8±0.8
Viable skin	2.1±1.2	1.0±0.1	3.0±0.6	2.9±0.6
Total in skin	5.7±0.8	3.8±0.7[b]	8.9±2.0	7.8±1.4
Total penetration	6.0±0.9	4.3±0.7[b]	10.2±2.2	9.9±1.6
Recovery	87.3±6.3	95.9±0.2	94.8±2.6	96.3±5.3

Values are the mean±SE for skin from 2 human subjects (3 replicates per skin sample).
[a] Percent of applied dose absorbed.
[b] Significantly different from 24-h value, $p<0.05$.

Table 2
In vitro percutaneous absorption of retinol in fuzzy rat skin using a gel vehicle

Recovery site	24 h	72 h
Receptor fluid	6.0±2.3[a]	12.9±3.9[b]
Stratum corneum	4.2±1.0	3.3±0.5
Viable skin	18.9±1.9	14.6±2.4
Total in skin	23.1±1.5	17.9±2.7
Total penetration	29.1±2.5	30.8±6.1
Recovery	93.6±5.2	98.5±2.0

Values are the mean±SE for 3 rats (3–4 replicates for each rat).
[a] Percent of applied dose absorbed.
[b] Significantly different from 24-h value, $p<0.05$.