DEVELOPMENT OF POLYETHOXYLATED RETINAMIDE AS AN ANTI-AGING AGENT

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Abstract

A novel retinol derivative, polyethoxylated retinamide (Medimin A) was synthesized, as an anti-aging agent. Collagen synthesis, skin permeation, stability, and toxicity of Medimin A were evaluated and compared with those of retinol and retinyl palmitate.

In vitro collagen synthesis was evaluated by quantitative assay of [^{3}H]-proline incorporation into collagenase sensitive protein in fibroblast cultures. For *in vitro* skin permeation experiments, Franz diffusion cells (effective diffusion area: 1.766 ㎠) and the excised skin of female hairless mouse aged 8 weeks were used. The stabilities of retinoids were evaluated at two different temperature (25 ℃ and 40 ℃) and under UV in solubilized state and in O/W emulsion. To estimate the safety, acute oral toxicity, acute dermal toxicity, primary skin irritation, acute eye irritation and human patch test were performed.

The effect of Medimin A on collagen synthesis was similar to that of retinol. The skin permeability of Medimin A was higher than those of retinol and retinyl palmitate. The Medimin A was more stable than retinol and retinyl palmitate. Medimin A was nontoxic in various toxicological tests.

These results suggest that Medimin A would be a good anti-aging agent for enhancing bioavailability and stability.

Key words

polyethoxylated retinamide (Medimin A), collagen synthesis, skin permeability, stability, toxicity

1. Introduction

Vitamin A and its derivatives are essential for normal skin development. Retinol is required for normal epidermal cell growth and differentiation and is an important regulator of keratinocyte

terminal differentiation (1). Retinol induces thickening of epidermis and thinning the stratum corneum and is effective for the treatment of skin disease (2). It is also known that retinol can alter or modulate total collagen synthesis (3-5). Also retinoic acid has demonstrated to alter the type of collagen synthesized (6). Retinol has the potential to alter the expression of protein molecules in both the epidermis and the dermis. All these effects of topically applied retinol tend to oppose changes that occur with aging, maintain the skin in more youthful condition and suggest its use in anti-aging products (7-9). The anti-aging effect on human skin after treatment with retinoic acid is convincingly documented in a number of open and controlled study (10-13).

Retinol is known as potential anti-aging agent. However, some problems that arose in using retinol in cosmetic formulation were its stability, permeability, and toxicity.

A main problem with the use of retinoids in cosmetics can be the side-effect of the dermatitis type after prolonged use (11). The known toxicity of this class of compounds(14) have prompted a continuing search for retinoids with decreased undesirable side effect. It has been published that retinoids are unstable in light, oxygen, temperature, lipid peroxide and water (15-17) and the poor stability of retinol does not allow its incorporation in cosmetic product (18). To increase the stability and decrease the toxicity of retinol, retinyl esters (such acetate, propionate, and palmitate) are employed in cosmetic formulation, as precursors of retinoic acid, but such retinol derivatives are so lipophilic that it is difficult to penetrate through skin (19). It can therefore not be expected to have clinical efficiency in treatment of wrinkles and other aging symptoms of the skin. Previous studies have pointed out that suitable substances such as complex polysaccharide or polyethylene glycol (PEG) have to used to increase the skin permeability of highly lipophilic substances (20-21). Conjugation with complex polysaccharide or PEG will enhance the skin permeability of lipophilic molecule and at the same time protect the substance against oxidation (degradation).

Polyethoxylated retinamide (Medimin A, average MW:831) has been developed by coupling retinoic acid with PEG to enhance skin permeability and stability.

In the present study, collagen synthesis, skin permeability, the stability, and skin toxicity of Medimin A were evaluated and compared with those of retinol and retinyl palmitate which are broadly used in cosmetics.

2. Experimental

2.1 Materials & Preparations

Retinol was purchased from Aldrich Chem. Co. (Belgium). Retinyl palmitate was purchased from Roche (Swiss). Medimin A was synthesized from LG chemical Ltd. (Korea). DMEM, fetal

bovine serum, streptomycin, penicillin were all purchased from GibcoBRL (USA). L-[2,3-^{3}H]-proline was from Amersham (USA). MTT, potassium phosphate, sodium chloride, and sodium phosphate were obtained from Sigma Co. (USA). HPLC grade methanol, methylene chloride, acetonitril were all purchased from J.T.Baker Chemical Co. (USA). Polyoxyethylene oleyl ether (Volpo 20) was from Croda Inc. (USA). Caprylic capryl triglyceride was obtained from Inolex Chemical Co. (USA). All other ingredients were reagent or cosmetic grade.

Caprylic capryl triglyceride solution containing 35mM of retinol, retinyl palmitate, or Medimin A were prepared for in vitro skin permeation experiments. Solubilized states and O/W emulsions containing 0.1 % of retinol, retinyl palmitate, or Medimin A were prepared for stability studies. Formulas are shown in table 1.

Table 1. Formulas for the Stability of Retinoids.

Solubilized states		**O/W Emulsion**	
POE(25) Octyldodecyl ether	3.0	Cetearyl alcohol	0.7
POE(40) Hydrogenated caster oil	1.0	Glyceryl stearate SE	1.5
Medimin A (or Retinyl palmitate)	0.1	Glyceryl stearate (and) PEG-100 stearate	1.0
Ethanol	10.0	Mineral oil	10.0
Water	to 100	Trioctanoin	2.0
		Polysorbate 60	1.5
		Sorbitan steatate	0.3
		Medimin A (or Retinyl palmitate or Retinol)	0.1
		Propylene glycol	5.0
		TEA	0.15
		Carbomer	0.12
		water	to 100

2.2 Collagen Synthesis

Determination of collagen synthesis was performed according to the method previously described (22). Human fibroblasts (WI-38 fibroblast) were cultured in T-75 flask (Felcon, USA) with DMEM (Dulbecco's Modified Eagle's Medium), supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 μg/ml) in a humidified atmosphere of 5 %

CO_2, 95 % air. The cells were initially seeded at 5 × 10^4 cells per well in microplates (Costar, USA) and incubated for 24 hrs. The cells were treated with retinoic acid or Medimin A in medium containing 3 μCi L-[2,3-^{3}H]-proline and the culture plates were incubated for further 24 hrs. At the end of incubation, culture supernatants were collected and treated with purified collagenase. Radioactivity incorporated into collagenase sensitive protein was measured over a 2 min period in a Beckman liquid scintillation counter.

2.3 Skin Permeation Studies

Vertically assembled Franz type diffusion cells (Microette transdermal diffusion system, Hanson Research Corporation, Chatsworth, CA, USA) were used for in vitro skin permeation experiments. The system consisted of Franz type diffusion cells with an effective diffusion area of 1.776 ㎠ and receptor volume of 7.0 ml, autosampler, and cell drive system with rpm controller. The fundamental experiments were performed according to the method given in our previous report (23). Briefly, the excised skin of female hairless mouse was obtained from 8-9 weeks old, 27-33 g animals. The skin was mounted on diffusion cell, and the receiver compartment was filled up with 7 ml of 50 mM phosphate buffer saline pH 7.4 (PBS) with 2 % Volpo 20 (a nonionic surfactant, HLB=16) and maintained at 32 ℃ by circulating water within a jacket around the lower chamber. Volpo 20 was used to insure that solubility in receptor solution would not limit penetration through skin. Diffusion characteristics of skin are unaffected by Volpo 20 exposure. Samples containing retinoids were applied in the donor compartment. Caprylic capryl triglyceride solution was uniformly distributed with a micropipette on the skin surface (20 μℓ). The receptor fluid was mixed by a magnetic stirrer throughout the experiment. The receptor fluid was collected from the receiver compartment at predetermined time (every 6 hours after sample application) and replaced by fresh fluid.

At the end of the experiment (24 hour after sample application), receptor fluid was collected, and donor compartment was washed with 500 μℓ of methanol three times. After completion of the preset time (24 hour), skin samples were taken out of diffusion cells. The skin was homogenized by 4 ml of methylene chloride to extract retinoids. After filtration on Millex filter FG (pore size: 0.2 ㎛, millipore), solutions were assessed by HPLC. Five hundred microliters of receptor fluid withdrawn from the receiver compartment at predetermined times was treated with 1 ml of methylene chloride and shaken by a vortex mixer. Following centrifugation (13,000 rpm), the supernatant was subjected to HPLC.

2.4 Stability Studies

To measure the temperature stabilities, retinol, retinyl palmitate, and Medimin A were prepared

in solubilized state and in O/W emulsion. Samples were stored at 25 ℃ and 40 ℃ up to 2 months. Periodically, 50 $\mu\ell$ aliquots of each sample were pipetted out and diluted with methanol. The amount of residual retinoids were measured by HPLC.

To measure the UV stabilities of retinol and Medimin A, 1 g aliquots of O/W emulsion containing retinol or Medimin A were divided into amount test tubes. UV was illuminated to the samples by artificial light generator (SUN TEST CPS, Heraeus Co.) for 30 min. The amount of residual retinoids were measured by HPLC.

2.5 High Performance Liquid Chromatography

The HPLC consisted of solvent delivery pump (Waters, 600 pump, Waters Co., MA, USA), UV detector (waters, 486 UV detector) and data process system (Waters millennium). Medimin A was analyzed with C_8 column (Waters Symmetry 5 μm, 4.6 × 150 mm, Millipore, USA), the mobile phase of 65 % acetonitril in 0.05 % phosphoric acid and at the flow rate of 1 ml/min. The absorbency at 350 nm was measured for the assay of Medimin A. The retention time was 10.5 min for Medimin A. For retinol and retinyl palmitate analysis, the mobile phase was 85% methanol in 0.05 % phosphoric acid, 100 % Methanol and flow rate was 1 ml/min, 1.4 ml/min for retinol and retinyl palmitate, respectively. C_{18} column (Waters Symmetry 5 μm, 4.6 × 150 mm, Millipore, USA) and wavelength of 326 nm was selected. The retention time was 9.6 min for retinol and 10.5 min for retinyl palmitate. Temperature of the column was kept at 40 ℃

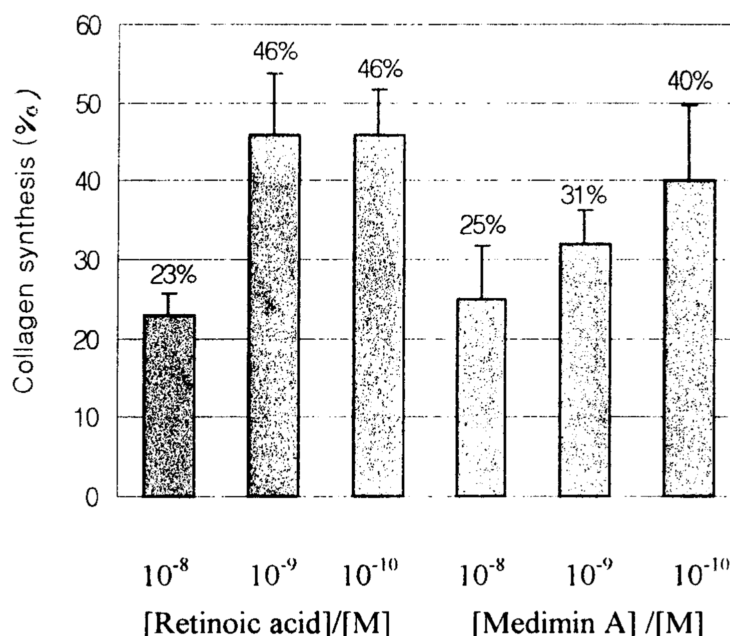
2.6 Safety

To estimate the safety, acute oral toxicity, acute dermal toxicity, primary skin irritation, acute eye irritation and human patch test were performed.

3. Results and Discussion

3.1 Collagen Synthesis

Figure 1 represents the increase in collagen synthesis by retinoic acid or Medimin A treatment. There were 23 %, 46 %, 46 % increases in collagen synthesis at 10^{-8} M, 10^{-9} M, and 10^{-10} M retinoic acid, respectively. There were 25 %, 31 %, 40 % increases, at 10^{-8} M, 10^{-9} M, and 10^{-10} M Medimin A, respectively. There was maximum 46 % increase at 10^{-10} M retinoic acid and 40 % increase at 10^{-10} M Medimin A. This result indicates that the effect of Medimin A on collagen synthesis is similar to retinoic acid and function of retinoic acid is remained.



10^{-8} 10^{-9} 10^{-10} 10^{-8} 10^{-9} 10^{-10}

[Retinoic acid]/[M] [Medimin A] /[M]

Figure 1. Collagen synthesis increased after retinoic acid or Medimin A treatment.

3.2 Skin Permeation Study of Retinoids

Table 2 shows the permeated amounts of retinol, retinyl palmitate, and Medimin A in oil (Caprylic capryl triglyceride) 24 hours after application to the excised hairless mouse skin. The skin permeability of Medimin A was higher than those of retinol and retinyl palmitate. The permeated amount of Medimin A was 3 times greater than that of retinol, and 6 times higher than retinyl palmitate. It was considered that this result was due to increase in partition of Medimin A to skin by PEG conjugation. It has already been reported that conjugation with PEG or a complex polysaccharide enhance the water solubility and the skin permeability of lipophilic molecule and at the same time protect the substance against oxidation (degradation) (19-20).

Table 2. Total Permeated Amount (%) of Retinoids Across the Excised Hairless Mouse 24 Hours After Application.

(nmole)

	Retinol	Retinyl palmitate	Medimin A
skin	23.38	18.73	54.17
Receptor Solution	11.48	0.48	62.72
total permeated amount	34.86	19.21	116.87
(%)	(5.0 %)	(2.5 %)	(15 %)

3.3 Stability Studies

Figure 2 and 3 demonstrate the stabilities of retinyl palmitate and Medimin A in solubilized state and in O/W emulsion during 2 months storage at 25 ℃ and 40 ℃. Medimin A was more stable than retinyl palmitate, a broadly known stable retinol derivative. Both retinyl palmitate and Medimin A were more stable in O/W emulsion than in solubilized solution. This indicates that retinoids are less stable in water.

Figure 4 shows stabilities of retinol, retinyl palmitate and Medimin A in O/W emulsion under UV treatment during 30 minutes. This results show the stability against light of retinol and Medimin A is low, However, Medimin A was more stable than retinol under UV. We assume that blocking of functional group enhances stability of molecule. It has already been reported that conjugation with fatty acid, complex polysaccharide or PEG protected the substance against degradation (20).

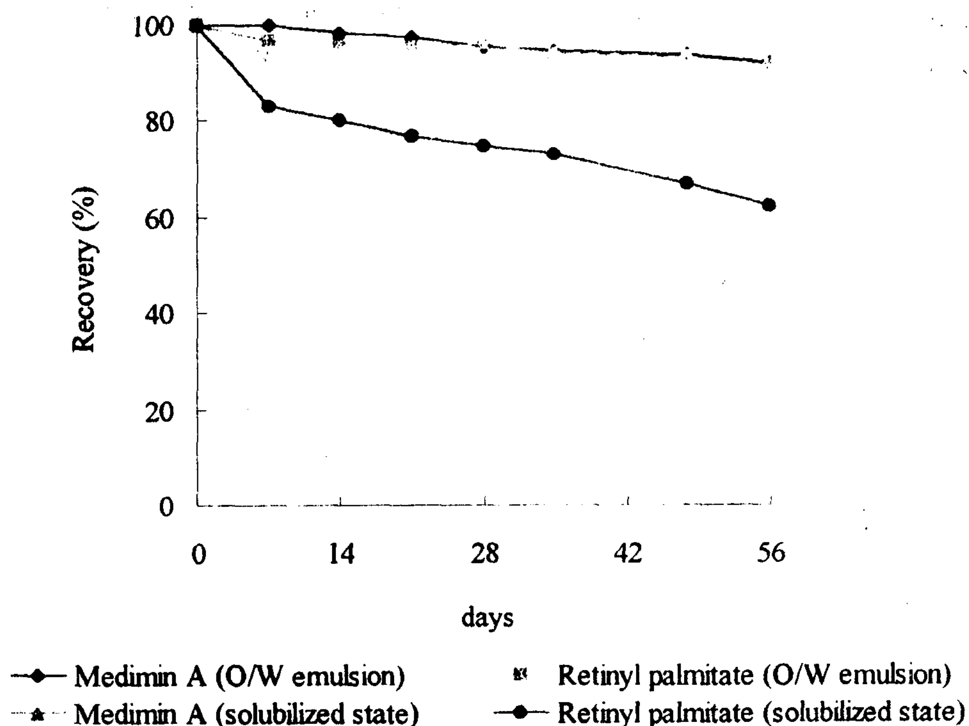


Figure 2. Recovery percent of Medimin A and retinyl palmitate in solubilized state and in O/W emulsion stored at 25 ℃.

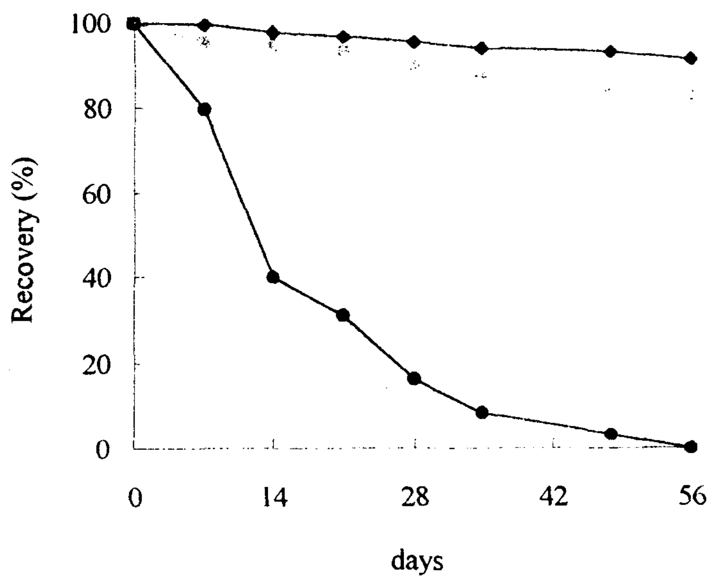


Figure 3. Recovery percent of Medimin A and retinyl palmitate in solubilized state and in O/W emulsion stored at 40 ℃.

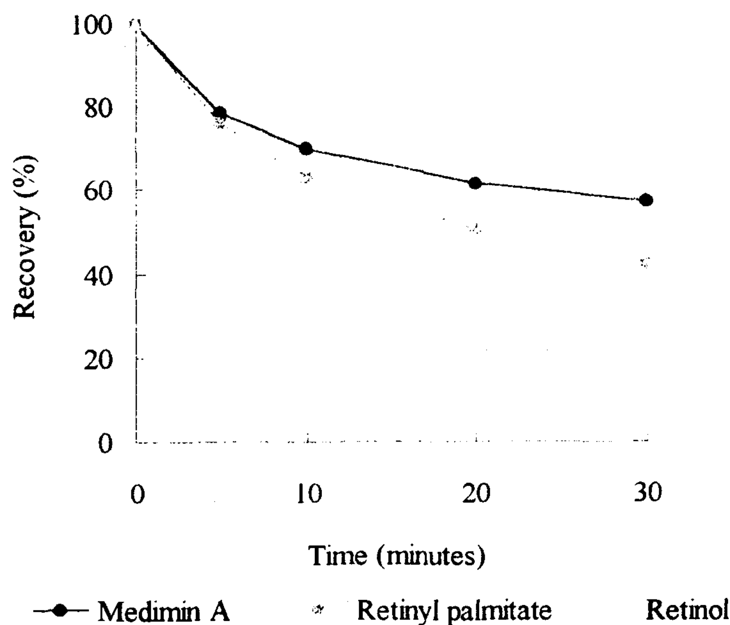


Figure 4. UV Stability of Medimin A, retinyl palmitate and retinol in O/W emulsion.

3.4 Safety

To estimate the safety of O/W emulsion containing 0.5 % Medimin A, various safety tests were carried out. Medimin A was safe in these tests. Medimin A was nontoxic in various toxicological tests proving that it can be safely introduced to the skin care formulations.

3.4.1 Acute Oral Toxicity in rats

When 10 g of O/W emulsion containing 0.5 % Medimin A was administered orally per kg of healthy Sprague-Dawley rats, neither observable symptom nor death was observed.

3.4.2 Acute Transdermal Toxicity in rats and rabbits

When 10 g/kg of O/W emulsion containing 0.5 % Medimin A was applied to the skin of selected healthy Sprague-Dawley rats and New Zealand white rabbits, no observable change was observed.

3.4.3 Primary Skin Irritation Test in rabbits

The PII (Primary Irritation Index by Draize) value was 0 in rabbit. Thus, the test material was practically non-irritant.

3.4.4 Ocular Irritation in rabbits

The M.O.I.(Mean ocular Irritation Index) of O/W emulsion containing 0.5 % Medimin A was "zero", hence Medimin A was non-irritant.

3.4.5 Skin Sensitization Test in Guinea Pigs

According to the Kligmam grading for skin sensitization, Medimin A scored grade 0, proving the Medimin A to be highly safe.

4. Conclusion

In conclusion, the effect of Medimin A on collagen synthesis was similar to that of retinol. The skin permeability of Medimin A was higher than those of retinol and retinyl palmitate. The Medimin A was more stable than retinol and retinyl palmitate. Medimin A was sufficiently safe for cosmetic use. We suggest that Medimin A could be a novel anti-aging agent with enhanced bioavailability and stability.

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