

SAFETY EVALUATION OF PHYTOSPHINGOSINE AND CERAMIDES OF PHARMACEUTICAL GRADE

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Synopsis

Ceramides are the most prominent lipids found in the stratum corneum and play a fundamental role in maintaining in healthy condition the skin barrier. Their decreasing levels, securely impairing this barrier, are accompanied by skin dryness, scaling, roughness that lead to pathological conditions.

For these reasons ceramides are considered important raw materials of pharmaceutical and cosmetic use. The aim of this study was to evaluate the toxicological and dermatological safety of phytosphingosine and ceramide-identical 1, 2, 3A, 3B and 6 produced by fermentation methodology.

Raw material' toxicological safety was performed using Umes test, acute oral and dermal toxicity (rat), eye skin irritancy and skin sensitization (rabbit) and human skin membrane irritancy.

Safety and efficacy of the cosmetic preparation, containing ceramide 6 and phytosphingosine with Vitamin C or Betacarotene, was controlled in a double blind randomized study on 30 volunteers (female subjects, 37 to 53 years old) and evaluated during a three months period using both clinical scores and the 3C System instrumental method. According to EEC Criteria (DIR 93/21, 27/4/93) the controlled ceramides have no obligatory labelling requirements for oral and dermal toxicity, eye irritation as well as for skin sensitization. The cosmetic preparations studied did not lead to any unwanted skin reactions and a progressive and significant ($p < 0.005$) increase of skin hydration (+87%), skin suppleness and firmness (from 23 to 42%) and fine wrinkle improvement (from 18 to 29%), with a TEWL decrease of 40% has been observed during the treatment period.

Riassunto

Le ceramidi rappresentano la parte predominante dei lipidi presenti a livello dello Strato Corneo. Una riduzione del loro livello sicuramente influenza l'integrità della barriera cutanea che può apparire secca, fessurata ed irritata, fino a raggiungere condizioni patologiche. Per queste ragioni le ceramidi sono considerate importanti materie prime d'uso sia farmaceutico che cosmetico.

Lo scopo di questo studio è stato di valutare la sicurezza tossicologica e farmacologica della fitosfingosina e delle ceramidi-identiche 1, 2, 3A, 3B e 6, prodotte per fermentazione batterica.

La sicurezza tossicologica di queste materie prime è stata valutata in-vitro mediante l' UMES Test, ed in-vivo, valutando la tossicità orale acuta, la tossicità dermica, quella oculare su animali, ed eseguendo le prove di irritabilità e di sensibilizzazione allergica sull'uomo.

La sicurezza e l'efficacia di un prodotto cosmetico finito, contenente ceramide-6 e fitosfingosina associate con Vitamina C o Betacarotene, è stata controllata a doppio cieco, su 30 volontarie donne di età compresa tra 37 e 53 anni, durante un periodo di 3 mesi, utilizzando sia la metodica del punteggio che il 3C System. In accordo con i criteri europei (DR 93/21, 27/4/93) le ceramidi utilizzate non richiedono un'etichettatura particolare. Le creme cosmetiche studiate si sono rivelate sicure nell'uso ed hanno mostrato di incrementare rispettivamente del 87% ($p < 0.005$) l'idratazione cutanea, dal 23 al 42% l'elasticità e di ridurre dal 18 al 29% le rughe sottili, riducendo la TEWL del 40% circa.

INTRODUCTION

Ceramides, a special class of sphingolipids, are the most prominent lipids found in the stratum corneum and play a fundamental role in maintaining in healthy condition the skin barrier. Epidermal sphingolipids represent 7,3% of total lipid in the basal layers, increasing to about 15% in the stratum granulosum, 30% in lower stratum corneum, and reaching 40% in the other stratum corneum. (1,2).

Their decreasing levels, moreover, impair the skin barrier function involving skin dryness, scaling, roughness and various skin disorders (3-6).

Decreased levels of ceramides have been also correlated with increasing age and seasonal variation (7-9). For these reasons ceramides are considered important raw material of pharmaceutical and cosmetic use.

AIMS

The aim of this study was to evaluate the toxicological safety and the dermatological efficacy of different kind of ceramides (1, 3, 3A, 3B, 6) and phytosphingosine obtained biotechnologically by fermentation methodologies and, therefore, having the same molecular configuration as human ceramides.

MATERIAL AND METHODS

Materials

Phytoceramide 1, Ceramide 3, 3A and 3B, 6 and Phytosphingosine were of pharmaceutical grade (Cosmoferm Gist-Brocades group NL).

TOXICITY STUDIES AND SAFETY EVALUATION

All the studies were performed according to international accepted guidelines (OECD/EU) and are in compliance with the principles of Good Laboratory Practice (FDA/OECD). (10-12)

The following safety studies were carried out:

Ames Test

The compounds were tested in the Salmonella typhimurium reverse mutation assay with two or four histidine-requiring strains of Salmonella typhimurium strains. Phytoceramide 1, was also tested in Escherichia coli reverse mutation assay with tryptophan-requiring strain of Escherichia coli WP2uvrA.

Two independent experiments were performed. Negative and positive controls were run simultaneously with tests.

Acute oral toxicity

The compounds were administered by oral gavage to three (or five) Wistar rats of each sex at 2000 mg/kg body weight (or 5000 mg/kg body weight, Ceramide 3).

Animals were subjected to daily observations and weekly determination of body weight. Macroscopic examination was performed after terminal sacrifice (day 8 or 15).

The result of this test is expressed in a LD50 value (lethal dose for 50% of test animals).

The obtained results are reported in Table I.

Primary skin irritation

Three rabbits were exposed to 0.5 grams of Ceramide, applied to the intact skin of the shaved area on one flank for 4 hours using a semi-occlusive dressing. Observations were made 1, 24, 48 and 72 hours after exposure.

The obtained results are reported in table 1.

Eye irritation

Single samples of the test substances were instilled into the eye of each of the three rabbits. Observation were made 1, 24, 48 and 72 hours after instillation. The obtained results are reported in table I.

Skin sensitisation

The potential of the compounds to cause delayed contact hypersensitivity in guinea-pigs was assessed

Table I

TOXICITY TESTS RESULTS

	Mutagenicity Ames Test	Acute oral toxicity	Primary skin irritation	Eye irritation	Skin sensitisation
<i>Phito Ceramide</i>	<u>Non mutagenic</u> SALMONELLA TYPHIMURIUM ESCHERICHIA COLI In presence and absence of S9 mix	<u>No mortality</u> Wistar Rat > 2000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT	<u>Non irritating</u> Rat > 2000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT	<u>Light irritation</u> Rabbits 28 mg NO OBLIGATORY LABELLING REQUIREMENT	<u>No sensitisation</u> Guinea-pig conc. 50% mg NO OBLIGATORY LABELLING REQUIREMENT
<i>Ceramide-3</i>	<u>Non mutagenic</u> SALMONELLA TYPHIMURIUM In presence and absence of S9 mix	<u>No mortality</u> Female Rats > 5000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT	<u>Non irritating</u> Rabbits > 5000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT	<u>Light irritation</u> Rabbits 27 mg NO OBLIGATORY LABELLING REQUIREMENT	<u>No sensitisation</u> Guinea-pig conc. 5/25% mg NO OBLIGATORY LABELLING REQUIREMENT
<i>Ceramide-3A</i>	<u>Non mutagenic</u> SALMONELLA TYPHIMURIUM In presence and absence of S9 mix	<u>No mortality</u> Rats > 2000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT	<u>Midly irritation</u> Albino Rabbits > 5000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT	<u>Light irritation</u> Rabbits 47 mg NO OBLIGATORY LABELLING REQUIREMENT	
<i>Ceramide-3B</i>	<u>Non mutagenic</u> SALMONELLA TYPHIMURIUM In presence and absence of S9 mix	<u>No mortality</u> Rats > 2000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT	<u>Non irritating</u> Albino Rabbits > 5000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT	<u>Light irritation</u> Rabbits 47 mg NO OBLIGATORY LABELLING REQUIREMENT	
<i>Ceramide-6</i>	<u>Non mutagenic</u> SALMONELLA TYPHIMURIUM In presence and absence of S9 mix	<u>No mortality</u> Rats > 2000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT	<u>Non irritating</u> Albino Rabbits > 5000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT	<u>Light irritation</u> Rabbits 51 mg NO OBLIGATORY LABELLING REQUIREMENT	
<i>Phyto Sphingosine</i>	<u>Non mutagenic</u> SALMONELLA TYPHIMURIUM In presence and absence of S9 mix	<u>No mortality</u> Rats > 2000 mg/kg	<u>Non irritating</u> Albino Rabbits > 5000 mg/kg NO OBLIGATORY LABELLING REQUIREMENT FOR PREPARATION CONTAINING 5% (v/v)	<u>Severely irritation</u> Rabbits 41 mg CARE SHOULD BE TAKEN WHEN HANDLING POWDER	

Table I.

by the Magnusson-Kligman Maximization Test. Test substance concentrations selected for the main study were based on the results of a preliminary study. The obtained results are reported in table 1.

HUMAN PATCH/TEST

The potential of the test compounds to cause irritation on the healthy human skin was assessed by the

Human patch test. A total of 44 subjects (males and females) were subjected to the test substances in 5% vaseline solution. 0.1 g of the test sample was placed in a finn chamber and 24-hour occlusive application on the flexor side of the subject's upper arm was concluded. After 24 hours, the test sample was removed and the skin condition was examined, both 1 hour and 24 hours after removal. Skin reactions as erythema and oedema were scored.

COSMETIC PREPARATION

A cosmetic formulation was formulated using liposomes composed of an high proportion of lipophilic phospholipids.

The liposomal fraction of the emulsion tested contained: ceramide-6 and Phytosphingosine, cholesterol, linoleic acid and ascorbic acid (active 1)¹ - cream A) or the same components plus betacarotene instead of vitamin C (active 2)² - cream B).

STUDY DESIGN

Thirty healthy volunteers (female subjects, age range 37 to 53) participated in the study. Each women had at least a moderate degree of photodamage as defined by an overall score of 5 with separate scores for each side of the face on a visual analogue scale 0 (none) to 10 (severe).

The number of wrinkling improvement were also counted. No topical retinoid use or other topical medications or cosmetic products were allowed for 1 month prior to study initiation.

The subjects were observed weekly for three months (september-november 1988) always by the same investigator. Each volunteer was supplied with two tube A (active 1) or B (active 2) together with a cleansing cream.

All were instructed to apply the test creams on their face twice a day (morning and evening) for three months and they were not allowed to use any other skin care product during the study period. Each subject was used as her own control; the test cream (A and B) being applied, always on a randomized basis, on the same right or left area of the face.

CLINICAL EVALUATION

Clinical evaluation were performed on the day 1 (baseline), and 2,4,6,8,10 and 12 weeks(end of treatment). Control of photoaging was controlled evaluating, by a visual analogue scale, the degree of fine wrinkles in the crow's feet area (lateral-periorbital area) and overall photoaging in the whole face right or left of the subjects. Finally the means for each

criterion was calculated.

The obtained results are reported on figure 4.

BIOPHYSICAL MEASUREMENTS

Skin surface lipids

The skin surface lipids were controlled by means of the computerized 3C System (13) (Dermotech, Roma, Italy). On the 1st day (baseline) and at 2,4,6,8,10 and 12 week. Determination is based on photometric measurement of light trasmission through a surface imprint obtained applying to the designed skin area a frosted plastic foil.

It allows adherence of skin lipids in a 1 cm² area. The obtained readings (4 means values) automatically converted into μ g/ cm² are reported in figure 1.

Skin hydration

Skin hydration of the horny layer was assessed by measuring electrical capacitance of the skin surface by the 3C System (13).

When the probe is applied to the skin (recording time 0.5 s.), the capacitance is displayed digitally in arbitrary 3C units. The results, expressed as means values of the measurements performed on 4 different right or left sites (cheek, forehead, chin and nose), are reported in figure 2.

Trans Epidermal Water Loss (TEWL)

Water evaporating from the skin (TEWL) was measured quantitatively by the 3C System methodology (13), after a 30-minute acclimatization period in a room at 22± 2°C and 50% humidity. Measurements were performed on the 1st day (baseline value) and after 2,4,6,8,10 and 12 week (end of treatment). The TEWL probe consists of a cylindrical open chamber measuring system diameter 14mm. height 10mm. and skin area 0.95 cm².

Two sensor units, containing thin capacitative film transducer are placed at 3 and 7 mm. distance from

1. Marketed under the name Idroskin C®/ Kera C®

2. Marketed under the name Kera A®

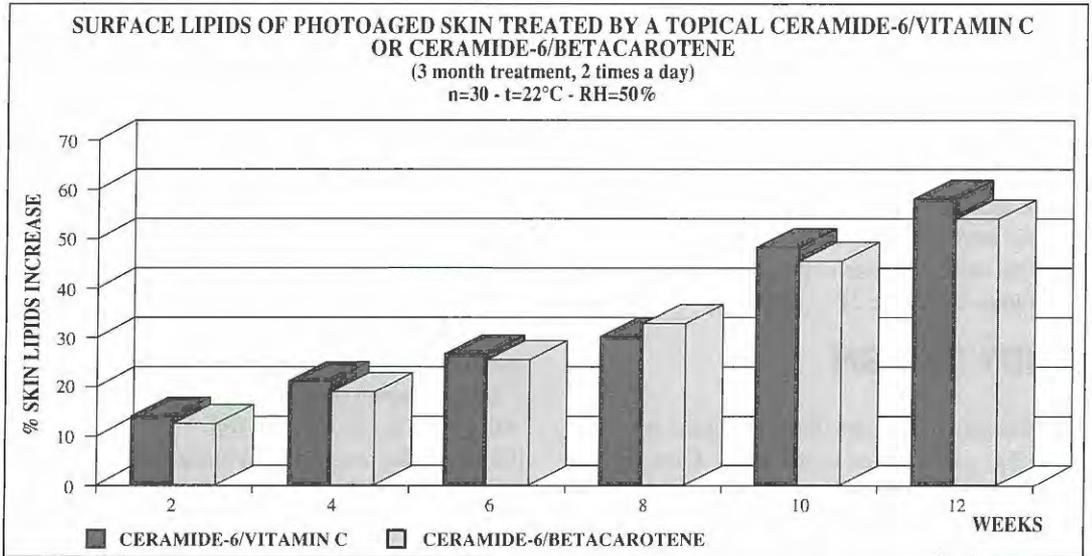


Fig. 1. All *p* values are highly significant ($p < 0.005$) as baseline values. VITAMIN C/CERAMIDE-6 versus CERAMIDE-6/BETACAROTENE not-significant.

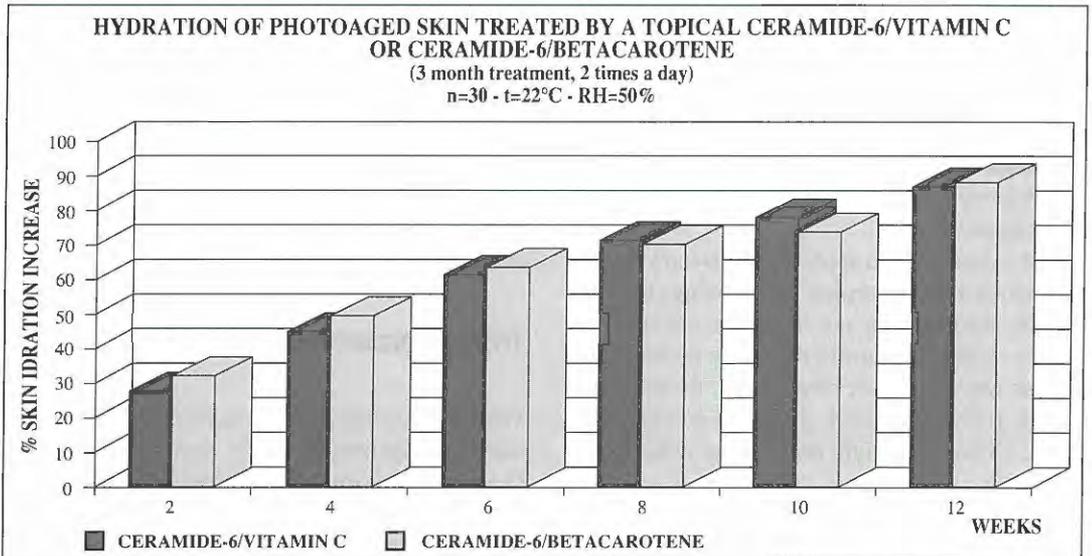


Fig. 2. All *p* values are highly significant ($p < 0.005$) as baseline values. VITAMIN C/CERAMIDE-6 versus CERAMIDE-6/BETACAROTENE not-significant.

the skin surface. TEWL is calculated automatically in g/ m² h.

The obtained results are reported in figure 3.

Skin firmness

Skin firmness was evaluated measuring the skin elasticity by the use of a torsional equipment (14). (Der-

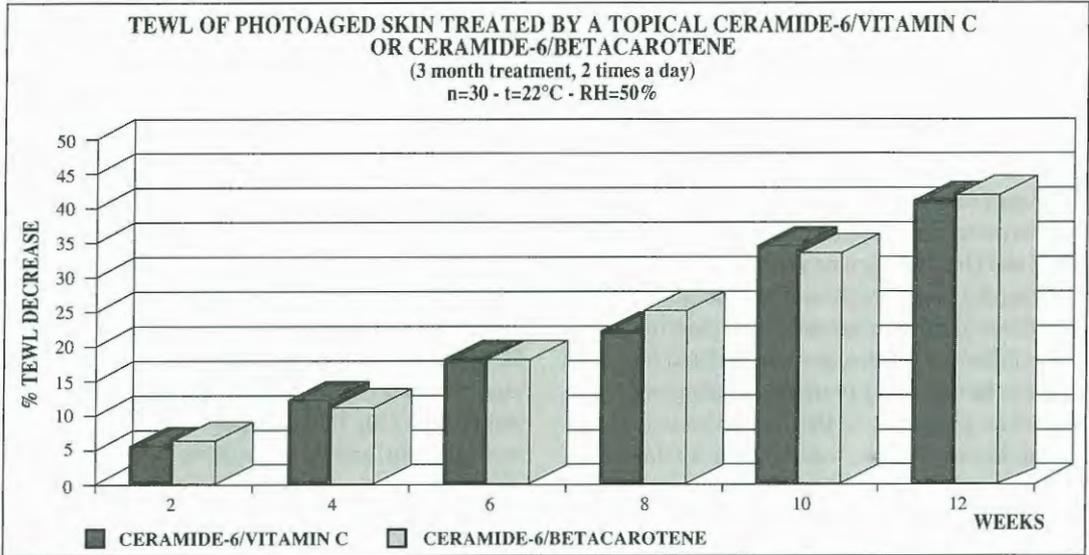


Fig. 3. All *p* values are highly significant ($p < 0.005$) as baseline values. VITAMIN C/CERAMIDE-6 versus CERAMIDE-6/BETACAROTENE not-significant.

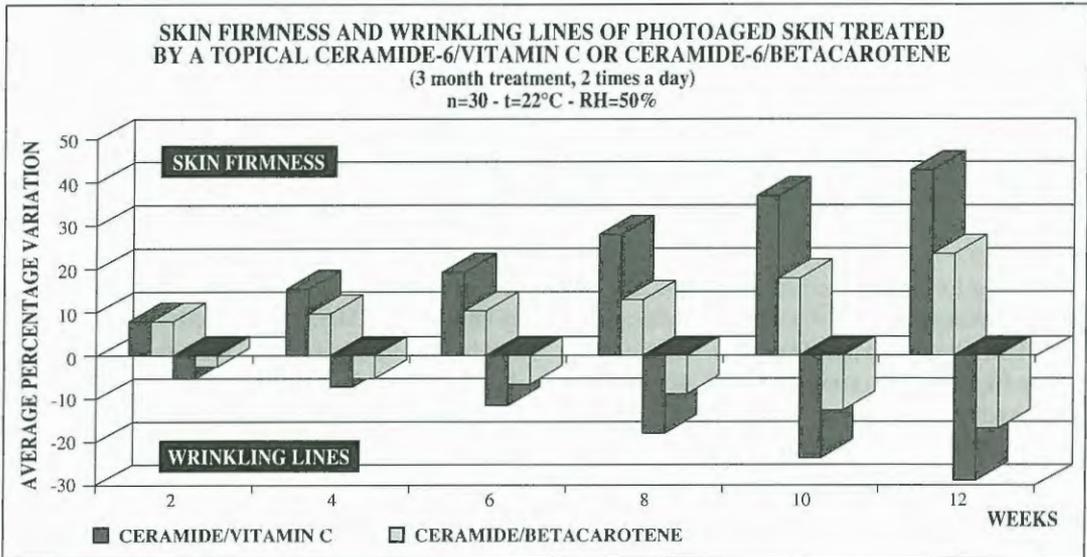


Fig. 4. All *p* values are highly significant ($p < 0.005$) as baseline values and as to groups. VITAMIN C/CERAMIDE-6 versus CERAMIDE-6/BETACAROTENE is also significant ($p < 0.005$).

mal Torque Meter, Diastrum, Ltd, Andover U.K.) Torsional equipment acts through a disk glued to the skin, which is rotated by a motor powered by a controlled voltage, thereby loading the peripheral

skin with a torque, the value of which can be evaluated.

The relative obtained signals are digitalized and a micro processor both computes the main parameters

and controls automatically the measurement phases. The obtained results are reported in figure 4.

RESULTS AND COMMENTS

None of the compounds tested was mutagenic in the Ames test.

Furthermore, the oral LD50 value of all ceramides tested and phytosphingosine was considered to exceed 2000 mg/kg body weight and the compounds were found not to be skin sensitizers or skin irritants. The studied ceramides, in unformulated form, are found to be minimally or mildly irritating to the eye. However, according to the EEC criteria for classification and labelling requirements for dangerous substances and preparations (Guidelines in Commission Directive 93/21/EEC), they do not have to be classified and have no obligatory labelling requirements for eye irritation.

Phytosphingosine (the pure, unformulated product), in the contrary, was considered to be a severely irritating to the rabbit eye.

Based on European legislation, however, preparations containing less than 5% (volume/volume) of Phytosphingosine need not to be classified and has no obligatory labelling requirement for eye irritancy (possible hazardous properties of other ingredients in the final preparation are not taken into account). See table 1.

According to the obtained results it appears also that ceramide-6 seems to be able to emoliorate the TEWL of photoaged people (fig. 3), when used in a right formulation in combine with l-ascorbic acid and or with betacarotene.

Moreover after the treatment with the emulsion A (Vitamin C-Cream) and B (Betacarotene-Cream), it has been obtained a significant ($p < 0.005$) increase of about 30% of skin hydration still in the second week of treatment (fig.2). This observed amelioration increases of about 40% at the week 12. Same results have been obtained with the surface skin lipids which increase from 15 to about 60% ($p < 0.005$) at week 12 (fig.1).

No differences have been noted between ceramide-6/vitamin C and ceramide-6/betacarotene treatment

in TEWL, skin hydration or surface lipids.

However, both the clinical evaluation of the number of the present face wrinkles, and the elasticity measurements, set in evidence how the addition of vitamin C notably increases these parameters unlike what happens with the betacarotene (fig.4).

Probably because the ascorbic acid, penetrating quickly through the skin like recently shown (15), immediately protects the cutaneous cells from the environmental pollutants and from the UV strengthening its abilities in recovering.

Moreover, according to recent studies, it seems that vitamin C plays a key role in the formation of SC barrier lipids (16) The tolerability of both formulations was excellent, and no undesirable side effects were observed.

Therefore, the use of ceramides in the future formulations will be surely increased when the role of the skin lipids will be better understood together with the role of antioxidant vitamins.

Clarified these roles, it will be certainly easier to formulate proper vehicles able to rebalance an altered cutaneous barrier, and/or to allow an easier and selective cutaneous absorption of the active principles of cosmetic use.

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