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Analysis of *in vitro* release through reconstructed human epidermis and synthetic membranes of multi-vitamins from cosmetic formulations

Simone Gabbanini [a], Riccardo Matera [b], Claudia Beltramini [a], Andrea Minghetti [a], Luca Valgimigli [b,*]

[a] *BeC S.r.l., R&D division, Via C. Monteverdi 49, 47100 Forlì, Italy*
[b] *University of Bologna, Faculty of Pharmacy, Dept. Organic Chemistry "A. Mangini", via San Giacomo 11, 40127 Bologna, Italy*

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ABSTRACT

A convenient method for *in vitro* investigation of the release of lipid- and water-soluble vitamins from cosmetic formulations was developed. The permeation of (*d*)-α-tocopherol (vitamin E), retinyl acetate (pro-vitamin A), ascorbic acid (vitamin C) and pyridoxine (vitamin B6) through SkinEthic® reconstructed human epidermis (RHE), and synthetic polyethersulfone and polycarbonate membranes was studied *in vitro* using a Franz-type diffusion apparatus, coupled either to a spectrophotometer for continuous reading (dynamic setting) or to HPLC-DAD analysis of the receptor medium (static setting). O/W and W/O emulsions were compared with simple aqueous solutions for their kinetic of vitamins release, to evaluate the influence of the cosmetic formulation on the bioavailability of active ingredients. Results indicate that synthetic membranes offer a limited barrier to the diffusion of vitamins, but may provide information on the release ability of the formulation. Penetration was more effective when water was the external phase of the formulation, i.e. W/O emulsions were less effective in the release of vitamins than O/W emulsion or aqueous solutions. RHE (17 days old) offered a significantly higher barrier to penetration of vitamins, as expected for native human epidermis. The relative ranking in coefficient of permeability (*Ps* (cm/h)) was: ascorbic acid > pyridoxine ≫ retinyl acetate > α-tocopherol ∼0, the absolute values depending on the formulation. The method herein described showed to be a practical and convenient tool for the quality-control and efficacy evaluation of cosmetic formulations.

1. Introduction

Vitamins are common ingredients of cosmetic formulations and a number of claims are based on the implicit assumption that these bio-active molecules are effectively released from the formulation into epidermis and possibly through epidermis into the derma and subcutaneous tissues. However studies assessing the skin absorption of vitamins released from cosmetic formulations are extremely rare in the literature and mostly limited to retinoids (vitamin A) [1] or vitamin E. [2] The lack of specific obligation for bioavailability studies and the prohibition of animal testing for cosmetic formulations, expressed in current EU regulations [3] do not stimulate the objective evaluation of these efficacy-related parameters. The term *cosmeceuticals* was coined in mid-eighties to indicate a particular category of personal-care products that could be placed at the border-line between cosmetics and pharmaceuticals [4]: cosmetic products containing active ingredients meant to have beneficial physiologic effects resulting from their pharmacologic properties [5]. Since there is no legal definition of cosmeceuticals, these products are often formally classified as cosmetics or in some cases as drugs, the distinction being sometimes arbitrary and varying from country to country. For instance skin-protectants (such as those based on vitamin E) are classified as drugs in USA and as cosmetics in Europe. This category of products has been exponentially increasing its importance during last two decades and a relevant percentage of products currently found in EU market could be classified as cosmeceuticals. However a scientifically sound evaluation of their efficacy is often lacking, as have been lamented by authoritative investigators [5,6].

In compliance with EU regulations and in line with OECD [7] and COLIPA guidelines [8] we aimed to set-up a convenient method, based on a custom designed Franz-type diffusion apparatus, for *in vitro* evaluation of the kinetic of release of vitamins form cosmetic/cosmeceutical formulations. In order to avoid the use of animal skin [9–12], which would conflict with the ethical principles of EU regulations, and to evaluate a convenient substitute for human epidermis from cadaver or surgery [11,13,14], we tested SkinEthic® reconstructed human epidermis (RHE). Previous studies

Abbreviations: RHE, reconstructed human epidermis; egf, epidermis growth factor; W/O, water in oil; O/W, oil in water; Ps, coefficient of permeability; LOD, limit of detection; LOQ, limit of quantitation; DTT, (*dl*)-Dithiothreitol; TFA, trifluoroacetic acid.

* Corresponding author. Tel.: +39 051 2095683; fax: +39 051 2095688.
E-mail address: luca.valgimigli@unibo.it (L. Valgimigli).

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have shown that RHE is reasonably similar to native human epidermis both in terms of morphology and lipid composition [15,16]. It was found less selective than native epidermis to the permeation of specific drugs [15], however it has been indicated as a valid substitute for *in vitro* testing of topical formulations [17,18,19]. Synthetic membranes have also been employed for release kinetics studies [20,21], so we compared RHE to synthetic polyethersulfone and polycarbonate membranes. Two different settings have been initially compared for the analysis of vitamins in the receptor fluid: continuous spectrophotometric reading by circulating the fluid through a flow cell (dynamic setting, see Appendix A), or discontinuous sampling followed by HPLC-DAD analysis (static setting). The former has lately been abandoned due to limited performance. Three different types of cosmetic formulation were considered in this investigation: O/W emulsions (creams), W/O emulsions (ointments) and aqueous solutions (lotions), to evaluate the influence of the formulation in the release of lipid- and water-soluble vitamins. Ascorbic acid (vitamin C), pyridoxine hydrochloride (vitamin B_6), retinol acetate (pro-vitamin A), and α-tocopherol (vitamin E) were chosen as representative exempla of commonly employed water- and lipid-soluble vitamins.

2. Materials and methods

2.1. Materials

The standard vitamins used for method validation were: ascorbic acid (99.9%), pyridoxine hydrochloride (99.9%), (*dl*)-α-tocopherol (99.6%), retinol acetate (97.4%) from Supelco (Bellefonte, PA, USA); trifluoroacetic acid (TFA, ≥99.0%), (*dl*)-Dithiothreitol (DTT; >99.0%), sodium phosphate dibasic anhydrous (≥99%), sodium chloride (≥99.5%), magnesium sulfate hexahydrate (>99.0%), isopropanol (≥99.8%), methanol (≥99.8%) from Sigma, (St. Louis, MO, USA); potassium dihydrogen phosphate (≥99.5%) from Merck (Darmstadt, Germany). Reconstituted Human Epidermis 12 days old 4.0 cm^2, and Growth Medium (1.5 mM calcium chloride, 25 mg/mL gentamycin, 5 mg/mL insulin, 1 ng/mL egf) were purchased from SkinEthic Laboratories (Nice, France). Polyethersulfone membranes (0.2 μm, Supor® 200, Pall corp., New York, USA) and polycarbonate membranes, (0.01 μm, Whatman, GE Healthcare, Uppsala, Sweden) were from FAVS s.n.c. (Bologna, Italy).

2.2. Apparatus and chromatographic conditions

2.2.1. Franz-type diffusion cells

Franz-Type diffusion cells (diffusion surface: 1.54 cm^2; internal volume: 14.8 ± 0.1 mL), designed in our labs to optimize the use of SkinEthic® RHE disks, were previously described [19] and are shown in Appendix A (Fig. S1). Homogenous concentration of the analytes within the receptor compartment was ensured during preliminary tests by monitoring the diffusion of blue-colored crystal violet solutions.

2.2.2. HPLC-DAD analysis

All analyses were carried out using a Thermo Accela Pump equipped with Accela Autosampler and Surveyor Photo-Diode Array detector (Thermo Scientific, San Jose, CA, USA). The LC was performed on a Synergi C18 Hydro-RP 4 μm column (150 mm × 4.6 mm I.D.) equipped with a guard column (C18, 4.0 mm × 3.0 mm) from Phenomenex (Torrance, CA) operating at 30 °C. Two solvent systems were employed: A = CH_3OH + TFA (0.25 mL/L) and B = H_2O + TFA (0.25 mL/L). The injection volume was 5 μL. Analyses of the lipid-soluble vitamins (method *LM*) were performed isocratically at 1.0 mL/min for 13 min with the mobile phase A-B (80:20, v/v). Retinol acetate and α-tocopherol were detected at 320 and 290 nm, respectively. Analyses of the water-soluble vitamins were performed with method *WM*, eluting at 1.0 mL/min in the following gradient program for 17 min: t = 0, A–B (0:100, v/v); t = 3, A–B (0:100, v/v); t = 10, A–B (10:90, v/v); t = 15, A–B (10:90, v/v); t = 17, A–B (0:100, v/v). Ascorbic acid and pyridoxine were detected at 245 and 290 nm, respectively.

2.3. Assay procedure

2.3.1. Calibration for HPLC-DAD analysis

Standard solutions in five levels from 0.001 to 0.100 mg/mL were analyzed in triplicate. Ascorbic acid and pyridoxine hydrochloride were dissolved in pH 7.4 phosphate buffered saline solution with (*dl*)-Dithiothreitol (DTT, 0.4 mg/mL), whereas (*dl*)-α-tocopherol and retinol acetate solutions were prepared in methanol. The LOD was determined from repeated analyses, as the concentration that yields a signal-to-noise (S/N) ratio of at least 3:1 and the LOQ was obtained as the concentration that yields S/N ratio of 10:1. Linearity and LOD/LOQ data are collected in Table 1.

2.3.2. Validation of the analytical procedure

Standard receptor medium solutions containing three-levels concentration of either lipid-soluble vitamins or water-soluble vitamins were subjected to five replicate analyses for three non-consecutive days (15 replicates for each level). Solutions were stored at +4 °C in the dark to minimize oxidation and thermal-photochemical degradation of vitamins. Accuracy was evaluated from % recovery with respect of theoretical content, while precision data were obtained from intra-day and inter-day % relative SD.

To test the robustness standard solutions were repeatedly analyzed (n = 5) intra-day using the method described in Section 2.2.2 (setting 1) and changing the chromatographic conditions (setting 2) as follows: T = 25 °C (−5 °C), flow rate 0.8 mL/min (−20%) and decreasing the content of TFA in the mobile phase to 0.20 mL/L (−20%). To test the selectivity, vitamins content was determined in the donor compositions (W/O emulsions, O/W emulsions and aqueous receptor medium) as detailed in Section 2.3.3.

The receptor medium was phosphate saline buffer pH 7.4 (8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4 and 1.15 g/L $NaHPO_4$ in bi-distilled water), 3% w/w of polysorbate-20 and 0.25% w/w of isopropanol and DTT 0.4 mg/mL [22].

2.3.3. Preparation and analysis of the cosmetic formulations (donor)

Four creams being Oil in Water or Water in Oil emulsions, each containing the water-soluble vitamins, or the lipid-soluble vitamins and DTT 0.4 mg/mL [22], were prepared as described in Appendix A and stored at 4 °C before use. The nominal vitamin C and B_6 content were 1.7% (w/w) each, whereas the vitamin A acetate and E were 1.8% and 3.0% (w/w), respectively. The exact amount of vitamin content was assessed by HPLC-DAD analyses. Briefly, water-soluble vitamins containing creams (100 mg) were suspended in CH_3OH (10 mL), then 1 mL of this suspension was diluted up to 10 mL with H_2O + TFA (0.25 mL/L). The suspension was filtered with a syringe through 0.45 μm syringe filter and analyzed. The lipid-soluble vitamins containing creams (100 mg) were suspended in CH_3OH (10 mL), diluted up to 10 mL with the same solvent and filtered (0.45 μm) prior to analysis.

2.3.4. Study of vitamins' stability in the donor and receptor media

Standard receptor medium solutions, W/O emulsions and O/W emulsions, each containing DTT 0.4 mg/mL and the water-soluble (B_6, C) or lipid-soluble (A, E) vitamins were stored at 4 °C (in the