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Improved procedures for *in vitro* skin irritation testing of sticky and greasy natural botanicals

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ABSTRACT

Skin irritation evaluation is an important endpoint for the safety assessment of cosmetic ingredients required by various regulatory authorities for notification and/or import of test substances. The present study was undertaken to investigate possible protocol adaptations of the currently validated *in vitro* skin irritation test methods based on reconstructed human epidermis (RhE) for the testing of plant extracts and natural botanicals. Due to their specific physico-chemical properties, such as lipophilicity, sticky/buttery-like texture, waxy/creamy foam characteristics, normal washing procedures can lead to an incomplete removal of these materials and/or to mechanical damage to the tissues, resulting in an impaired prediction of the true skin irritation potential of the materials. For this reason different refined washing procedures were evaluated for their ability to ensure appropriate removal of greasy and sticky substances while not altering the normal responses of the validated RhE test method. Amongst the different procedures evaluated, the use of a SDS 0.1% PBS solution to remove the sticky and greasy test material prior to the normal washing procedures was found to be the most suitable adaptation to ensure efficient removal of greasy and sticky in-house controls without affecting the results of the negative control. The predictive capacity of the refined SDS 0.1% washing procedure, was investigated by using twelve oily and viscous compounds having known skin irritation effects supported by raw and/or peer reviewed *in vivo* data. The normal washing procedure resulted in 8 out of 10 correctly predicted compounds as compared to 9 out of 10 with the refined washing procedures, showing an increase in the predictive ability of the assay. The refined washing procedure allowed to correctly identify all *in vivo* skin irritant materials showing the same sensitivity as the normal washing procedures, and further increased the specificity of the assay from 5 to 6 correct predictions out of 7 non irritants as compared to the normal washing procedures. In addition, when exposed to non-irritant oily and viscous materials, tissues rinsed with 0.1% SDS generally showed increased viabilities accompanied by decreased variabilities as compared to the normal washing procedures. Similar results were obtained when testing typical in-house natural botanical ingredients. In conclusion, the use of a refined washing procedure making use of SDS 0.1% in PBS was found a suitable procedure to ensure efficient removal of greasy and sticky materials, leading to an increased predictive capacity and decreased variability of the tissue responses while maintaining its sensitivity and not affecting untreated tissues morphology and viability.

1. Introduction

Information about a substance's potential to cause skin irritation is required by international regulations and testing guidelines for the safety assessment of chemicals and mixtures (REACH, EU CLP, Cosmetics directive). Until the last decade, the rabbit Draize dermal irritation test has been the method traditionally used for this purpose (OECD TG 404, 2002; Draize et al., 1944). However, this animal test has major drawbacks such as different physiological characteristics as compared to human skin, lack of reproducibility (Spielmann and Reinhardt, 1996; Weil and Scala, 1971; OECD, 2010a), and the suffering of animals exposed to severe irritants and corrosives. Since the 1980s the European Commission promotes the reduction of laboratory animals for safety testing as soon as scientifically valid alternative methods are available (Council Directive on the protection of animals used for scientific purposes 86/609/EEC revised as 2010/63/EU, EC, 2010). The 7th Amendment to the Cosmetics Directive (Directive 2003/15/EC taken up by Regulation 1223/2009) went even further and implemented a complete ban on animal testing for finished

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cosmetics products from 2004, and for cosmetic ingredients from 2009, for all the human health-related effects (EC, 2003, 2009a).

Skin irritation refers to the production of reversible damage to the skin following the application of a test substance (United Nations-Economic Commission for Europe (UN/ECE), 2009; OECD, 2002). Several validated *in vitro* methods for skin corrosion and irritation were adopted by the OECD and by the European Union during the last decade (Eskes et al., 2012). In the EU, these assays allow the full replacement of animal testing for identifying and classifying compounds as skin corrosives, skin irritants, and non irritants. In particular, the test method B.46 for skin irritation testing was adopted by the EU in 2009 and by the OECD in 2010 as TG 439 (EC, 2009b; OECD, 2010b). Within the EU, test method B.46 can be used to determine the skin irritancy hazard of chemicals as a stand-alone replacement for the assessment of acute dermal irritation test within a tiered testing strategy and/or in a weight of evidence approach (EC, 2009b). The adopted *in vitro* skin irritation method is based on reconstructed human epidermis (RhE) and measures the initiating events in the cascade of irritation. Three commercially available RhE models were endorsed as scientifically valid to be used within the framework of the test guidelines B.46 and OECD TG 439, i.e., the EpiSkin™ Skin Irritation Test (SIT), the EpiDerm™ EPI-200-SIT and the SkinEthic™ SIT^{42bis} models.

During the last decade, the use of botanicals and plant extracts from natural origin has attracted strong interest from industries searching for novel pharmaceuticals, natural fungicides, insecticides, food colorants, flavouring agents, and natural cosmetic ingredients. For a new botanical cosmetic ingredient and/or plant extract to be placed in the market, it is important to characterize its toxicological profile in order to ensure consumers protection. However, materials from natural origin have often specific physico-chemical properties which can represent a challenge for testing, especially in terms of solubility, lipo- or hydrophilicity, texture (waxes, foam) and rheology and they tend to be, upon our experiences, highly lipophilic, sticky and have a buttery-like texture.

When using the validated protocols to test plant extracts and botanicals, difficulties can be observed in particular during the rinsing of the test material, which tends to stick to the epidermis surface. This could lead to extended exposure conditions and/or mechanical damage to the tissues which could affect the tissue viability, resulting in turn into an impaired prediction of the true skin irritation potential of the tested material. Indeed, the current guidelines require that the test chemical should be carefully washed from the epidermis surface (OECD, 2010b).

The present study was undertaken to investigate possible adaptations of the currently validated and adopted protocols in order to ensure reliable predictive ability of materials having greasy and sticky physico-chemical properties similar to natural botanicals and plant extracts with which we are experienced. For this purpose, different refined washing procedures were assessed in a first step for their ability to ensure appropriate removal of in-house greasy and sticky control substances while not altering the normal responses of the untreated tissues. The predictive capacity of the most suitable adapted protocol was then further assessed by using a set of twelve greasy and sticky compounds having known skin irritation effects supported by high quality *in vivo* data. Finally, the most suitable adapted protocol was applied to test typical in-house natural botanical and plant extract materials.

2. Materials and methods

2.1. Tested substances

Phosphate buffer solution (PBS without Ca++ and Mg++) was used as the reference negative control as indicated in the Standard Operating Procedures (SOP) of the validated RhE model. In addition, vaseline (or petroleum jelly, Cooper, CAS 8009-03-8) and tallow propylene polyamine (Ceca, CAS 68911-79-5) were used as in-house negative (non irritant) and borderline (no category under EU CLP/GHS and R38 under EU DSD) controls respectively for sticky and greasy substances.

In order to evaluate the predictive capacity of the optimal refined washing procedures to correctly identify irritant and non-irritant sticky and greasy materials, a set of twelve substances having viscous and/or oily properties and well characterized skin irritation effects supported by high quality raw and/or peer-reviewed *in vivo* data were used. To retrieve these substances, an extensive search was carried out based on publically available scientific literature, annexes V and VI of the EU Cosmetic Directive and the ECETOC database (EC, 2009a; ECETOC, 1995). A total of about 400 substances were screened from these data sources looking for their MSDS in order to identify those substances described as being viscous and/or oily. Regrettably only 15 substances having viscous and/or oily characteristics were retrieved, out of which 3 could not be purchased from readily accessible commercial sources. The total resulting 12 substances included 9 liquids and 3 solid materials which were distributed across the 4 categories of skin toxicity as: 5 non-classified substances, 2 GHS Category 3, 3 GHS Category 2 and 2 GHS Category 1C substances. Due to the small number of substances of interest retrieved, and in order to assess whether the adapted protocol is also able to identify borderline corrosive materials for using the assay in a first step of a bottom-up testing strategy (not generating a false negative prediction of borderline skin corrosives), it was decided to assess all twelve substances. Table 1 shows the details on the 12 selected viscous and/or oily substances having raw and/or peer-reviewed *in vivo* data.

2.2. Reconstructed human epidermis

The reconstructed human epidermis (RhE) model used was the validated SkinEthic™ SIT^{42bis} assay (SkinEthic Laboratories, France). The model consists of normal human-derived keratinocytes cultured for 17-days on an inert polycarbonate filter at the air–liquid interface using chemically defined medium, to form a differentiated three-dimensional epidermis comprising suprabasal, spinous and granular cell layers as well as a *Stratum corneum* (Rosdy and Clauss, 1990; Rosdy et al., 1993). The reconstructed 0.5 cm^2 epidermis is received on day 18 of culture, and maintained overnight in a nutrient medium at 37 °C, 5% CO_2, according to the manufacturer's prescriptions. Each batch of tissue is quality controlled by the manufacturer for viability, barrier function, histology and safety data and the results reported on a quality control data sheet provided within the batch.

RhE tissues were topically exposed to undiluted liquids (16 ± 0.5 μL i.e. 32 μL/cm^2) or solids (16 ± 2 mg i.e. 32 mg/cm^2) for 42 min at room temperature according to the formally validated protocol. Prior to applying solids, 10 ± 0.5 μL (20 μL/cm^2) of distilled water was spread on the tissue surface to favor contact of the solid substances with the tissues. For liquid and viscous test substances, a nylon mesh (7.5 mm diameter, provided by SkinEthic™) was applied onto the test substance as a spreading aid. Finally, sticky and greasy substances were weighted 16 ± 2 mg (i.e. 32 mg/cm^2) and spread on the nylon mesh which was then applied with the coated side of the mesh turned to the epidermal surface, as advised in the validated SOP.

2.3. Refined washing procedures

The validated SkinEthic™ RHE washing procedures require that after exposure, excess of product is removed, that the RhE tissues are rinsed 25 times with 1 mL each time of sterile PBS without