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Use of HPLC/UPLC-spectrophotometry for detection of formazan in *in vitro* Reconstructed human Tissue (RhT)-based test methods employing the MTT-reduction assay to expand their applicability to strongly coloured test chemicals

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ABSTRACT

A number of *in vitro* test methods using Reconstructed human Tissues (RhT) are regulatory accepted for evaluation of skin corrosion/irritation. In such methods, test chemical corrosion/irritation potential is determined by measuring tissue viability using the photometric MTT-reduction assay. A known limitation of this assay is possible interference of strongly coloured test chemicals with measurement of formazan by absorbance (OD). To address this, Cosmetics Europe evaluated use of HPLC/UPLC-spectrophotometry as an alternative formazan measurement system. Using the approach recommended by the FDA guidance for validation of bio-analytical methods, three independent laboratories established and qualified their HPLC/UPLC-spectrophotometry systems to reproducibly measure formazan from tissue extracts. Up to 26 chemicals were then tested in RhT test systems for eye/skin irritation and skin corrosion. Results support that: (1) HPLC/UPLC-spectrophotometry formazan measurement is highly reproducible; (2) formazan measurement by HPLC/UPLC-spectrophotometry and OD gave almost identical tissue viabilities for test chemicals not exhibiting colour interference nor direct MTT reduction; (3) independent of the test system used, HPLC/UPLC-spectrophotometry can measure formazan for strongly coloured test chemicals when this is not possible by absorbance only. It is therefore recommended that HPLC/UPLC-spectrophotometry to measure formazan be included in the procedures of *in vitro* RhT-based test methods, irrespective of the test system used and the toxicity endpoint evaluated to extend the applicability of these test methods to strongly coloured chemicals.

Abbreviations: R^2, Coefficient of Determination; CV, Coefficient of Variation; EIT, Eye Irritation Test; EIVS, Eye Irritation Validation Study; DG SANCO, European Commission Directorate General for Health and Consumer Protection; EURL ECVAM, European Union Reference Laboratory for Alternatives to Animal Testing; FDA, Food and Drug Administration; HPLC, High Performance Liquid Chromatography; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; LLOQ, Lower Limit of Quantification; ME, Matrix Effect; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt; NSC, non-specific colour in living tissues; NSC_{killed}, non-specific colour in killed tissues; NSMTT, non-specific MTT reduction; OD, Optical Density; OECD, Organisation for Economic Co-operation and Development; QC, Quality Control; RHE, Reconstructed Human Epidermis; RhT, Reconstructed human Tissue; SCCS, Scientific Committee on Consumer Safety; SD, Standard Deviation; SOP, Standard Operating Procedure; TG, Test Guideline; UPLC, Ultra High Performance Liquid Chromatography; ULOQ, Upper Limit of Quantification; UN GHS, United Nations Globally Harmonized System of Classification and Labelling of Chemicals.

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1. Introduction

In recent years, a number of *in vitro* test methods based on Reconstructed human Tissues (RhT) have been developed, validated and accepted by regulatory authorities for evaluation of skin corrosion and skin irritation. For skin corrosion these are EpiDerm™ Skin Corrosion Test (EURL ECVAM, 2000; Liebsch et al., 2000; ICCVAM, 2002), EpiSkin™ (EURL ECVAM, 1998; Fentem et al., 1998; Alépée et al., 2014a), SkinEthic™ Reconstructed Human Epidermis (RHE) (EURL ECVAM, 2006; Kandárová et al., 2006a; Alépée et al., 2014b) and epiCS (Hoffmann et al., 2005; EURL ECVAM, 2009) in OECD Test Guideline (TG) 431 (OECD, 2013a)). For skin irritation these are EpiDerm™ Skin Irritation Test (Kandárová et al., 2004, 2005, 2009; Spielmann et al., 2007), EpiSkin™ (Cotovio et al., 2007; Spielmann et al., 2007), SkinEthic™ RHE 42bis (Kandárová et al., 2006b; Alépée et al., 2010; Tornier et al., 2010a,b) and LabCyte EPI-MODEL 24 SIT (Kojima et al., 2012, 2014) in OECD TG 439 (OECD, 2013b)). These test methods allow identification of Irritant (UN GHS Cat 2), Corrosive (UN GHS Cat 1) or Not-Classified (NC).

In all these RhT test methods, skin irritation or skin corrosion potential of a test chemical is determined by measuring tissue viability in treated tissues after topical application onto the tissue surface. Tissue viability is determined by enzymatic reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) tetrazolium salt to purple reduced MTT (formazan) (Mosmann, 1983). Formazan is then quantified photometrically with the results being expressed as % viability of the test chemical treated tissues relative to negative control treated tissues and converting this to a classification using a prediction model based on a percentage (%) viability cut-off value.

A known limitation of the photometric MTT-reduction assay is the possible interference of coloured test chemicals with the absorbance measurement of formazan. This limitation has been recognised by the European Commission Directorate General for Health and Consumer Protection (DG SANCO) Scientific Committee on Consumer Safety (SCCS) which provides scientific advice to the Commission on issues related to non-food topics. In 2010, the SCCS published a "Memorandum (addendum) on the *in vitro* test EPISKIN™ for skin irritation testing" (SCCS, 2010). In this memorandum, the SCCS expressed the opinion that for hair dye substances/coloured chemicals there was insufficient evidence that the MTT-reduction assay can be used as a suitable endpoint when coloured ingredients/hair dye substances are tested for their potential skin irritant properties. The SCCS also expressed the opinion that "for coloured substances, a different endpoint, not involving optical density quantification, should be envisaged. Analytical methods such as HPLC/UPLC might be more appropriate to detect formazan in the *in vitro* assay (McNamee et al., 2009)". It is also recognised that this limitation of using standard photometry as the endpoint detection system for evaluation of coloured chemicals interfering with the MTT-reduction assay is relevant to any RhT-based test method that uses Optical Density (OD) measurement of formazan (RhT/MTT-based test methods).

To address this limitation and respond to SCCS concerns, Cosmetics Europe undertook a study to establish and evaluate the use of an analytical method – High Performance Liquid Chromatography/Ultra High Performance Liquid Chromatography combined with spectrophotometric detection (hereafter referred to as HPLC/UPLC-spectrophotometry) to measure formazan from RhT extracts.

This paper describes the Cosmetics Europe study to determine the relevance and reproducibility of the analytical HPLC/UPLC-spectrophotometry detection system in RhT/MTT-based test methods as compared to the regulatory accepted standard absorbance (OD) measurement. Three representative RhT/MTT-based test methods covering the toxicity endpoints of serious eye damage/eye irritation, skin irritation and skin corrosion were used for this purpose. For serious eye damage/eye irritation this was the EpiOcular™ Eye Irritation Test (EIT) (in connection with the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)/Cosmetics Europe RhT test methods Eye Irritation Validation Study (EIVS) (Freeman et al., 2010; Pfannenbecker et al., 2013; Alépée et al., 2013). For skin irritation this was EpiSkin™ as representative of OECD TG 439 (OECD, 2013b) and for skin corrosion this was SkinEthic™ RHE as representative of OECD TG 431 (OECD, 2013a). Since reproducibility was an integral aspect of this evaluation, the inter-laboratory reproducibility was assessed in three independent laboratories.

2. Material and methods

2.1. Establishment and qualification of the HPLC/UPLC-spectrophotometry systems

The approach used to qualify the HPLC/UPLC-spectrophotometry systems is based on the Federal Drug Administration (FDA) guidance for industry from May 2001 on Bio-analytical Method Validation (FDA, 2001). Within the FDA guidance, validation of a bio-analytical method encompasses all of the procedures that demonstrate a particular bio-analytical method used for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for the intended use. The fundamental parameters for such a validation include: (1) selectivity; (2) precision and accuracy; (3) Matrix Effect; (4) carryover; (5) reproducibility and (6) stability. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method. The information provided in the FDA guidance applies to bio-analytical procedures including HPLC/UPLC-spectrophotometry and is relevant for validation activities including full, partial and cross validation. As such, determination of the reliability and reproducibility of HPLC/UPLC-spectrophotometry as an alternative endpoint for detection of formazan in the MTT-reduction assay in *in vitro* RhT test methods for serious eye damage/eye irritation, skin irritation and skin corrosion falls within the scope of the FDA guidance document.

Using the approach in the FDA guidance document as the basis, key parameters, with associated acceptance criteria, were applied to the qualification of HPLC/UPLC-spectrophotometry systems for measurement of formazan to demonstrate acceptability of this analytical technique as an alternative endpoint detection system in RhT/MTT-reduction assay based test methods. The key parameters, with associated acceptance criteria, from the FDA guidance document that were applied in this study, are summarized in the first three columns of Table 1.

Specific HPLC/UPLC-spectrophotometry conditions were established in each laboratory for measurement of formazan using different analytical systems (Table 2). The laboratories that participated in this project were L'Oréal Research & Innovation (R&I) (Aulnay sous Bois, France), Pierre Fabre Laboratories (Castres, France) and VITO (Mol, Belgium).

2.2. Test chemicals selection

Primary selection criteria were applied to the choice of test chemicals with the aim of achieving a balanced representation of: (1) coloured test chemicals anticipated to produce colour