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Ex vivo human skin permeation of Methylchloroisothiazolinone (MCI) and Methylisothiazolinone (MI)

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Abstract

Methylchloroisothiazolinone (MCI) and methylisothiazolinone (MI) are biocides used in many types of products such as cosmetics, paints, and cleaning agents. Skin contact is often encountered when using these products. Although MCI and MI are strong allergens and cause skin irritation, no scientific skin permeation study has been reported except for some unpublished data. Therefore, this study assessed the permeation of MCI and MI both separately and as a mixture through freshly dermatomed human skin (800 µm) in a flow-through diffusion cell system. Different concentrations of aqueous standards (1.5/1, 70/50, 150/35, and 750/175 µg/mL of MCI/MI) and various commercial products were assessed after 15 to 20 hours of exposure. In parallel, the dose dependent irritant effects of MCI/MI and MI were estimated by histology following 6-h or 24-h exposure. Overall results show that MI in formulations or in aqueous standard solutions quickly permeated the skin with time lags less than 15 minutes while MCI was much slower (>3.5 h). MCI in formulations had permeation rates up to five times greater than for MI in the same product, and in two tested creams were not found to permeate skin. Some signs of irritation were observed by histology; especially at the highest MCI/MI concentrations (750/205 µg/mL) in aqueous solutions. This confirms that MCI reacts readily with skin and may induce local irritation. The MCI and MI permeations are also greatly influenced by the topical vehicle. It is therefore more relevant to test exposures to formulations than aqueous standard solutions.

Key words

Methylchloroisothiazolinone (MCI), methylisothiazolinone (MI),, dermal penetration, skin irritation, human skin allergens

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Introduction

Isothiazolinones are extensively used and have been since the 1970s (Douglas 2010). They are frequently used in various industrial products (e.g., paints, oils, glues, adhesives, detergents, inks, polishes, water-cooling systems), leave-on and rinse-off cosmetics, and aqueous-based household products (e.g., water-based paints, cleaning and washing agents) (Ackermann et al. 2011; Alexander 2002; Castanedo-Tardana and Zug 2013; Devos et al. 2015). A solution of methylchloroisothiazolinone (MCI) and methylisothiazolinone (MI) mixed in a 3:1 ratio and MI alone are both commonly used as preservatives in products. In the Danish Product Register Database (PROBAS), the MCI/MI mixture was the third most frequent isothiazolinone (611 different products) after MI (884 different products) and benzisothiazlinone; and before MCI (474 different products) (Friis et al. 2014). MCI/MI concentrations are far greater in industrial products (1.5% - 13.5%) than in cosmetics (7.5 ppm or µg/mL) or domestic products (15 ppm or µg/mL) (Castanedo-Tardana and Zug 2013; Reinhard et al. 2001; Urwin et al. 2015). Hairdressers, beauticians, mechanics, repairmen, machinists, painters, paint factory workers, and restaurant workers have extensive exposures (Vauhkala et al. 2015).

Like all isothiazolinone compounds, MCI and MI have a heterocyclic sulphur with an activated N-S bond making them electrophilic (see the physico-chemical properties in Table 1) (Alexander 2002; Alvarez-Rivera et al. 2012). This electrophilic N-S bond reacts with nucleophilic cell material and oxidizes compounds containing thiols (Alvarez-Rivera et al. 2012). It is this electrophilic property that gives them a powerful antimicrobial activity against a broad-spectrum of bacteria, yeasts, fungi, and algae; even at very low concentrations and over very broad pH ranges (Ackermann et al. 2011; Alvarez-Rivera et al. 2012; De Bethizy et al. 1986).

MCI and MI are considered high potent contact allergens and are one of the most common causes of contact allergy and dermatitis induced by preservatives (Braun-Falco et al. 2008; Castanedo-Tardana and Zug 2013). Several studies have reported airborne contact dermatitis after recent exposure to water-based paint containing MCI and MI (e.g., Bohn et al. (2000); Braun-Falco et al. (2008); Breuer et al. (2015); Lundov et al. (2014); Reinhard et al. (2001); Wright and Cahill (2015)). Furthermore, various studies and case reports have described allergic contact dermatitis (ACD) following skin exposure (e.g., Ackermann et al. (2011); Carlsen et al. (2008); Cleenewerck (2008); Lundov et al. (2011); Mendonca et al. (2014); Puangpet et al. (2015)). Although no clear thresholds are defined, skin effects are dose-dependent where low concentrations can result in ACD and high concentrations can induce severe burns as well as sensitization (Bayraktar and Ozcan 2007; Cleenewerck 2008). In the early 2000s, MCI was

recognized as an extreme skin sensitizer, while MI was considered as a weak sensitizer. This has resulted in an increase of MI use as it the MCI/MI mixture. However, MI was later deemed as a strong sensitizer (Devos et al. 2015). This substitution was also made for cosmetic products which authorized a MI concentration limit of 100 µg/mL (Castanedo-Tardana and Zug 2013; Devos et al. 2015). Even after this substitution was made, the number of ACD cases kept increasing as did the prevalence of sensitization due to MCI/MI and MI. Occupational ACD caused by MCI/MI or/and MI are especially frequent among beauticians, hairdressers and healthcare workers as described in the retrospective study by Gameiro et al. (2014). This study reported with an increase in prevalence from <1% in 2005 to 10.9% in 2013 of which 51.4% were related to professional activities. Furthermore, Urwin et al. (2015) reported an increase in incidence of 4.1% per annum from 1996 to 2012. MI is now considered a strong sensitizer (Devos et al. 2015).

Despite their extensive use, MCI and MI exposure data are lacking. No study was identified in the scientific literature on MCI/MI skin permeation rate; although, some unpublished data from Rohm and Haas on skin permeation rates in animals or in *post mortem* human skin from Rohm and Haas have been reported by Burnett et al. (2010) and SCCS (2009). In the present study, MCI and MI permeation properties were assessed separately, and as a mixture in standard solutions or in commercial formulations using viable human skin ex vivo, mounted on a flow-through diffusion cell system. Structural changes in the epidermis were determined by histology when full thickness viable human skin was used and a quantitative dose-response was obtained for irritation.

Materials and methods

Chemicals and supplies

The methylchloroisothiazolinone (5-chloro-2-methyl-4-isothiazolin-3-one; MCI; CAS 26172-55-4; purity of 99%) and the methylisothiazolinone (2-methyl-4-isothiazolin-3-one; MI; CAS 2682-20-4; purity of 98.2%) were bought from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The internal standard 6-deuterated isoproturon (isoproturon-d6, CAS 217487-17-7, 99.8% purity) was obtained from Sigma Aldrich (Pestanal®, Buchs, Switzerland). In addition, ammonium acetate (>98% purity) were purchased from Merk (Zug, Switzerland) and sodium chloride (NaCl) (>99% purity) from Fluka (Sigma-Aldrich, Buchs, Switzerland). For skin irritation appraisal test, RPMI 1640 media (HEPES Modification, with 25 mM HEPES, without L-glutamine) was purchased from Sigma-Aldrich (Buchs, Switzerland). Methanol (MeOH) was analytical grade and purchased from Sigma-Aldrich (Buchs, Switzerland). All stock and working solutions were prepared in purified water (TKA GenPure water treatment system (TKA Wasseraufbereitungsszsteme GmbH, Niederelbert, Germany). Solid-phase extraction cartridges (Isolute ENV+, 100 mg, 3 mL) were obtained from Biotage (Uppsala, Sweden). The following four commercially available products were kindly provided by the manufacturers: a fungicide to treat swimming pool water (Revaguard® Plus, Mareva, Saint-Martin de Crau, France;14% MCI/MI mixture (3:1)), liquid used to preserve a broad range of water based products (Parmetol® K40, Schülke & Mayr AG, Zürich, Switzerland; 1.7% MCI/MI mixture (3:1)), two moisturizing creams for occupational use (MR Extra®, Sorein-fabrik GmbH, Pfäffikon, Switzerland and Priva Care®, Minatol Werke AG, Kloten, Switzerland). No information was provided on the composition of the two creams.

Human skin

Human abdominal full thickness skin from Caucasian females (age range 37-54 years) was obtained as surgical waste from abdominoplasty surgeries from the Lausanne university hospital (CHUV) and the Department of Musculoskeletal Medicine (DAL) biobank, under anonymous donation, in accordance with its regulation and accepted by the cantonal ethics committee under protocol 264/12. The full thickness skin was collected immediately following surgery, rinsed with physiological water (saline water; 0.9% sodium chloride (NaCl) (>99% purity, Sigma-Aldrich, Buchs, Switzerland) in purified water, dermatomed to a thickness of 0.8 mm using an electrical dermatome (Acculan®II, B. Braun/Aesculap, Sempach, Switzerland),

and cut into circular discs. No more than 2 hours elapsed from the surgery-end to the skin was mounted on the flow-through diffusion cells. Full thickness skin was used for the irritation appraisal.

Flow-through diffusion cell experiments

A rack of six jacketed flow-through diffusion cells (PermGear®, SES Analytical System, Bechenheim, Germany) was used. Receptor compartments (12 mL) were filled with physiological solution and kept at 32°C using a recirculating water bath and a jacket surrounding each cell. The system was operated at a rate of 2.4 mL/h by a peristaltic pump (8 channels, Ismatec IPC-N, IDEX Health & Science GmbH, Wertheim-Mondfeld, Germany). Six skin discs (skin area exposed was 1.77 cm^2) from each donor were stabilized in the system for approximately 30 minutes prior to topical applications of standard solutions or commercial products. The skin barrier integrity was assessed by measuring the transepidermal water loss (TEWL; mean measured value was 7.5 ± 2.7 $g/m^2/h$) using a VapoMeter (Delfin Technologies Ltd., Kuopio, Finland). Skin discs with values above 11 $g/m^2/h$ were deemed damaged and replaced before starting the experiment. Infinite doses (i.e., the added volume of the tested compounds were sufficient for the complete experiment time) of MCI and MI standard solutions (from cosmetic up to industrial product range concentrations) as well as four different products, pure or diluted in water were applied, separately (Table 2 summarizes the tested products and the doses applied). MCI/MI standard solutions were prepared by adding and mixing MCI and MI stock solutions (100 µL of each stock solution) and then diluting in 9.8 mL of purified water. To quantify the dose of the cream applied on the top of the skin, plastic graded pipettes were weighted, filled with approximately 1 mL of cream, and then re-weighted. The difference in weight was used as the amount of cream applied. The applied cream was spread by rubbing the pipette on the skin thus simulating the use of skin cream. Lastly, the pipette was reweighted to quantify the dose applied on the skin. For both creams CMI and MI were listed on the ingredient label, but no quantitative information was available. To calculate a dose, an assumption of 15 ppm was made which is the maximal concentration recommended by the European Cosmetic Products legislation (Directive 76/768/EEC, subsequently Regulation (EC) 1223/2009).

The exposed skin area was 1.77 cm^2. Skin samples were not occluded. The reservoir liquid was automatically sampled by a fraction collector (FC 204, Gilson Inc., Middleton, WI, USA) before experiment started (contamination check) and at 2, 4, 6, 8, 10, 12, 15 and 20 h after product application. All assays were performed in agreement with the Organization for Economic Co-

operation and Development (OECD) guidelines 28 and 428 (OECD 2004a; OECD 2004b). These guidelines do not include procedures for washing and applied dose estimates for creams.

MCI and MI quantification

MCI and MI were extracted from the reservoir liquid using solid phase extraction (SPE) cartridges. First, the cartridges were washed, (2 mL of MeOH, followed with 2 mL of ammonium acetate (50 mM, pH 6)) then a 2-mL aliquot of reservoir liquid spiked with 100 µL of isoproturon 6-d internal standard (52 µg/mL in MeOH) was passed through the SPE cartridge. The cartridges were washed with 1 mL of purified water and dried using a positive pressure manifold (Biotage® Pressure + 48, Biotage, Uppsala, Sweden) at a flow rate of 2 mL/min. Washed (with 0.5 mL of MeOH) 45 µm PTFE filters were added directly to the SPE cartridge, before eluting MI/MCI with 1 mL of MeOH. Analytes were then eluted from the column, filtrated and collected in the glass vials for injection.

MCI and MI were quantified using a liquid chromatography (Ultimate 3000 system - pump, autosampler and column compartment, Dionex Softron GmbH, Germering, Germany) – electrospray ionization ion trap tandem mass spectrometry (LC/ESI-MS/MS). Analytical parameters were adapted from the method of Speksnijder et al. (2010).

A 10-µL of the extract was injected on the analytical column Agilent C18 Zorbax Eclipse plus (Rapid Resolution HT, Agilent Technologies, Morges, Switzerland; 4.6 x 50 mm, 1.8 µm). The column was maintained at 30 °C. The mobile phase consisted of 100% purified water (eluent A) and 100% MeOH (eluent B). The following solvent gradient program were used for elution in 15 min: 20% eluent B ramping to 80% in 6 min, maintained at 80% for 4 min before returning to initial conditions of 20% from 10.1 to 12 min, and then the column was stabilized at 20% eluent B for 3 minutes. The ESI interface operated in positive mode for MCI ((m/z 150), MI (m/z 116) and isoproturon 6-d (m/z 212→171), no fragmentation for MCI and MI. Under these conditions, the retention times were 4.2, 2.1 and 8.2 min for MCI, MI, and isoproturon-6d, respectively. Quantification was based on peak area and calculated from standard calibration curves of the compound adjusted by the internal standard area (working range 0.1 to 50 µg/mL for MCI and MI). The limit of detection (LOD) was established on the basis of the signal to noise (s/n) ratio, and was 0.1 µg/mL for MCI and 1 µg/mL for MI.

Data analysis

Skin permeation curves were created based on the MCI and MI concentrations in the reservoir liquid. Permeation rates (J), lag times (T_{lag}), and permeability coefficients (Kp) were calculated. As steady-state conditions were not reached in the experimental set-up except for MI in the cream MR Extra experiments, the apparent permeation coefficients were estimated from cumulative amount of MCI or MI absorbed per unit skin area per time course for each permeation cell. Means and standard deviations were calculated for the replicates. Individual permeation rates or J (µg/cm^2/h) were determined by calculating the slope from the steepest linear part of the curve. T_{lag} (h) was obtained by extending the slope of the steepest linear part of the curve to the time axis. Kp (ng/cm) was calculated by dividing the J with the initial applied MCI or MI concentration (ng/cm^3). The total amount of MCI or MI in the donor chamber at the end of the experiment, or the amount retained in the skin after washing were not quantified, thereby a percentage of absorption could not be calculated.

Skin irritation test

Epidermis changes following MCI and MI exposure were determined in full thickness viable skin exposed to MCI/MI at one of the following concentrations: 75/25, 150/50, 375/125, and 750/250 µg/mL, and 500 µg/mL of MI. All experiments exposed the skin for 6 h (assuming 6-h exposure in a day) or 24 h (OECD guidelines). Full thickness skin as removed from patient, was divided in six large pieces, and placed in separate containers filled at the bottom with saline water 0.9% or RPMI media. A donor chamber from the diffusion cell (exposure area of 1.77 cm^2) was placed on top of each skin piece where a finite dose was applied. By using the donor chamber as a template the exposure area could be defined. MCI/MI or MI solutions (n=5) were added (1 mL) directly in the donor chamber. Saline water or RPMI media were applied (1 mL) onto one skin piece which served as a negative control. After exposure, three skin biopsies were collected for each exposed area for histology preparation and interpretation. This experiment was repeated three times, each time with a different donor.

Histology interpretation

A Nikon 90i microscope was used to analyze the histological hematoxylin and eosin (H&E) stained skin slides. Each slide was randomly divided into ten section images. In each image, changes in the epidermis and in cell morphology were qualitatively assessed for spongiosis (accumulation of fluid between the keratinocytes and intercellular edema), vacuolization of the

basal layer (epidermolysis), apoptotic cells (bloated cells and shrunken nuclei (pyknosis) with a condensation of the chromatin), and suffering cells (big and ballooned nucleus) (Miles et al. 2014). The intensity of the irritation was defined based on the criteria defined in Table 3 and as described in Miles et al. (2014). The exposed skin was compared to the negative controls from the same experiment to control potential damages due to experimental conditions.

Results

Percutaneous permeation characteristics

The permeation coefficients calculated for MCI and MI are presented in Table 4. MCI was not detected in the reservoir liquid during the 20h experiment when it was applied as a standard diluted in water except at the greatest concentration applied (750 µg/mL) and then only after a long delay (T_{lag} > 8h). MCI was not detected in the reservoir liquid when applied as a cream. In commercial liquid formulations, MCI was detected between 3.5 and 5h; depending on the commercial formulations. MCI concentrations in the commercial products (pure vs diluted) did not influence permeation rates (J) nor T_{lag} as shown in Fig. 1 and Table 4. Overall, these results show that constituents in the formulations influence the percutaneous permeation of MCI.

For MI, the cumulative mass permeation curves, J, and T_{lag} obtained for the commercial formulations and the aqueous solutions were quite similar, except for one commercial product (Parmetol® K40) (Fig. 2). For formulations and standard solutions, the permeation rates and delays (T_{lag}) were 0.5-1.5 µg/cm^2/h and 0-0.2 h, respectively. This was also true following topical application of MI in the moisturizing cream and the hand cleaning gel (Table 4). From the cream MR Extra®, the cumulative mass permeation curve tapered off after 10 h indicating an apparent steady-state. Regarding dilution, it did not influence permeation rates or T_{lag} for MI. Permeability was inversely related with concentrations since J is not concentration-dependent; as indicated by a Kp value higher at lower concentrations and lower at higher concentrations. This was also observed for MCI. Overall, MI percutaneous permeation characteristics were distinctively different from MCI; constituents in the formulations did not seem to influence MI permeation through human skin as was observed for MCI, and MI permeated faster through human skin compared to MCI.

Skin irritation

Table 5 presents the irritation intensity results of skin samples exposed to different MCI/MI aqueous solutions or formulations based on histological criteria presented in Table 3. No sign of irritation was observed for full thickness skin exposed to the two lowest concentrations (75/25, 150/50 µg/mL) of MCI/MI for either 6 or 24 h, regardless of the medium used (physiological water or RPMI). Some mild signs of irritation were noted at the 375/125 µg/mL concentration; again for both exposure times and media. Moderate signs were noted at 750/250 µg/mL with the presence of apoptotic (a small, condensed and eosinophilic nucleus as chromatin is completely condensed inside it) and suffering (big and ballooned nucleus) cells (Fig. 3). No sign of irritation was observed for the greatest concentration of MI applied (500 µg/mL).

Regarding skin recuperated after the flow-through diffusion cell experiments (800 µm thickness), some moderate irritation signs were noted for the undiluted formulation, Revaguard® and only mild irritation signs were observed when the formulation was diluted to 50% in water (Table 5). No irritation signs were observed for the cream tested (Priva Care®). We did not perform irritation tests on the other formulations due to monetary constraints.

The skin barrier integrity was not affected by MCI/MI exposure, although moderate signs of irritation were identified by histology. TEWL measurements at the end of the experiments remained unchanged from the measurements taken at the start. Change in skin barrier integrity were only noted for the positive controls (skin exposed to sodium lauryl sulfate (SLS) 10%), which had strong signs of irritation using the histological criteria.

Discussion

MI permeated through human fresh skin in less than 15 min (Tlag ≈ 0-0.2 h; Fig. 2) and with a relatively high permeation rate (J = 0.5-1.5 ug/cm^2/h) regardless of the nature of the topical vehicle. On the contrary, MCI as a constituent in cream did not permeate skin and only after several hours in aqueous formulations. Thus, MCI permeation depends on type of vehicle (Table 4). The severity of irritation caused by the MCI/MI mixture after 6h-exposure was dose dependant; producing moderate irritation at the highest dose (750/205 µg/mL), mild at the medium dose (375/125 µg/mL), and no irritation at the lowest dose (75/25 µg/mL).

Unpublished data from Rohm and Haas (1989; 2003; 2005a; 2005b; 2005c) describe permeation of MCI and MI in *in vitro* experiments using rat or human post mortem skin occluded for 24h. A summary of the Rohm and Haas results has been compiled in Table 6 for an easier comparison (unpublished data reported by Burnett et al. (2010) and SCCS (2009)). These authors found that MI permeated easily though the skin. MCI in body lotion, facial cream, or at low concentrations in aqueous solutions (11.4 or 22.5 µg/mL) did not permeate through the skin after 24-h exposure. These findings are in accordance with the present study. However, MI permeation rates were 10 to 100 times faster in this study (J = 0.46 to 1.48 µg/cm^2/h) compared to reported values from the Rohm and Haas studies (J = 0.07 to 0.037 µg/cm^2/h). In addition to skin occlusion and skin thickness, experimental parameters can also influence the permeation rates, but since no information was provided in the report of Burnett et al. (2010) and SCCS (2009) for the Rohm and Haas's unpublished studies, only general interpretations can be provided here. In general, chemicals permeate frozen skin faster compared to viable skin (Barbero and Frasch 2016), which was not what we observed for MI. All studies have shown that the permeation rate increased with increasing MI concentration applied as is to be expected for small water soluble molecules (Wester and Maibach 2010).

MCI permeation was influenced by the topical vehicle while MI was not. MCI in shampoo, body lotion or cream did not permeate skin as was observed in both Rohm and Haas and this study. Aqueous solutions probably disrupt the SC intercellular lipid lamellae and create corneocyte separations as is known from *ex vivo* skin contact with water (Warner et al. 2003). The hydrogen bonding between MCI and MI with water can be an enhancer, which is reduced when added to cream. This is especially true for MCI with a higher K_{ow} than MI (2.5 vs 0.3, respectively; Table 1). The vehicle thus plays a role in skin permeation as previously reported by other studies (Gujjar and Banga 2014; Kanikkannan et al. 2000).

In vivo rat studies from Rohm and Haas (1982) and De Bethizy et al. (1986) highlighted a persistence of the two compounds in the skin application site for at least 96 h with an especially long elimination half-life for MCI (13.1 days). Rohm and Haas (1989; 2003; 2005a; 2005b; 2005c) measured an important dose of MCI and a relatively high dose of MI in epidermis, suggesting that both compounds were retained in this skin layer (Table 6). However, Rohm and Haas studies noted no difference in the MCI doses measured in human SC or human epidermis, they remained similar for all tested solutions (around 8.9% and 50.3% of dose recovered for SC and epidermis, respectively). According to De Bethizy et al. (1986), it could be possible that the MCI retained in epidermis was not available for the systematic blood circulation and would be eliminated by normal desquamation of the epithelial cells.

The mechanism of action regarding the reactivity toward nucleophilic materials for MCI and MI are fairly different, and this may explain the difference in permeation observed in this study. Both MCI and MI react with proteins. MCI reacts rapidly with most nucleophiles to form different stable protein adducts. MI can only react with thiol-containing peptides to form unstable protein adducts (Alvarez-Sánchez et al. 2004; Devos et al. 2015; Divkovic et al. 2005; Jayjock et al. 1996; Mutschler et al. 2009). Thus stable MCI protein would retain the MCI in the skin and thus not allow it to permeate, while unstable MI only formed unstable protein adducts and could therefore permeate the skin.

The hallmark of isothiazolinones is to kill microorganisms by interacting and oxidizing accessible cellular thiols (Du et al. 2002). By destroying the protein thiol groups, the cell undergoes cell death and produces free radicals (Williams 2007). Consequently, it is possible that MCI and MI interact with GSH of keratinocytes as GSH being the major source of free thiol group in the cells. This may in turn induce cell death by apoptosis or necrosis (Devos et al. 2015; Ettorre et al. 2003). Anselmi et al. (2002) reported apoptosis 24-h after exposure to 0.001 and 0.1% of MCI/MI in aqueous solutions, and necrosis at concentrations of 0.1 to 0.5% in their *in vitro* experiment using the human promyelocytic leukemia cell line (HL60). Similar results were observed in the study from Ettorre et al. (2003) using primary basal keratinocytes obtained from skin samples of patients during dermatologic operations. They observed an increase in reactive oxygen species (ROS) production related to an increase of MCI/MI concentrations. The authors suggested that low concentrations of MCI/MI induce apoptotic mechanisms while high concentrations activate necrotic mechanisms; and concluded that apoptosis was probably a keratinocytic response to less severe injuries than those inducing necrosis. This could explain our histology results as we observed focal spongiosis at 375/125 µg/mL of MCI/MI and suffering

and apoptotic cells at the highest concentration 750/250 µg/mL of MCI/MI in aqueous water (Table 5). Spongiosis development is characterized by apoptosis of single keratinocytes (Kerstan et al. 2011).

Moreover, Williams (2007) emphasized that various adjuvants and surfactants added in formulations may increase the extent of cell death. This may explain the results for Revaguard®, pure or diluted, which differed significantly from the other commercial formulations tested. No skin irritation was observed in the histological evaluation of skin exposed to 71/35 µg/mL of MCI/MI in aqueous solutions for 24h, while moderate irritation was noticed for pure Revaguard® (70/22 µg/mL of MCI/MI) and mild irritation for the 50% dilution of this product (35/11 µg/mL of MCI/MI). Consequently, the vehicle may have some influence on the permeation of MCI and MI through the skin as well as on skin irritation effects.

Metabolism may occur in viable skin (Bronaugh 2005). MI metabolism has been observed in a rat study after administration of MI by gavage where main urinary metabolites detected were N-methyl malonamic acid (NMMA), 3-mercapturic acid conjugate of 3-thiomethyl-N-methyl-propionamide, and N-methyl-3-hydroxyl-propionamide (Burnett et al. 2010). Whether or not skin can metabolize MI is unknown and was not assessed in our skin permeation study. No studies on MCI metabolism were identified in the scientific literature. MCI and MI skin metabolism need to be addressed in future studies.

MCI and MI are considered to be haptens; molecules with low molecular weight with electrophilic properties and chemically reactive that can form covalent bonds with nucleophilic materials thereby inducing sensitization (Alvarez-Sánchez et al. 2004; Devos et al. 2015; Divkovic et al. 2005). As of 2015, MCI is classified as an extreme skin sensitizer and MI a strong sensitizer (Devos et al. 2015; Roberts 2013). For this reason, the legislations do not allow the use of MCI and MI in rinse-off products at higher concentrations than 15 µg/mL of MCI/MI and up to 7.5 µg/mL for leave-on products. Although no sign of skin irritation was observed at these concentrations in this study, allergic reactions cannot be excluded as they can be induced at any concentrations. Since MI permeated through the skin quite easily and with a very short $T_{lag,}$ it is recommended to limit exposure to MI and MCI. This is especially pertinent where topical vehicles may increase permeation.

To attempt to relate permeation experiments and histology interpretations, several different concentrations of MCI/MI in aqueous water were tested. A quantitative dose response for the structural changes in the epidermis was obtained; however, the experiments did not include assays testing MI and MCI separately. This was due to the limited amount of skin samples that

we had access to perform the assays. Consequently, an influence of MCI or MI on the permeation coefficients when used in mixtures may not be excluded. Further studies should be performed to assess MCI and MI permeation separately as mixture ingredients may affect permeation profile of a compound by modifying properties of the stratum corneum and leading to an enhanced permeation of the compound (Ghafourian et al. 2010).

Conclusion

MI in formulations quickly permeated the skin with time lags less than an hour while MCI was much slower (>8h). MCI in formulations had permeation rates up to five times greater than for MI in the same product. Standard aqueous solutions of MCI/MI made in the laboratory, did not reflect formulation permeation characteristics; MI permeated skin slowly and after less than an hour exposure, while MCI was not detected after 20h of exposure. MCI in the two tested creams were not found to permeate skin. Some signs of irritation were observed by histology; especially at the highest MCI/MI concentrations (750/205 µg/mL) in aqueous solutions. The topical vehicle greatly influenced the MCI and MI permeations. It is therefore more relevant to test exposures to formulations than aqueous standard solutions. As allergic skin reactions may occur at any concentrations, it is recommended to limit exposure to MI and MCI.

References

Ackermann L, Aalto-Korte K, Alanko K, et al. (2011) Contact sensitization to methylisothiazolinone in Finland—a multicentre study. Contact Dermatitis 64(1):49-53 doi:10.1111/j.1600-0536.2010.01811.x

Alexander BR (2002) An assessment of the comparative sensitization potential of some common isothiazolinones. Contact Dermatitis 46(4):191-6

Alvarez-Rivera G, Dagnac T, Lores M, et al. (2012) Determination of isothiazolinone preservatives in cosmetics and household products by matrix solid-phase dispersion followed by high-performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1270:41-50

Alvarez-Sánchez R, Basketter D, Pease C, Lepoittevin J-P (2004) Covalent binding of the 13C-labeled skin sensitizers 5-chloro-2-methylisothiazol-3-one (MCI) and 2-methylisothiazol-3-one (MI) to a model peptide and glutathione. Bioorganic & Medicinal Chemistry Letters 14(2):365-368 doi:http://dx.doi.org/10.1016/j.bmcl.2003.11.002

Anselmi C, Ettorre A, Andreassi M, Centini M, Neri P, Di Stefano A (2002) In vitro induction of apoptosis vs. necrosis by widely used preservatives: 2-phenoxyethanol, a mixture of isothiazolinones, imidazolidinyl urea and 1,2-pentanediol. Biochemical Pharmacology 63(3):437-453 doi:http://dx.doi.org/10.1016/S0006-2952(01)00910-8

Barbero AM, Frasch HF (2016) Effect of Frozen Human Epidermis Storage Duration and Cryoprotectant on Barrier Function Using Two Model Compounds. Skin Pharmacology and Physiology 29(1):31-40

Bayraktar AMD, Ozcan MMD (2007) An Unusual Case: Burn Following an Accidental Exposure to Methylchloroisothiazolinone/Methylisothiazolinone. Journal of Burn Care & Research January/February 28(1):195-197

Bohn S, Niederer M, Brehm K, Bircher AJ (2000) Airborne contact dermatitis from methylchloroisothiazolinone in wall paint. Abolition of symptoms by chemical allergen inactivation. Contact Dermatitis 42(4):196-201 doi:10.1034/j.1600-0536.2000.042004196.x

Braun-Falco M, Knott E, Huss-Marp J, Ring J, Hofmann H (2008) Alkalization of wall paint prevents airborne contact dermatitis in patients with sensitization to isothiazolinones. Contact Dermatitis 59(2):129-31 doi:10.1111/j.1600-0536.2008.01356.x

Breuer K, Uter W, Geier J (2015) Epidemiological data on airborne contact dermatitis – results of the IVDK. Contact Dermatitis 73(4):239-247 doi:10.1111/cod.12455

Bronaugh RL (2005) In vitro diffusion cell studies in dermal absorption models in toxicology and pharmacology. In: Riviere JEE (ed) Dermal Absorption Models in Toxicology and Pharmacology. CRC Press, Boca Raton, FL, USA, p pp. 21-27

Burnett CL, Bergfeld WF, Belsito DV, et al. (2010) Final report of the safety assessment of methylisothiazolinone. International journal of toxicology 29(4 Supplement):187S-213S

Carlsen BC, Menne T, Johansen JD (2008) Associations between baseline allergens and polysensitization. Contact Dermatitis 59(2):96-102 doi:10.1111/j.1600-0536.2008.01400.x

Castanedo-Tardana MP, Zug KA (2013) Methylisothiazolinone. Dermatitis 24(1):2-6 doi:10.1097/DER.0b013e31827edc73

Cleenewerck M-B (2008) Occupational Cutaneous Contact Allergy from Isothiazolinones. Dermatitis November/December 19(6):354

De Bethizy JD, Longacre SL, Steigerwalt RB, et al. (1986) Absorption and disposition of 14C-labelled Kathon biocide, a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one, following intravenous or dermal administration to male Sprague-Dawley rats. Food Chem Toxicol 24(1):43-9

Devos FC, Pollaris L, Van Den Broucke S, et al. (2015) Methylisothiazolinone: dermal and respiratory immune responses in mice. Toxicology letters 235(3):179-188

Divkovic M, Pease CK, Gerberick GF, Basketter DA (2005) Hapten-protein binding: from theory to practical application in the in vitro prediction of skin sensitization. Contact Dermatitis 53(4):189-200

Douglas JD (2010) Allergic Contact Dermatitis: Topical Preservatives, Part II. Dermatology Nursing May/June 22(3):43-46

Du S, McLaughlin B, Pal S, Aizenman E (2002) In Vitro Neurotoxicity of Methylisothiazolinone, a Commonly Used Industrial and Household Biocide, Proceeds via a Zinc and Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase-Dependent Pathway. The Journal of Neuroscience 22(17):7408-7416

Ettorre A, Neri P, Di Stefano A, Andreassi M, Anselmi C, Andreassi L (2003) Involvement of Oxidative Stress in Apoptosis Induced by a Mixture of Isothiazolinones in Normal Human Keratinocytes. Journal of Investigative Dermatology 121(2):328-336 doi:http://dx.doi.org/10.1046/j.1523-1747.2003.12360.x

Friis UF, Menne T, Flyvholm MA, et al. (2014) Isothiazolinones in commercial products at Danish work places. Paper presented at the 12th Congress of the European Society of Contact Dermatitis, Barcelona, Spain, June

Gameiro A, Coutinho I, Ramos L, Gonçalo M (2014) Methylisothiazolinone: second ‘epidemic’ of isothiazolinone sensitization. Contact Dermatitis 70(4):242-243 doi:10.1111/cod.12200

Ghafourian T, Samaras EG, Brooks JD, Riviere JE (2010) Modelling the effect of mixture components on permeation through skin. International Journal of Pharmaceutics 398(1–2):28-32 doi:http://dx.doi.org/10.1016/j.ijpharm.2010.07.014

Gujjar M, Banga AK (2014) Vehicle influence on permeation through intact and compromised skin. Int J Pharm 472(1-2):362-8 doi:10.1016/j.ijpharm.2014.06.050

Jayjock MA, Hazelton GA, Lewls PG, Wooder MF (1996) Formulation effect on the dermal bioavailability of isothiazolone biocide. Food and Chemical Toxicology 34(3):277-282 doi:10.1016/0278-6915(95)00114-x

Kanikkannan N, Kandimalla K, Lamba SS, Singh M (2000) Structure-activity Relationship of Chemical Penetration Enhan-cers in Transdermal Drug Delivery. Current Medicinal Chemistry 7(6):593-608 doi:http://dx.doi.org/10.2174/0929867003374840

Kerstan A, Bröcker E-B, Trautmann A (2011) Decisive role of tumor necrosis factor-α for spongiosis formation in acute eczematous dermatitis. Archives of Dermatological Research 303(9):651-658 doi:10.1007/s00403-011-1149-5

Lundov MD, Kolarik B, Bossi R, Gunnarsen L, Johansen JD (2014) Emission of Isothiazolinones from Water-Based Paints. Environmental Science & Technology 48(12):6989-6994 doi:10.1021/es500236m

Lundov MD, Krongaard T, Menné TL, Johansen JD (2011) Methylisothiazolinone contact allergy: a review. British Journal of Dermatology 165(6):1178-1182 doi:10.1111/j.1365-2133.2011.10523.x

Mendonca FMI, Prosper MMS, Pichardo ARPR, Bravo BGBG, Martinez FCMC (2014) Kathon CG allergic contact dermatitis epidemic in Seville. Contact Dermatitis 70:69

Miles A, Berthet A, Hopf NB, et al. (2014) A new alternative method for testing skin irritation using a human skin model: A pilot study. Toxicology in Vitro 28(2):240-247 doi:http://dx.doi.org/10.1016/j.tiv.2013.10.022

Mutschler J, Giménez-Arnau E, Foertsch L, Gerberick GF, Lepoittevin J-P (2009) Mechanistic assessment of peptide reactivity assay to predict skin allergens with Kathon® CG isothiazolinones. Toxicology in Vitro 23(3):439-446 doi:http://dx.doi.org/10.1016/j.tiv.2009.01.014

OECD (2004a) Guidance Document for the Conduct of Skin Absorption Studies OECD Series on Testing and Assessment Number 28. Paris, Organisation for Economic Co-operation and Development

OECD (2004b) OECD guideline 428 for the testing of chemicals. Skin absorption: in vitro method OECD Series on Testing and Assessment Number 428. Paris, Organisation for Economic Co-operation and Development

Puangpet PMD, Chawarung AMD, McFadden JPMD (2015) Methylchloroisothiazolinone/Methylisothiazolinone and Methylisothiazolinone Allergy. Dermatitis March/April 26(2):99-102

Reinhard E, Waeber R, Niederer M, Maurer T, Maly P, Scherer S (2001) Preservation of products with MCI/MI in Switzerland. Contact Dermatitis 45(5):257-264

Roberts DW (2013) Methylisothiazolinone is categorised as a strong sensitiser in the Murine Local Lymph Node Assay. Contact Dermatitis 69(5):261-262 doi:10.1111/cod.12167

Rohm, Haas (1982) 14C Kathon 886 disposition after percutaneous application to male rats. In: Chemicals RH (ed) Report No 82R-21. Rohm & Haas Chemicals, LLC

Rohm, Haas (1989) 14C-Kathon™ biocide: In vitro dermal absorption study in male rat skin. In: Chemicals RH (ed) Report No 86R-156. Rohm & Haas Chemicals, LLC

Rohm, Haas (2003) 2-methyl-4-isothiazolin-3-one: in vitro per cutaneous absorption through rat skin. In: Chemicals RH (ed) LLC Report 06R-1002. Rohm & Haas Chemicals, LLC, p 3 pages

Rohm, Haas (2005a) 2-Methyl-4-isothiazolin-3-one (MI): in vitro absorption from water and three formulations through human epidermis. In: Chemicals RH (ed) LLC Report 06R-1002. Rohm & Haas Chemicals, LLC, p 2 pages

Rohm, Haas (2005b) 5-Chloro-2-methyl-4-isothiazolin-3-one (CMI)/2-Methyl-4-isothiazolin-3-one (MI) in a 3:1 w/v Mixture: In vitro absorption of CMI from aqueous solutions through human epidermis. In: Chemicals RH (ed) LLC Report 04RC-067. Rohm & Haas Chemicals, LLC

Rohm, Haas (2005c) 5-Chloro-2-methyl-4-isothiazolin-3-one (CMI)/2-Methyl-4-isothiazolin-3-one (MI): In vitro absorption of CMI from an aqueous solution and three formulations through human epidermis. In: Chemicals RH (ed) LLC Report 05RC-055. Rohm & Haas Chemicals, LLC

SCCS (2009) Opinion on the mixture of 5-chloro-2-methylisothiazolin-3(2H)-one and 2-methylisothiazolin-3(2H)-one. In: (SCCS) SCoCS (ed). European Commission, Brussels, p 45 pages

Speksnijder P, van Ravestijn J, de Voogt P (2010) Trace analysis of isothiazolinones in water samples by large-volume direct injection liquid chromatography tandem mass spectrometry. J Chromatogr 1217(32):5184-9

Urwin R, Warburton K, Carder M, Turner S, Agius R, Wilkinson SM (2015) Methylchloroisothiazolinone and methylisothiazolinone contact allergy: An occupational perspective. Contact Dermatitis 72(6):381-386

Vauhkala A-R, Pesonen M, Suomela S, Kuuliala O, Suuronen K, Aalto-Korte K (2015) Occupational contact allergy to methylchloroisothiazolinone/methylisothiazolinone and methylisothiazolinone. Contact Dermatitis 73(3):150-156 doi:10.1111/cod.12413

Warner RR, Stone KJ, Boissy YL (2003) Hydration disrupts human stratum corneum ultrastructure. J Invest Dermatol 120(2):275-84 doi:10.1046/j.1523-1747.2003.12046.x

Wester RC, Maibach H (2010) Methodology: In vivo Methods for Percutaneous Absorption Measurements Percutaneous absorptio: Drugs, Cosmetics, Mechanisms, Methodology Fourth Edition. Informa Healthcare, New York, USA, p 257-263

Williams TM (2007) The Mechanism of Action of Isothiazolone Biocide. 2006/1/1/

Wright AM, Cahill JL (2015) Airborne exposure to methylisothiazolinone in paint causing allergic contact dermatitis: An Australian perspective. The Australasian journal of dermatology doi:10.1111/ajd.12384

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical standards

The manuscript does not contain clinical studies or patient data.

Human abdominal skin was obtained as surgical waste of abdominoplasty from DAL biobank, under anonymous donation, in accordance with its regulation and the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The protocol was accepted by the State Ethics Commission under protocol 264/12. General consent of patients was obtained by the surgeon for the use of the removed skin in our experiments and the skin samples were deidentified before collection.

Research involving human participants and/or animals

This article does not contain any studies with human participants or animals performed by any of the authors.

Figure captions

Fig. 1 – Cumulative amount of MCI (µg/cm^2) measured in the receptor fluid over time (hours) following skin exposure to pure (Revaguard®, Permatol® K40) or diluted formulations (Revaguard®).

Fig. 2 – Cumulative amount of MI (µg/cm^2) measured in the receptor fluid over time (hours) following skin exposure to aqueous solutions (standard shown as solid triangles) and four different formulations (Permatol® K40 shown with solid diamonds, Revaguard® with white circle (pure) and black circle (diluted), Priva care® with black square and MR Extra® cream with white square).

Fig. 3 – Irritation intensity results of skin sample exposed to 750/250 µg/mL of MCI/MI for 24 h compared to negative and positive controls based on histological criteria (spongiosis, epidermolysis, apoptotic and suffering cells). a) histological results for skin sample exposed to 750/250 µg/mL of MCI/MI for 24 h (x10 enlargement); b) histological results for a negative control (skin exposed to physiological water for 24 h) (x40 enlargement); and c) histological results for a positive control (skin exposed to 10% of sodium lauryl sulphate (SLS) for 24 h) (x100 enlargement).