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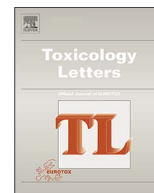
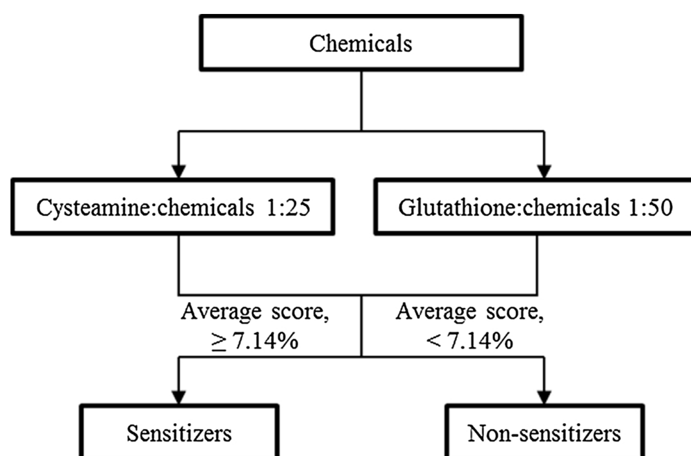


A simple *in chemico* method for testing skin sensitizing potential of chemicals using small endogenous molecules

Mahesh Raj Nepal, Rajina Shakya, Mi Jeong Kang, Tae Cheon Jeong*

College of Pharmacy, Yeungnam University, Gyeongsan 38541, South Korea

GRAPHICAL ABSTRACT



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ABSTRACT

Among many of the validated methods for testing skin sensitization, direct peptide reactivity assay (DPRA) employs no cells or animals. Although no immune cells are involved in this assay, it reliably predicts the skin sensitization potential of a chemical *in chemico*. Herein, a new method was developed using endogenous small-molecular-weight compounds, cysteamine and glutathione, rather than synthetic peptides, to differentiate skin sensitizers from non-sensitizers with an accuracy as high as DPRA. The percent depletion of cysteamine and glutathione by test chemicals was measured by an HPLC equipped with a PDA detector. To detect small-size molecules, such as cysteamine and glutathione, a derivatization by 4-(4-dimethylaminophenylazo) benzenesulfonyl chloride (DABS-Cl) was employed prior to the HPLC analysis. Following test method optimization, a cut-off criterion of 7.14% depletion was applied to differentiate skin sensitizers from non-sensitizers in combination of the ratio of 1:25 for cysteamine:test chemical with 1:50 for glutathione:test chemical for the best predictivity among various single or combination conditions. Although overlapping HPLC peaks could not be fully resolved for some test chemicals, high levels of sensitivity (100.0%), specificity (81.8%), and accuracy (93.3%) were obtained for 30 chemicals tested, which were comparable or better than those achieved with DPRA.

Abbreviations: LLNA, local lymph node assay; ECVAM, European centre for the validation of alternative methods; DPRA, direct peptide reactivity assay; HTS-DCYA, high-throughput dansyl-cysteamine; DABS-C1, 4-(4-dimethylaminophenylazo) benzenesulfonyl chloride; ROC, receiver operating curve

* Corresponding author at: 280 Daehak ro, College of Pharmacy, Yeungnam University, Gyeongsan, 38541, South Korea.
E-mail address: taecheon@ynu.ac.kr (T.C. Jeong).

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1. Introduction

Allergic contact dermatitis, a common occupational and environmental health hazard, is a hapten-specific T cell-mediated delayed-type hypersensitivity (Adam et al., 2011). In the sensitization phase, antigenic adducts of a hapten with an endogenous carrier are presented by skin dendritic cells (i.e., epidermal Langerhans cells) to cause the activation of hapten-specific $CD8^+$ and $CD4^+$ T cells (Agüero et al., 2012). Upon a hapten re-exposure in the sensitized individual, effector T cells trigger the inflammatory process leading to clinical symptoms, such as pruritus and erythema (Kimber et al., 2002). Haptens are non-immunogenic, low-molecular-weight chemicals that bind via their electrophilic residues to the nucleophilic part of carrier molecules, mostly epidermal peptides and proteins (Kimber et al., 2002). Meanwhile, these reactive haptens also extensively bind to endogenous small molecules and defensive molecules, such as cysteamine and glutathione, existing in all tissues (Kerksick and Willoughby, 2005; Sariozkan et al., 2015).

Previously, one of the most common alternative methods to determine the skin sensitization potential of chemicals has been local lymph node assay (LLNA). LLNA involves the measurement of T cell proliferation in draining lymph nodes after the repeated topical application of test chemicals on the ear skin of mice. In the assay, increased T cell proliferation by a test chemical is compared with a control by calculating the stimulation index, and the chemical is classified as either a sensitizer or a non-sensitizer (Kern et al., 2010; Kimber and Weisenberger, 1989; Natsch et al., 2009). However, with the ban on animal testing for cosmetics and their ingredients and the implementation of the European regulation on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), alternative methods excluding animals are being explored extensively (Hartung et al., 2003). Although several alternative methods with cell cultures have been developed or are under validation by many authorities, including the European Centre for the Validation of Alternative Methods (ECVAM), none of these methods accurately classify chemicals as sensitizers or non-sensitizers. Therefore, the development of much simpler and reasonably acceptable *in vitro* methods is needed.

Accordingly, we initially decided to use the direct peptide reactivity assay (DPRA) because it is an *in chemico* method to measure the depletion of peptides following their co-incubation with a test chemical for 24 h at room temperature without any cell cultures (OECD, 2015). Cysteine- and lysine-containing synthetic peptides are used to measure only the chemical reactivity, which would be reasonable because skin sensitizers are required to be bound to carrier proteins for being an immunogen (Gerberick et al., 2007, 2004). Based on this principle, various similar alternative methods have been developed (Fujita et al., 2014; Jeong et al., 2013; Natsch and Gfeller, 2008; Troutman et al., 2011). Although none of them perfectly fulfilled the ideal criteria of chemical categorization according to the *in vivo* skin sensitization potential, DPRA allowed reasonably high predictivity when compared with other methods using cell cultures (Stokes et al., 2012). However, DPRA has some drawbacks. One of the major drawbacks of DPRA is its cost because the two different hepta-peptides used are required to be chemically synthesized with high purity (Bray, 2003). In addition, it is difficult to control the reaction and extraction steps following the incubation of a peptide with a test chemical prior to HPLC detection. This could lead to the misinterpretation of the HPLC results. A time restriction must be applied between sample preparation and HPLC analysis to prevent discrepancies in the results between the first and last samples. Moreover, most test chemicals have their UV absorption maxima at around 220 nm, at which DPRA is performed. This may interfere with the absorption maxima of a number of test chemicals leading to false results. To improve these problems associated with DPRA, we developed a new method by using simple endogenous chemicals, such as cysteamine and glutathione.

Cysteamine and glutathione are small-sized molecules abundantly present in the human body (Martin and Teismann, 2009; Szwergold, 2006; Wu et al., 2004). Reactive functional groups, such as thiol and amino groups, present in these chemicals exhibit prominent nucleophilic characteristics, and have been proved to sufficiently react with electrophiles (Ketterer et al., 1983; Szwergold, 2006). Studies also have reported their antioxidant and cytoprotective activities against chemical toxicity by detoxifying electrophilic compounds (Kessler et al., 2008; Masella et al., 2005; Sonni et al., 2011). In addition, the role of glutathione in allergic contact dermatitis has been proved *in vivo,* by examining the changes in the level of glutathione and glutathione disulfide in the mouse skin and *in vitro* in several studies (Gerberick et al., 2007, 2004; Schmidt and Chung, 1992). Meanwhile, cysteamine, an amino-thiol and a by-product of amino acid cysteine, also acts as a precursor for the biosynthesis of homo-taurine and taurine (Theofanopoulos and Lau-Cam, 1998). Thiol and amino groups in cysteamine are exposed to reactive haptens owing to their high reactivities toward electrophiles (Hamilton et al., 1979). Moreover, in a fluorescence-based high-throughput dansyl-cysteamine (HTS-DCYA) assay, a clear correlation in reactivity between skin sensitizing chemicals and DCYA derivative was seen (Avonto et al., 2015).

In the present study, a novel but simple *in chemico* method using cysteamine and glutathione is proposed to rapidly identify skin sensitizers. The developed method would not only be robust and cost-effective, but also as reliable as any existing alternative methods for skin sensitization. In this study, either cysteamine or glutathione was allowed to react with test chemicals for 24 h at room temperature, and the percent depletion of either cysteamine or glutathione was measured. For quantitative analysis, 4-(4-dimethylaminophenylazo) benzenesulfonyl chloride (DABS-Cl) was used as a derivatizing agent to form a complex with either cysteamine or glutathione remained after incubation with test chemicals, followed by the quantitation of complexes at 436 nm using HPLC equipped with a PDA detector. Although the depletion of either cysteamine or glutathione alone gave excellent predictivity, a model with the combination of both is also proposed.

2. Materials and methods

2.1. Materials

Test chemicals used in the present study with their suppliers are listed in Table 1. DABS-Cl (CAS No. 56512-49-3) was obtained from Supelco (Bellefonte, PA, USA) and 2,4-dinitro fluorobenzene (CAS No. 70-34-8) was obtained from TCI (Tokyo, Japan). Acetonitrile for HPLC analysis was obtained from J. T. Baker (Phillipsburg, NJ, USA) and that for sample preparation from MERCK (Darmstadt, Germany). Trifluoroacetic acid was obtained from Alfa Aesar (Lancashire, UK). Cysteamine (≥98%) and glutathione (≥98%) were purchased from Sigma Chemical Company (St. Louis, MO, USA). All chemicals were used as received.

2.2. Incubation conditions

Solvent selection for each chemical was based on the OECD-DPRA guideline (OECD, 2015). Stock solutions (150 mM) of all test chemicals, except 2-mercaptobenzothiazole, imidazolidinyl urea, and tetramethylthirum disulfide, were prepared in acetonitrile. 2-Mercaptobenzothiazole was solubilized in a 1:9 mixture of DMSO and acetonitrile, imidazolidinyl urea was dissolved in pure distilled water, and tetramethylthirum disulfide was dissolved in a 1:1 mixture of DMSO and acetonitrile. In an Eppendorf tube, 375 µl of 1 mM of either cysteamine or glutathione stock solution in 0.1 M sodium acetate buffer (pH 4.5) was mixed with 125, 62.5, or 31.25 µl of 150 mM stock solutions of test chemicals. HPLC grade acetonitrile was added to make-up the volume to 0.5 ml/tube for the preparation of 1:50, 1:25, and 1:12.5 molar ratios of either cysteamine or glutathione to the test chemical, respectively. Separate controls were prepared for each solvent used.

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