Compatibility Determination of Alternative Solvents in the human Cell Line Activation Test (h-CLAT) for the Assessment of Skin Sensitization

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Abstract 3312

Abstract

A skin sensitizer refers to a substance leading to an allergic reaction after repeated skin contact. The prediction of skin sensitization is recognized as an important endpoint in toxicology, required by regulatory agencies for hazard identification, as well as used for risk assessment. A battery of *in vitro* tests have been developed based on the current knowledge of the key biological events leading to skin sensitization. The h-CLAT assesses the activation of monocytes and dendritic cells by determining the expression of cell surface markers, CD86 and CD54, in a human monocytic leukemia cell line (THP-1). Although the h-CLAT has been found to be applicable to chemicals with diverse functional groups, its predictive performance is limited to substances that are soluble in the assay solvents (DMSO and Saline) specified in the validated method. Therefore, we aimed to determine the compatibility of various solvents with the h-CLAT and to evaluate its predictive capacity. The h-CLAT was performed as previously described (OECD TG 442E, 2018[1]). In addition to the standard solvents, Acetone (ACE), Acetonitrile (ACN), Ethanol (EtOH), Methanol (MeOH), and Isopropanol (ISO), were evaluated. All the solvents were determined to be non-Sensitizers and non-Cytotoxic. Two test substances were selected for assessment of their sensitization potential: the proficiency chemical lactic acid (LA) and benzyl salicylate (BS), a known "difficult to work" chemical in h-CLAT. LA was found to be soluble in all the test solvents while BS was found to be soluble in all the solvents except for Saline. LA was correctly predicted as a non-Sensitizer when prepared in EtOH, ACE and MeOH, however, it was miss predicted in ACN and ISO. BS was classified as a non-Sensitizer in DMSO, EtOH, MeOH while it was classified as a Sensitizer in ACE, ACN and ISO. BS is regarded as a Sensitizer based on *in vivo* data, however, results from human experiments suggest this is a false positive. Based on the LA results, EtOH, ACE and MeOH appear to be appropriate alternative solvents for the h-CLAT. Furthermore, EtOH and MeOH, classified BS consistently in line with human data which is the most relevant dataset to compare our results to. Our data indicate that the selected solvent has a profound effect on the results of the h-CLAT. The inclusion of EtOH and MeOH as primary solvents may improve and extend the applicability domain of the h-CLAT.

Introduction

Poorly soluble test substances are difficult to assess *via* flow cytometry, may produce inconclusive results and miss classification of the sensitization potential. Our goal was to determine if other solvents besides DMSO or Saline can be used, and thereby expand the applicability of the h-CLAT. The five solvents selected for testing were anticipated to be non-cytotoxic based on their use in other applications. Our test substances, Benzyl Salicylate (BS) and Lactic Acid (LA), were chosen based on their reported sensitization potential and solubility. BS is used as a fragrance ingredient which is poorly soluble in water with a log K_{ow} (n-octanol–water partition coefficient) of 4.31. This is higher than the 3.5 threshold applicable to the h-CLAT. BS is soluble in alcohols and thus better solubility was achieved using some of our test solvents. BS is a predicted sensitizer based on *in vivo* and *in vitro* studies. However, in human clinical trials, BS has been determined as a non-sensitizer. The positive results are likely a false-positive due to challenges with preclinical test approaches. Therefore, BS was considered as non-sensitizer for the current study. LA is a non-sensitizer, included as a proficiency chemical for h-CLAT, and soluble in most polar solvents. LA can react with alcohols in an esterification reaction, which may effect the h-CLAT results, and thus, was included as a reference test substance.

Chemical Name	*In Vivo*	*In Vitro*				Human Clinical Trials	
	LLNA	DPRA	Keratino Sens™	h-CLAT	Sens-is™	HRIPT	HMT
Benzyl Salicylate (BS)	Moderate	No	Yes	No	Yes	No	No

Table 1. Known Sensitization Potential for BS. BS has been regarded as a sensitizer based on Local Lymph Node Assay (LLNA) data. BS has also been classified as sensitizer in Sens-IS™. However, BS is classified as non-sensitizer using the "2 out of 3" approach for the *in vitro* assays: Direct Peptide Reactivity (DPRA), KeratinoSens™, and h-CLAT. BS is determined as non-sensitizer in the Human Repeat Insult Patch Test (HRIPT) and the Human Maximization Test (HMT)[2, 3].

Materials & Methods

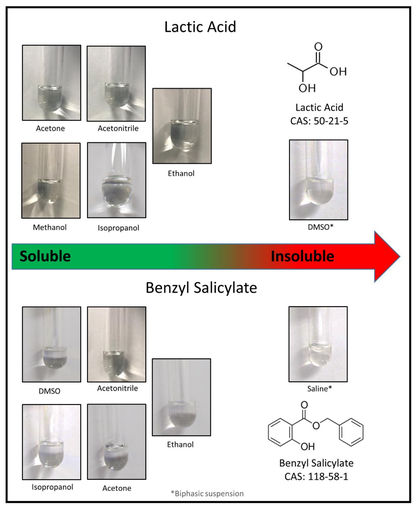


Figure 1. Solubility determination of Lactic Acid and Benzyl Salicylate in the testing solvents. All test substances were prepared at a top stock concentration of 500 mg/mL in the selected solvent. The mixtures were vortexed for 1 minute before physical description was determined. A test article was considered soluble if it produced a clear colorless solution after vortexing.

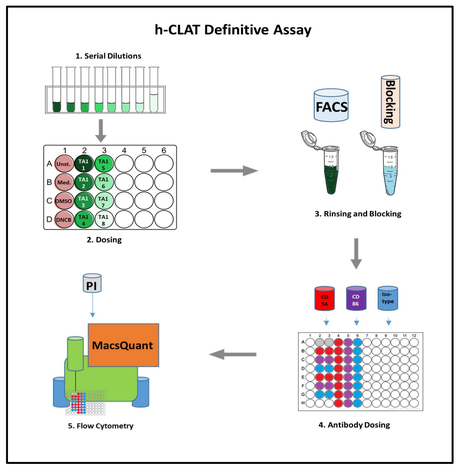


Figure 2. Methods for the h-CLAT definitive assay[1]. The h-CLAT was performed according to the OECD TG 442E, 2018. The test substances were diluted in the selected solvents and then a total of 8 serial dilutions were prepared. A dose range finding assay was performed to establish the doses used in the definitive assays (not shown). THP-1 cells were seeded into a 24-well plate at a final density of $1x10^6$ cells/mL after dosing. The cells were dosed with the working test substance solutions and then incubated for 24 hours. Then, the cells were rinsed with FACS buffer for 3 total washes. A blocking solution was added to the cells and they were incubated at 4°C for 15 minutes. The cells were then plated on a 96 well plate and added with 3 different FITC-conjugated antibodies (CD54, CD86, and isotype control). After a 30 minutes incubation the cells were rinsing and then the surface marker expression was determined *via* the MacsQuant flow cytometer.

Results

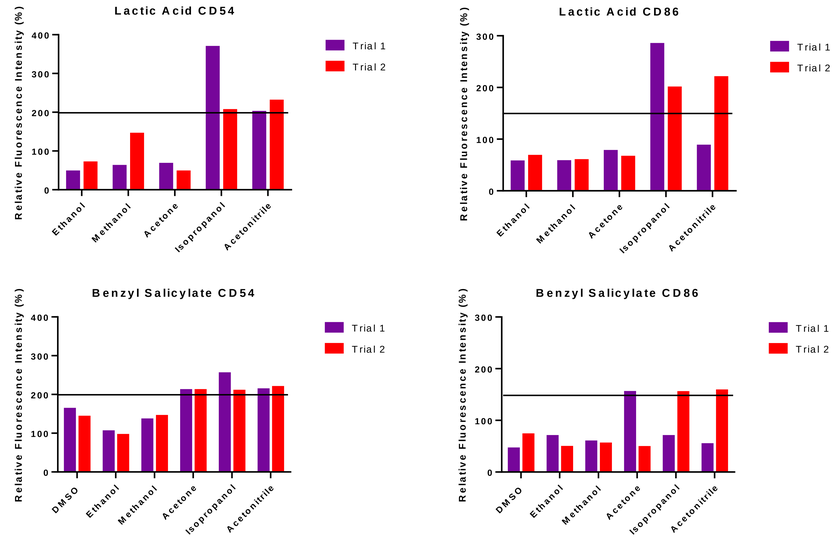
Solvent	Viability (%)	CD86 RFI (%)	CD54 RFI (%)
Ethanol	95.3	82.2	90.1
Acetonitrile	96.6	102.4	92.8
Acetone	96.8	96.7	107.2
Methanol	96.5	89.7	80.7
Isopropanol	95.6	96.7	85.6

Table 2. Relative Fluorescence Intensity (RFI) and cell viability results of the selected solvents in the h-CLAT. The solvents were tested at a single final concentration of 0.2%. The presented values are the average of two independent trials. In order for a solvent to be used in the h-CLAT the following three criteria must be meet: a) the RFI for CD54 must be less than 200%; b) the RFI for CD86 must be less than 150%; and c) cell viability must be greater than 90%. The selected solvents were considered usable in the h-CLAT since they meet the three criteria.

Solvent	Lactic Acid	Benzyl Salicylate
Saline	-	Not Soluble
DMSO	Not Soluble	-
Acetone	-	+
Acetonitrile	+	+
Ethanol	-	-
Methanol	-	-
Isopropanol	+	+
Expected Skin Sensitization Potential	-	-

Table 3. LA and BS skin sensitization potential results obtained in the h-CLAT for the test solvents. Based on two h-CLAT independent definitive trials, a (+) indicates that the test substance was predicted as a sensitizer and a (-) indicates that the test substance was predicted as a non-sensitizer. The expected skin sensitization potential based on human reported data[2, 3] is shown un grey. The results highlighted in green match the expected results while the red highlight indicate miss prediction. The result for LA in saline is based on historical data.

Figure 3. Expression of CD54 and CD86 for Benzyl Salicylate and Lactic Acid in the test solvents. Two independent definitive trials were performed (shown as purple and red bars, respectively). For a test substance to be determined as a sensitizer the CD54 RFI must be higher than 200% and/or the CD86 RFI must be higher than 150%, and the results must be unequivocal in two independent trials. The black line across the graphs indicates the threshold for a test substance to be considered a sensitizer.



Conclusions & Future Directions

- Ethanol and Methanol allowed to correctly classify the skin sensitization potential of the test substances in the h-CLAT.
- Isopropanol and Acetonitrile lead to an incorrect sensitization prediction of the test substances in the h-CLAT.
- Acetone correctly predicted Lactic Acid as a non-sensitizer while it miss predicted Benzyl Salicylate as a sensitizer.
- Overall, our results indicate that Ethanol and Methanol are promising solvents to be used in the h-CLAT. Further testing is recommended to corroborate the results in a larger set.
- The methodology presented may useful for the screening of novel solvents to be used on the h-CLAT.

References

1. OECD TG 442E. (2018) *In vitro* Skin Sensitization: Human Cell Line Activation Test (h-CLAT).
2. Bergal M., *et al.* (2020) *In vitro* testing strategy for assessing the sensitizing potential of "difficult to test" cosmetic ingredients. Toxicology in Vitro. 65, 104781.
3. Basketter D.A., *et al.* (2014) Categorization of chemicals according to their relative human skin sensitizing potency. Dermatitis. 25 (1), 11-21.