

Tradename: AC Kerazyme®

Code: 16594

CAS #: 69430-36-0 & 999999-99-4

Test Request Form #: 2260

Lot #: 47371P

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Maureen Danaher

Principal Investigator: Jennifer Goodman

Test Performed:

OECD TG 442C: *In Chemico* Skin Sensitization – Direct Peptide Reactivity Assay (DPRA)

Introduction

A skin sensitizer is a substance that will lead to an allergic response following skin contact¹. Haptenation is the covalent binding of a hapten, or low-molecular weight substance or chemical, to proteins in the skin. This is considered the prominent mechanism which defines a chemical as a sensitizer. Haptenation is described as a "molecular initiating event" in the OECD Adverse Outcome Pathway (AOP) for skin sensitization which summarizes the key events known to be involved in chemically-induced allergic contact dermatitis². The direct peptide reactivity assay (DPRA) is designed to mimic the covalent binding of electrophilic chemicals to nucleophilic centers in skin proteins by quantifying the reactivity of chemicals towards the model synthetic peptides containing cysteine and lysine. The DPRA is able to distinguish sensitizers from non-sensitizer with 82% accuracy (sensitivity of 76%; specificity of 92%)³.

This assay was conducted to determine skin sensitization hazard of **AC Kerazyme®** in accordance with European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and OECD Test Guideline 442C.

Assay Principle

The DPRA is an *in chemico* method which addresses peptide reactivity by measuring depletion of synthetic heptapeptides containing either cysteine or lysine following 24 hours incubation with the test substance. The peptide is a custom material containing phenylalanine to aid in detection. Depletion of the peptide in the reaction mixture is measured by HPLC with gradient elution and UV detection at 220 nm. Cysteine and lysine peptide percent depletion values are then calculated and used in a prediction model which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

1. United Nations Economic Commission (UNECE) (2013) Global Harmonized System of Classification and Labelling of Chemicals (GHS) 5th Revised Edition
2. OECD (2012). The Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168
3. EC EURL ECVAM (2012) Direct peptide reactivity assay (DPRA) validation study report; pp 1 -74.

Materials

- A. Equipment:** HPLC-UV (Waters Breeze - Waters 2998 Photodiode Array Detector); Pipettes; Analytical balance
- B. HPLC/Guard Columns:** Agilent Zorbax SB-C18 2.1 mm x 100 mm x 3.5 µm; Phenomenex Security Guard C18 4 mm x 2 mm
- C. Chemicals:** Trifluoroacetic acid; Ammonium acetate; Ammonium hydroxide; Acetonitrile; Cysteine peptide (Ac-RFAACAA-COOH); Lysine peptide (Ac-RFAAKAA-COOH); Cinnamic aldehyde
- D. Reagents/Buffers:** Sodium phosphate buffer (100 mM); Ammonium acetate buffer (100 mM)
- E. Other:** Sterile disposable pipette tips

Methods

A. Solution Preparation

- 0.667 mM Cysteine Peptide in 100 mM Phosphate Buffer (pH 7.5)
- 0.667 mM Lysine Peptide in 100 mM Ammonium Acetate Buffer (pH 10.2)
- 100 mM Cinnamic Aldehyde in Acetonitrile
- 100 mM* **AC Kerazyme®** in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as **AC Kerazyme®**, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution.

B. Reference Controls

- Reference Control A: For calibration curve accuracy
- Reference Control B: For peptide stability over analysis time of experiment
- Reference Control C: For verification that the solvent does not impact percent peptide depletion

C. Sample, Reference Control, and Co-Elution Control Preparation

- Once these solutions have been made they should be incubated at room temperature, protected from light, for 24±2 hours before running HPLC analysis.
- Each chemical should be analyzed in triplicate.

1:10 Ratio, Cysteine Peptide 0.5 mM Peptide, 5 mM Test Chemical	1:50 Ratio, Lysine Peptide 0.5 mM Peptide, 25 mM Test Chemical
750 µL Cysteine Peptide Solution - Or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls 200 µL Acetonitrile 50 µL Test Chemical Solution - Or Acetonitrile for Reference Controls	750 µL Lysine Peptide Solution - Or 100 mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls 250 µL Test Chemical Solution - Or Acetonitrile for Reference Controls

D. Calibration Curve

- Standards are prepared in a solution of 20% Acetonitrile:Buffer
 - For the Cysteine peptide using the phosphate buffer, pH 7.5
 - For the Lysine peptide using the ammonium acetate buffer, pH 10.2

	<u>Standard 1</u>	<u>Standard 2</u>	<u>Standard 3</u>	<u>Standard 4</u>	<u>Standard 5</u>	<u>Standard 6</u>	<u>Standard 7</u>
mM Peptide	0.534	0.267	0.1335	0.0667	0.0334	0.0167	0.000

E. HPLC Analysis

- HPLC-UV system should be equilibrated at 30°C with 50% Mobile Phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% Mobile Phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for 2 hours
- Absorbance is measured at 220 nm
- Flow Conditions:

<i>Time (minutes)</i>	<i>Flow (mL/min)</i>	<i>% A</i>	<i>% B</i>
0	0.35	90	10
10	0.35	75	25
11	0.35	10	90
13	0.35	10	90
13.5	0.35	90	10
20	End Run		

F. Calculations

- Percent peptide depletion is determined by the following equation:

$$\text{Percent Peptide Depletion} = \left[1 - \left(\frac{\text{Peptide Peak Area in Replicate Injection}}{\text{Mean Peptide Peak Area in Reference Controls C}} \right) \right] \times 100$$

Data and Reporting

A. Acceptance Criteria

- The following criteria must be met for a run to be considered valid:
 - Standard calibration curve should have an $r^2 > 0.99$.
 - Mean percent peptide depletion values of three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69% for the lysine peptide and the maximum standard deviation should be < 14.9 for the percent cysteine depletion and < 11.6 for the percent lysine depletion.
 - Mean peptide concentration of reference controls A should be 0.50 ± 0.05 mM and the coefficient of variable of the peptide peak areas for reference B and C in acetonitrile should be $< 15.0\%$.
- The following criteria must be met for a test chemical's results to be considered valid:
 - Maximum standard deviation should be < 14.9 for percent cysteine depletion and < 11.6 for percent lysine depletion.
 - Mean peptide concentration of the three reference control C should be 0.50 ± 0.05 mM.

B. Prediction Model

- Cysteine 1:10/Lysine 1:50 Prediction Model

Cysteine 1:10/Lysine 1:50 Prediction Model		
<i>Mean of Cysteine and Lysine % Depletion</i>	<i>Reactivity Class</i>	<i>Prediction</i>
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitizer
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitizer

- b. If co-elution occurs with the lysine peptide, then the cysteine 1:10 prediction model can be used:

Cysteine 1:10 Prediction Model		
<u>Mean of Cysteine and Lysine % Depletion</u>	<u>Reactivity Class</u>	<u>Prediction</u>
0% < Cys % Depletion < 13.89%	Minimal Reactivity	Non-sensitizer
13.89% < Cys % Depletion < 23.09%	Low Reactivity	Sensitizer
23.09% < Cys % Depletion < 98.24%	Moderate Reactivity	Sensitizer
98.24% < Cys % Depletion < 100%	High Reactivity	Sensitizer

Results

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated. The measured values of percent depletion, reactivity class, and prediction for each assay are outlined in Table 1 and Table 2.

Table 1. Cysteine 1:10/Lysine 1:50 Prediction Model.

Cysteine 1:10/Lysine 1:50 Prediction Model		
<u>Mean of Cysteine and Lysine % Depletion</u>	<u>Reactivity Class</u>	<u>Prediction</u>
3.26	Minimal Reactivity	Non-sensitizer
3.24	Minimal Reactivity	Non-sensitizer
3.28	Minimal Reactivity	Non-sensitizer

Table 2. Cysteine 1:10 Prediction Model.

Cysteine 1:10 Prediction Model		
<u>Mean of Cysteine and Lysine % Depletion</u>	<u>Reactivity Class</u>	<u>Prediction</u>
3.16	Minimal Reactivity	Non-sensitizer
3.11	Minimal Reactivity	Non-sensitizer
3.17	Minimal Reactivity	Non-sensitizer

Discussion

Based on HPLC-UV analysis of **AC Kerazyme® (16594)** we can determine this product is not classified as a sensitizer and is not predicted to cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.20% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.