High Performance Liquid Chromatography

Analysis of DPRA (Direct Peptide Reactivity Assay) for Skin Sensitization Testing Using Prominence™-i

Animal bioassay involving the use of guinea pigs or mice was once the mainstream method of skin sensitization testing. However, a total ban on animal testing of cosmetics enacted in the EU in 2013 made it necessary to establish alternative testing methods which do not use animals. DPRA (direct peptide reactivity assay) is one of these alternative methods used to evaluate the potency of skin sensitization by examining the reactivity between a test chemical and peptides. This method uses two types of peptides, one containing cysteine and another containing lysine, which are mixed and reacted with the test chemical for 24 hours before using HPLC to determine the percent peptide depletion. The skin sensitization of the test chemical is evaluated from the determined percent depletion value of each peptide.

In this article, we performed an analysis based on "TG 442C OECD GUIDELINE FOR THE TESTING OF CHEMICALS In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)". The Prominence-i integrated high performance liquid chromatograph (PDA model) was used as the analytical instrument. The rack in the autosampler of this instrument was used to perform 24-hour incubation of the test chemical and peptides. The Shim-pack™ HR-ODS (100 mm L × 2.1 mm i.d., 3 μm) was used for the column.

Test Chemicals

Three test chemicals were selected for use from the proficiency substances listed in Annex 2 of the above guideline. Testing was performed on the sensitizers of formaldehyde (strong), benzylideneacetone (moderate), and the non-sensitizer of lactic acid to determine the percent depletion values of the cysteine peptide and the lysine peptide.

Sample Solution Preparation

A sample of 100 mmol/L of each test chemical was prepared using acetonitrile. Both the cysteine peptide solution and lysine peptide solution were adjusted to a 0.667 mmol/L concentration in the buffer immediately prior to analysis. Each solution was mixed as shown in Tables 1 and 2. The concentrations of these solutions were, with respect to a 0.5 mmol/L peptide concentration, 5 mmol/L for the cysteine peptide solution (1:10 concentration ratio), and 25 mmol/L for the lysine peptide solution (1:50 concentration ratio). These solutions were put into glass vials and incubated for 24 hours in the rack of the autosampler set to 25 °C.

Reference controls A to C were prepared to confirm no increases or decreases in peptides due to operational factors, such as incubation or analysis. Reference control A was measured at the start of consecutive analysis to verify the suitability of the HPLC system. Reference control B was measured before and after measuring the test solution to verify the stability of reference controls over the analysis time. Reference control C was measured together with the test solution to verify that the sample solvent did not affect the percent peptide depletion. The co-elution controls are samples for confirming no elution of impurities at the peptide elution time. Cinnamic aldehyde was used as the positive control. This compound has a known percent peptide depletion value and is used to verify that the sequence of operations and analysis are performed correctly.

Table 1 Preparation of Sample Solutions for Cysteine Peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Solution</th>
<th>pH7.5 Phosphate Buffer</th>
<th>Acetonitrile</th>
<th>Test Chemical Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference control</td>
<td>750 μL</td>
<td>-</td>
<td>250 μL</td>
<td>-</td>
</tr>
<tr>
<td>Test solution</td>
<td>750 μL</td>
<td>-</td>
<td>200 μL</td>
<td>50 μL</td>
</tr>
<tr>
<td>Coelution control</td>
<td>-</td>
<td>750 μL</td>
<td>200 μL</td>
<td>50 μL</td>
</tr>
</tbody>
</table>

Table 2 Preparation of Sample Solutions for Lysine Peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Solution</th>
<th>pH10.5 Ammonium Acetate Buffer</th>
<th>Acetonitrile</th>
<th>Test Chemical Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference control</td>
<td>750 μL</td>
<td>-</td>
<td>250 μL</td>
<td>-</td>
</tr>
<tr>
<td>Test solution</td>
<td>750 μL</td>
<td>-</td>
<td>-</td>
<td>250 μL</td>
</tr>
<tr>
<td>Coelution control</td>
<td>-</td>
<td>750 μL</td>
<td>-</td>
<td>250 μL</td>
</tr>
</tbody>
</table>
Evaluation of Acceptance Criteria and Results of Proficiency Testing

An evaluation of acceptance criteria (Table 4) was performed from the results of the analysis sequence. We found that the criteria were satisfied for all items in this analysis. Figs. 3 and 4 show the chromatograms of reference control C and positive control which compose the peptide standard solutions in addition to the analysis result of each test solution (data of each first analysis). Analysis of each test solution was repeated three times and the mean of each percent peptide depletion values was calculated from the analysis results (Table 5). The percent peptide depletion values indicated in the proficiency testing in Annex 2 were within the reference range.

Table 4 Acceptance Criteria and Results

<table>
<thead>
<tr>
<th>Cysteine Peptide</th>
<th>Lysine Peptide</th>
<th>Criteria</th>
<th>Judgment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.999</td>
<td>0.999</td>
<td>&gt;0.990</td>
<td>OK</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cinnamic aldehyde</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean percent</td>
<td>71.7 %</td>
<td>51.2 %</td>
<td></td>
</tr>
<tr>
<td>peptide depletion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.8 %</td>
<td>1.2 %</td>
<td></td>
</tr>
<tr>
<td>percent peptide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>depletion of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>three replicates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference control</td>
<td>0.49 mmol/L</td>
<td>0.50 mmol/L</td>
<td>0.50±0.05 mmol/L</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean peptide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>concentration of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>three replicates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference controls</td>
<td>B and C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E and C</td>
<td>3.7 %</td>
<td>0.3 %</td>
<td>&lt;15.0 %</td>
</tr>
<tr>
<td>Coefficient of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>variation of peak</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>areas of nine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>replicates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test chemical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>0.6 %</td>
<td>0.1 %</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of percent peptide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>depletion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test chemical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzylideneaceton</td>
<td>0.3 %</td>
<td>0.3 %</td>
<td>&lt;14.9 %</td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of percent peptide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>depletion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test chemical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
<td>1.8 %</td>
<td>0.1 %</td>
<td>&lt;14.9 %</td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of percent peptide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>depletion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference control</td>
<td>0.50 mmol/L</td>
<td>0.51 mmol/L</td>
<td>0.50±0.05 mmol/L</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean peptide</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>concentration of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>three replicates</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5 Proficiency Testing

<table>
<thead>
<tr>
<th>Cysteine Peptide</th>
<th>Lysine Peptide</th>
<th>Criteria</th>
<th>Judgment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Percent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide Depletion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Listed in Annex 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OECD/OCDE TG 442C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>41.7</td>
<td>30 - 60</td>
<td>1.7</td>
</tr>
<tr>
<td>(strong sensitizer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzylideneaceton</td>
<td>92.7</td>
<td>80 - 100</td>
<td>3.8</td>
</tr>
<tr>
<td>(moderate sensitizer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
<td>5.5</td>
<td>0 - 7</td>
<td>1.2</td>
</tr>
<tr>
<td>(non-sensitizer)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3 HPLC Analytical Conditions

- **System**: Prominence-i
- **Column**: Shim-pack HB-ODS (*1)
- **Mobile Phase**: A: 0.1 %TFA-Water, B: 0.085 %TFA-Acetonitrile
- **Time Program**: B Conc. 10 % (0 min) → 25 % (10 min) → 90 % (11 min →13 min) →10 % (13.5 min →20 min)
- **Flow Rate**: 0.35 mL/min
- **Column Temp.**: 30 °C
- **Injection Volume**: 7 μL
- **Detection**: PDA220 nm

*1 We recommend using a guard column.

Fig. 3 Chromatograms of Sample Solutions for Cysteine Peptide

Fig. 4 Chromatograms of Sample Solutions for Lysine Peptide

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