Insulin

DEFINITION
Insulin is a protein that affects the metabolism of glucose. It is obtained from the pancreas of healthy bovine or porcine animals, or both, used for food by humans. Its potency is NLT 26.5 USP Insulin Units/mg, calculated on the dried basis; Insulin labeled as purified contains NLT 27.0 USP Insulin Units/mg, calculated on the dried basis. The proinsulin content is NMT 10 ppm, determined by a validated method.

NOTE—One USP Insulin Unit is equivalent to 0.0342 mg of pure Insulin derived from beef or 0.0345 mg of pure Insulin derived from pork.

IDENTIFICATION

A. The retention time of the major peak for insulin in the Sample solution corresponds to that of the appropriate species in the Identification solution, as obtained in the Assay.

NOTE—It may be necessary to inject a mixture of Sample solution and Identification solution.

B. PEPTIDE MAPPING

Sulfate buffer: 2.0 M ammonium sulfate and 0.5 M sulfuric acid (1:1)
Enzyme solution: 500 units/mL of Staphylococcus aureus V-8 protease activity in water
HEPES buffer: 0.1 M HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid). Adjust with 5 M sodium hydroxide to a pH of 7.5 before diluting with water to a final volume.

Solution A: Acetonitrile, water, and Sulfate buffer (100:700:200)
Solution B: Acetonitrile, water, and Sulfate buffer (400:400:200)

Mobile phase: See Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>60</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>65</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>70</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>71</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>86</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Standard digest solution: 2 mg/mL of USP Insulin RS of the appropriate species in 0.01 N hydrochloric acid. Transfer 500 µL of the resulting solution to a clean vial. Add 2.0 mL of HEPES buffer and 400 µL of Enzyme solution, and incubate at 25°C for 6 h. Quench the digestion by adding 2.9 mL of Sulfate buffer.

Sample digest solution: 2 mg/mL of Insulin in 0.01 N hydrochloric acid, mix to dissolve. Transfer 500 µL of the resulting solution to a clean vial. Add 2.0 mL of HEPES buffer and 400 µL of Enzyme solution, and incubate at 25°C for 6 h. Quench the digestion by adding 2.9 mL of Sulfate buffer.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 214 nm
Column: 4.6-mm x 10-cm; packing L1
Column temperature: 40°C
Flow rate: 1 mL/min

System suitability
Sample: Standard digest solution

Suitability requirements
Chromatogram comparability: The chromatogram of the Standard digest solution corresponds to that of the reference chromatogram provided with USP Insulin RS of the appropriate species.

Resolution: NLT 1.9 between digest fragments II and III.

NOTE—Fragment I elutes at the same time in insulin derived from pork and Insulin Human; Fragment II elutes at the same time in Insulin Human and insulin derived from beef and pork; and Fragment III elutes at the same time in insulin derived from beef and pork.

Tailing factor: NMT 1.5

Analysis
Samples: Standard digest solution and Sample digest solution

Using the gradient program, run a blank. Separately inject equal volumes of the Standard digest solution and the Sample digest solution, and record the responses of each peak.

Acceptance criteria: The chromatographic profile of the Sample digest solution corresponds to that of the Standard digest solution.

ASSAY

Change to read:

• PROCEDURE

Solution A: Dissolve 28.4 g of anhydrous sodium sulfate in 1000 mL of water. Pipet 2.7 mL of phosphoric acid into the solution, and adjust with ethanolamine to a pH of 2.3, if necessary.

Mobile phase: Acetonitrile and Solution A (26:74)

NOTE—The acetonitrile is warmed to a temperature NLT 20°C to avoid precipitation.

System suitability solution: 1.5 mg/mL of Insulin in 0.01 N hydrochloric acid. Allow to stand at room temperature for NLT 3 days to obtain a solution containing NLT 5% of A-21 desamido insulin.

Identification solution: 0.6 mg/mL of USP Insulin Pork RS and 0.6 mg/mL of USP Insulin Beef RS in 0.01 N hydrochloric acid.

Standard solution: 1.5 mg/mL of USP Insulin RS of the appropriate species. *either USP Insulin Beef RS or USP Insulin Pork RS,* (RB 1-Feb-2013) in 0.01 N hydrochloric acid.

For insulin of mixed species prepare a solution containing 1.3 mg/mL of USP Insulin Beef RS and 0.25 mg/mL of USP Insulin Pork RS in 0.01 N hydrochloric acid. *For insulin of mixed species prepare a solution containing 1.3 mg/mL of USP Insulin Beef RS and 0.25 mg/mL of USP Insulin Pork RS in 0.01 N hydrochloric acid.* (RB 1-Feb-2013)

Sample solution: 1.5 mg/mL of Insulin in 0.01 N hydrochloric acid

NOTE—The Identification solution, Standard solution, and Sample solution may be stored at room temperature for up to 12 h or in a refrigerator for up to 48 h.

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Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 214 nm
Column: 4.6-mm × 15-cm; packing L1
Column temperature: 40°
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Samples: System suitability solution and Standard solution
Suitability requirements
Resolution: NLT 2.0 between insulin and A-21 desamido insulin, System suitability solution
Tailing factor: NMT 1.8 for the insulin peak, System suitability solution
Relative standard deviation: NMT 1.6%, Standard solution
Analysis
Samples: Identification solution, Standard solution, and Sample solution
Measure the peak responses for insulin and A-21 desamido insulin, using the chromatogram of the Identification solution to identify the insulin peaks.
For Insulin derived from a single species, calculate the potency on the undried basis, in USP Insulin Units/mg, of the insulin in the Sample solution:

\[
\text{Result} = \left( \frac{\Sigma r_i}{\Sigma r_0} \times \frac{C_i}{C_0} \right)
\]

\(\Sigma r_i\) = sum of the peak responses of insulin and A-21 desamido insulin from the Sample solution
\(\Sigma r_0\) = sum of the peak responses of insulin and A-21 desamido insulin from the Standard solution
\(C_i\) = concentration of USP Insulin RS of the appropriate species in the Standard solution (USP Insulin Units/mL)
\(C_0\) = concentration of Insulin in the Sample solution (mg/mL)

For Insulin derived from a mixture of beef insulin and pork insulin, calculate the total potency as the sum of the potencies of the beef-derived insulin and pork-derived insulin, determined separately.

Acceptance criteria: NLT 26.5 USP Insulin Units/mg on the dried basis; Insulin labeled as purified contains desamido insulin NLT 27.0 USP Insulin Units/mg on the dried basis.

IMPURITIES

Change to read:

- Organic Impurities

Solution A: Dissolve 28.4 g of anhydrous sodium sulfate in 1000 mL of water. Pipet 2.7 mL of phosphoric acid into the solution, and adjust with ethanolamine to a pH of 2.3, if necessary.
Solution B: Acetonitrile and Solution A (18:82)
Solution C: Acetonitrile and Solution A (50:50)
Mobile phase: See Table 2.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution B (%)</th>
<th>Solution C (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>81</td>
<td>19</td>
</tr>
<tr>
<td>60</td>
<td>81</td>
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<td>64</td>
</tr>
<tr>
<td>92</td>
<td>81</td>
<td>19</td>
</tr>
</tbody>
</table>

System suitability solution: 1.5 mg/mL of Insulin in 0.01 N hydrochloric acid. Allow to stand at room temperature for NLT 3 days to obtain a solution containing NLT 5% of A-21 desamido insulin.

Standard solution A: 3.75 mg/mL of USP Insulin RS of the appropriate species, either USP Insulin Beef RS or USP Insulin Pork RS.

For insulin of mixed species prepare a solution containing 3.2 mg/mL of USP Insulin Beef RS and 0.6 mg/mL of USP Insulin Pork RS in 0.01 N hydrochloric acid.

Standard solution B: Pipet 1 mL of Standard solution A into a 10-mL volumetric flask, dilute with 0.01 N hydrochloric acid to volume, and mix (0.375 mg/mL).

Standard solution C: Pipet 1 mL of Standard solution B into a 10-mL volumetric flask, dilute with 0.01 N hydrochloric acid to volume, and mix (0.0375 mg/mL).

NOTE—The three Standard solutions may be stored at room temperature for up to 12 h and in a refrigerator for up to 48 h.

Sample solution: 3.75 mg/mL of Insulin in 0.01 N hydrochloric acid. Prepare the solution in a capped vial, cap the vial, and shake gently to dissolve. Store the solution for NMT 2 h at room temperature or for NMT 12 h in a refrigerator.

Chromatographic system
(See Chromatography (621), System Suitability.)
Mode: LC
Detector: UV 214 nm
Column: 4.6-mm × 25-cm; packing L1
Column temperature: 40°
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Samples: System suitability solution, Standard solution A, Standard solution B, and Standard solution C

NOTE—Adjust the Mobile phase composition and the duration of the isocratic elution to obtain a retention time of about 31 min for insulin, with the A-21 desamido insulin eluting just prior to the start of the gradient elution phase.

Suitability requirements for the System suitability solution
Resolution: NLT 2.0 between insulin and A-21 desamido insulin
Tailing factor: NMT 1.8 for the insulin peak

Suitability requirements for the Standard solutions
Calculate the factor \(X_1\):

\[X_1 = \left( \frac{r_B}{r_A} \right) \times D\]

\(r_A\) = peak response from Standard solution A
\(r_B\) = peak response from Standard solution B
\(D\) = dilution factor, 10
Result: Between 0.91 and 1.09

Calculate the factor \(X_2\):

\[X_2 = \left( \frac{r_C}{r_A} \right) \times D\]

\(r_C\) = peak response from Standard solution C
\(r_A\) = peak response from Standard solution A
\(D\) = dilution factor, 100
Result: Between 0.7 and 1.3

Analysis
Sample: Sample solution
Calculate the percentage of insulin, A-21 desamido insulin, and other impurities in the portion of Insulin taken:
Calculate the percentage of Insulin (%):

\[\text{Result} = \left( \frac{r}{r_A} \right) \times 100\]
\[ r_i = \text{peak response of insulin} \]
\[ r_T = \text{sum of the responses of all the peaks} \]

Calculate the percentage of A-21 desamido insulin (%D):
\[ \text{Result} = \left( \frac{r_D}{r_T} \right) \times 100 \]

\[ r_0 = \text{peak response of A-21 desamido insulin} \]
\[ r_r = \text{sum of the responses of all the peaks} \]

Calculate the percentage of other insulin related compounds:
\[ \text{Result} = 100 - (\%I + \%D) \]

Acceptance criteria: NMT 10.0% of A-21 desamido insulin, and NMT 5.0% of other insulin related compounds.

For Insulin derived from a single species, measure the responses of any peaks corresponding to beef insulin or pork insulin, and calculate their concentration as a percentage of \( r_r \). The amount of cross-contamination is NMT 1.0%.

**LIMIT OF HIGH MOLECULAR WEIGHT PROTEINS**

Solution A: 1 mg/mL of L-arginine

Mobile phase: Solution A, acetonitrile, and glacial acetic acid (65:20:15)

Resolution solution: 4 mg/mL of Insulin containing NLT 0.4% high molecular weight proteins in 0.01 N hydrochloric acid. Store in a refrigerator, and use within 7 days.

[NOTE—Insulin containing NLT 0.4% high molecular weight proteins may be prepared by allowing Insulin to stand at room temperature for about 5 days.]

Sample solution: 4 mg/mL of Insulin in 0.01 N hydrochloric acid. Store in a refrigerator, and use within 7 days.

Chromatographic system

(See Chromatography (621), System Suitability.)

Detector: UV 276 nm

Column: 7.8-mm × 30-cm; packing L20

Flow rate: 0.5 mL/min

Injection volume: 100 µL

System suitability

Sample: Resolution solution

Suitability requirements

Retention times: Between 13 and 17 min for the polymeric insulin complexes, about 17.5 min for the covalent insulin dimer, and between 18 and 22 min for the insulin monomer, with salts eluting after the insulin monomer.

Peak-to-valley ratio: The ratio of the height of the covalent insulin dimer peak to the height of the valley between the covalent insulin dimer peak and the insulin monomer peak is NLT 2.0.

**Analysis**

Sample: Sample solution

Disregard any peaks having retention times greater than that of the insulin monomer. Calculate the percentage of high molecular weight proteins in the portion of Insulin taken:

\[ \text{Result} = 100 \times \frac{\sum nr_h}{(\sum nr_h + nr_m)} \]

\[ nr_h = \text{sum of the responses of all peaks having retention times less than that of the insulin monomer} \]

Acceptance criteria: NMT 10.0%

**SPECIFIC TESTS**

- **INSULIN ASSAYS, BIOIDENTITY TEST (121):** Meets the requirements of the Bioidentity Test.
- **LOSS ON DRYING (731):**
  - Sample: 200 mg
  - Analysis: Dry the sample at 105° for 16 h.
  - Acceptance criteria: NMT 10.0%
- **ZINC DETERMINATION (591):**
  - Sample: 10 mg
  - Acceptance criteria: NMT 1.0% on the dried basis.
- **BACTERIAL ENDOTOXINS TEST (85):** NMT 10 USP Endotoxin Units/mg of insulin.
- **MICROBIAL ENUMERATION TESTS (61):**
  - Tests for Specified Microorganisms (62): The total bacterial count does not exceed 3 × 10^2 cfu/g, the test being performed on a portion of about 0.2 g, accurately weighed.

**ADDITIONAL REQUIREMENTS**

- **PACKAGING AND STORAGE:** Preserve in tight containers. Store, protected from light, in a freezer.
- **LABELING:** Label it to indicate the one or more animal species to which it is related, as pork, beef, or a mixture of pork and beef. If the Insulin is purified, label it as such.

**Change to read:**

**USP REFERENCE STANDARDS (11)**

- USP Endotoxin RS
- USP Insulin Beef RS
- USP Insulin Pork RS