Reference solution (a). Dissolve the contents of a vial of insulin aspart CRS in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml. Maintain the solution at 2-8 °C and use within 48 h.

Reference solution (b). Mix equal volumes of reference solution (a) and water R. Maintain the solution at 2-8 °C and use within 48 h.

Resolution solution. Use an appropriate solution with a content of B3Asp insulin aspart and A21Asp insulin aspart of not less than 1 per cent. This may be achieved by storing reference solution (a) at room temperature for about 1-3 days. Maintain the solution at 2-8 °C and use within 72 h.

Column:
- size: l = 0.25 m, Ø = 4 mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 µm),
- temperature: 40 °C.

Mobile phase:
- mobile phase A: dissolve 142.0 g of anhydrous sodium sulphate R in water R; add 13.5 ml of phosphoric acid R and dilute to 5000 ml with water R; adjust to pH 3.6, if necessary, with strong sodium hydroxide solution R; filter and degas; mix 9 volumes of the solution with 1 volume of acetonitrile for chromatography R; filter and degas;
- mobile phase B: mix equal volumes of water R and acetonitrile for chromatography R; filter and degas;

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile phase A (per cent V/V)</th>
<th>Mobile phase B (per cent V/V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 35</td>
<td>58 → 20</td>
<td>42 → 80</td>
</tr>
<tr>
<td>35 - 40</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>45 - 46</td>
<td>20 → 58</td>
<td>80 → 42</td>
</tr>
<tr>
<td>46 - 60</td>
<td>58</td>
<td>42</td>
</tr>
</tbody>
</table>

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 10 µl.

Relative retention with reference to insulin aspart (retention time = 20-24 min): B28isoAsp insulin aspart = about 0.9; B3Asp insulin aspart plus A21Asp insulin aspart (generally coeluted) = about 1.3; B3isoAsp insulin aspart = about 1.5.

System suitability: resolution solution:
- resolution: minimum 2.0 between the peak due to insulin aspart and the peak due to A21Asp insulin aspart and to B3Asp insulin aspart.

Calculate the content of insulin aspart C_{254}H_{377}N_{65}O_{75}S_{6} plus B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart using the areas of the corresponding peaks in the chromatograms obtained with the test solution and reference solution (a) and the declared content of insulin aspart plus B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart in insulin aspart CRS.

STORAGE
In an airtight container, protected from light, at or below 18 °C until released by the manufacturer. When thawed, insulin aspart is stored at 5 ± 3 °C and used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, insulin aspart must be at room temperature for about 1-3 days. Maintain the solution at 2-8 °C and use within 72 h.

LABELLING
The label states, where applicable, that the substance is free from bacterial endotoxins.

01/2005:1637 corrected

**INSULIN, BOVINE**

**Insulinum bovinum**

\[H\cdot Gly\cdot Ile\cdot Val\cdot Glu\cdot Gin\cdot Cys\cdot Cys\cdot Ala\cdot Ser\cdot Val\cdot Cys\cdot Ser\cdot Val\cdot Tyr\cdot Gin\cdot Leu\cdot Glu\cdot Asn\cdot Tyr\cdot Cys\cdot Asn\cdot OH\]

\[H\cdot Phe\cdot Val\cdot Asn\cdot Gin\cdot His\cdot Leu\cdot Cys\cdot Gly\cdot Ser\cdot His\cdot Leu\cdot Val\cdot Glu\cdot Ala\cdot Leu\cdot Tyr\cdot Leu\cdot Val\cdot Cys\cdot Gly\cdot Glu\cdot Arg\cdot Gly\cdot Phe\cdot\]

\[Phe\cdot Tyr\cdot Thr\cdot Pro\cdot Lys\cdot Ala\cdot OH\]

**DEFINITION**

Bovine insulin is the natural antidiabetic principle obtained from beef pancreas and purified. The content of bovine insulin C_{254}H_{377}N_{65}O_{75}S_{6} plus A21 desamido bovine insulin is not less than 93.0 per cent and not more than 105.0 per cent, calculated with reference to the dried substance.

By convention, for the purpose of labelling insulin preparations, 0.0342 mg of bovine insulin is equivalent to 1 IU of insulin.

**PRODUCTION**

The animals from which bovine insulin is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority. The manufacturing process is validated to demonstrate suitable inactivation or removal of any contamination by viruses or other infectious agents.

**CHARACTERS**

Appearance: white or almost white powder.

Solubility: practically insoluble in water and in ethanol. It dissolves in dilute mineral acids and with decomposition in dilute solutions of alkali hydroxides.

**IDENTIFICATION**

A. Examine the chromatograms obtained in the assay. Results: the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with reference solution (c).

B. Peptide mapping.

Test solution. Prepare a 2.0 mg/ml solution of the substance to be examined in 0.01 M hydrochloric acid and transfer 500 µl of this solution to a clean tube. Add 2.0 ml of HEPES buffer solution pH 7.5 R and 400 µl of a 1 mg/ml solution of Staphylococcus aureus strain V8 protease R. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 2.9 ml of sulphate buffer solution pH 2.0 R.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using bovine insulin CRS instead of the substance to be examined. Examine the digests by liquid chromatography (2.2.29).
Insulin, bovine

EUROPEAN PHARMACOPOEIA 5.0

Insulin, bovine

1.0 per cent of the total area of the peaks. Disregard any peak with a retention time greater than that of the insulin peak.

2.2.30

Liquid chromatography (2.2.29) as described under Assay, following the elution conditions as described in the table below:

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<td>42</td>
<td>58</td>
</tr>
<tr>
<td>30 - 44</td>
<td>42 → 11</td>
<td>58 → 89</td>
</tr>
<tr>
<td>44 - 50</td>
<td>11</td>
<td>89</td>
</tr>
</tbody>
</table>

Maintain the solutions at 2-10 °C and use within 24 h. Perform a system suitability test (resolution, linearity) as described under Assay. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Inject 20 µl of reference solution (c) and 20 µl of the test solution. If necessary, adjust the injection volume to between 10 µl and 20 µl in accordance with the results obtained in the test for linearity as described under Assay. Record the chromatograms for approximately 50 min. In the chromatogram obtained with reference solution (c), A21 desamido bovine insulin appears as a small peak after the principal peak and has a relative retention of about 1.3 with reference to the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido bovine insulin is not greater than 3.0 per cent of the total area of the peaks; the sum of the areas of all the peaks, apart from those due to bovine insulin and A21 desamido bovine insulin, is not greater than 3.0 per cent of the total area of the peaks.

TESTS

Impurities with molecular masses greater than that of insulin. Size-exclusion chromatography (2.2.30): use the normalisation procedure.

Test solution. Dissolve 4 mg of the substance to be examined in 1.0 ml of 0.01 M hydrochloric acid.

Resolution solution. Use a solution of insulin (approximately 4 mg/ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of 6 M hydrochloric acid, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in 0.01 M hydrochloric acid, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days.

Maintain the solutions at 2-10 °C and use within 7 days. If an automatic injector is used, maintain the temperature at 2-10 °C.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

Equilibration: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Injection: 50 µl.

System suitability: the chromatograms obtained with the test solution and the reference solution are qualitatively similar to the chromatogram of bovine insulin digest supplied with bovine insulin CRS. In the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III. The symmetry factor of the peaks due to fragments II and III is not greater than 1.5, and the resolution between the 2 peaks is at least 1.9.

Results: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

NOTE: The retention time of fragment I is the same for porcine insulin and for human insulin. The retention times of fragments II and IV are the same for all insulins. The retention time of fragment III is the same for bovine insulin and for porcine insulin.

Related proteins. Liquid chromatography (2.2.29) as described under Assay, following the elution conditions as described in the table below:

<table>
<thead>
<tr>
<th>Time (min)</th>
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<tr>
<td>44 - 50</td>
<td>11</td>
<td>89</td>
</tr>
</tbody>
</table>

Maintain the solutions at 2-10 °C and use within 24 h. Perform a system suitability test (resolution, linearity) as described under Assay. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Inject 20 µl of reference solution (c) and 20 µl of the test solution. If necessary, adjust the injection volume to between 10 µl and 20 µl in accordance with the results obtained in the test for linearity as described under Assay. Record the chromatograms for approximately 50 min. In the chromatogram obtained with reference solution (c), A21 desamido bovine insulin appears as a small peak after the principal peak and has a relative retention of about 1.3 with reference to the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido bovine insulin is not greater than 3.0 per cent of the total area of the peaks; the sum of the areas of all the peaks, apart from those due to bovine insulin and A21 desamido bovine insulin, is not greater than 3.0 per cent of the total area of the peaks.
Bovine proinsulin-like immunoreactivity (PLI): maximum 10 ppm, calculated with reference to the dried substance.

Use a suitably sensitive immunochemical method (2.7.1) such as radio-immunooassay, using the International Reference Reagent for bovine proinsulin to calibrate the method.

Zinc: maximum 1.0 per cent, calculated with reference to the dried substance.

Atomic absorption spectrometry (2.2.23, Method I).

**Test solution.** Dissolve 50.0 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid. Dilute if necessary to a suitable concentration (for example, 0.4 µg to 1.6 µg of Zn per millilitre) with 0.01 M hydrochloric acid.

**Reference solutions.** Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting zinc standard solution (5 mg/ml Zn) R with 0.01 M hydrochloric acid.

**Source:** zinc hollow-cathode lamp.

**Wavelength:** 213.9 nm.

**Flame:** air-acetylene flame of suitable composition (for example, 11 litres of air and 2 litres of acetylene per minute).

**Loss on drying (2.2.32):** maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 100-105 °C for 24 h.

**Sulphated ash (2.4.14):** maximum 2.5 per cent, determined on 0.200 g and calculated with reference to the dried substance.

**Bacterial endotoxins (2.6.14):** less than 10 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

**ASSAY**

Liquid chromatography (2.2.29).

**Test solution.** Dissolve 40.0 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 10.0 ml with the same solvent.

**Reference solution (a).** Dissolve the contents of a vial of human insulin CRS in 0.01 M hydrochloric acid to obtain a concentration of 4.0 µg/ml.

**Reference solution (b).** Dissolve the contents of a vial of porcine insulin CRS in 0.01 M hydrochloric acid to obtain a concentration of 4.0 µg/ml.

**Reference solution (c).** Dissolve 40.0 mg of bovine insulin CRS in 10.0 ml of 0.01 M hydrochloric acid.

**Reference solution (d).** Dilute 1.0 ml of reference solution (c) to 10.0 ml with 0.01 M hydrochloric acid.

**Resolution solution.** Mix 1.0 ml of reference solution (a) and 1.0 ml of reference solution (b).

Maintain the solutions at 2-10 °C and use within 48 h. If an automatic injector is used, maintain the temperature at 2-10 °C.

**Column:**

- **size:** I = 0.25 m, Ø = 4.6 mm.
- **stationary phase:** octadecylsilyl silica gel for chromatography R (5 µm).

**Mobile phase:** mix 42 volumes of mobile phase A and 58 volumes of mobile phase B, adjusting the composition of the mixture if necessary.

Prepare and maintain the following solutions at a temperature of at least 20 °C:

- **mobile phase A:** dissolve 28.4 g of anhydrous sodium sulphate R in water R and dilute to 1000 ml with the same solvent; add 2.7 ml of phosphoric acid R; adjust to pH 2.3, if necessary, with ethanolamine R; filter and degas;
- **mobile phase B:** mix 550 ml of mobile phase A with 450 ml of acetonitrile R. Warm the solution to a temperature of at least 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas.

**Flow rate:** 1 ml/min.

**Detection:** spectrophotometer at 214 nm.

**System suitability:**

- **resolution:** inject 20 µl of the resolution solution and 20 µl of reference solution (b). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (b) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks corresponding to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.
- **linearity:** inject 20 µl each of reference solutions (c) and (d). The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (c) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d). If this test fails, adjust the injection volume to between 10 µl and 20 µl, in order that the responses are within the linearity range of the detector.

**Injection:** 20 µl of the test solution.

Calculate the content of bovine insulin C_{22}H_{37}N_6O_{17}S_5 plus A21 desamido bovine insulin from the area of the principal peak and the area of the peak corresponding to A21 desamido bovine insulin in the chromatograms obtained with the test solution and reference solution (c) and the declared content of bovine insulin plus A21 desamido bovine insulin in bovine insulin CRS.

**STORAGE**

In an airtight container, protected from light, at −20 °C until released by the manufacturer. When thawed, insulin may be stored at 5 ± 3 °C and used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, the insulin must be at room temperature.

**LABELLING**

The label states where applicable, that the substance is free from bacterial endotoxins.