

<121.1> PHYSICOCHEMICAL ANALYTICAL PROCEDURES FOR INSULINS

INTRODUCTION

Several physicochemical procedures are used for the assessment of the quality attributes of Insulin Human, various insulin analogues, and animal derived insulins, hereafter called insulins. Of these procedures, peptide mapping and determination of high molecular weight proteins are similar for the various insulin drug substances and drug products. This chapter describes the tests for peptide mapping and for quantitative analysis of high molecular weight proteins that can be used for the various insulins.

Specific instructions that deviate from the general procedures outlined here are given in the respective drug substance or drug product monographs for the different insulins.

PEPTIDE MAPPING

Insulin digestion is performed using *Staphylococcus aureus* strain V8 (*S. aureus* V8) protease (also referred to as Endoproteinase Glu-C), a serine endoproteinase that cleaves at the C-terminal side of glutamyl and aspartyl residues in phosphate buffer at pH 7.8. This protease is specific for Glu-C digestion in ammonium bicarbonate or other non-phosphate-containing buffers at pH 7.8. The presence of proline on the carboxy side of the peptide bond inhibits cleavage. The buffer system used should cleave all glutamyl bonds in insulin without cleavage of the aspartyl bonds.

In general, insulin is digested into four peptides in the buffer system described in the *Sample solution* below. The following show the amino acid differences of the insulin analogues compared to Insulin Human and the *in silico* differences in the fragments obtained upon digestion with *S. aureus* V8 protease.

Figure 1 shows the four fragments of Insulin Human following digestion with *S. aureus* V8 protease.

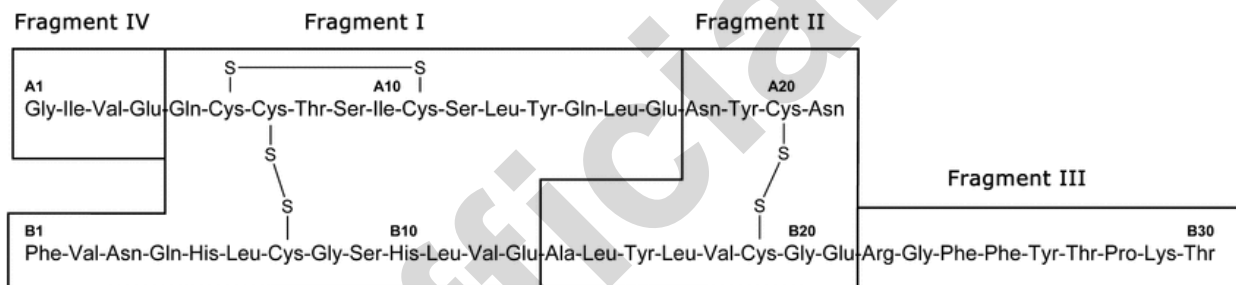


Figure 1. Insulin Human: *S. aureus* V8 protease digest fragments.

Table 1 shows the amino acid differences between insulin analogues, animal derived insulins, and Insulin Human.

Table 1. Amino Acid Differences between Insulin Analogues, Animal Derived Insulins, and Insulin Human

Insulin	Amino Acid Differences from Insulin Human
Insulin aspart	B28 Asp
Insulin glargine	A21 Gly, two Arg added to C-terminus of B chain
Insulin glulisine	B3 Lys, B29 Glu
Insulin lispro	B28 Lys, B29 Pro
Insulin porcine	B30 Ala
Insulin bovine	A8 Ala, A10 Val, B30 Ala

Table 2 shows specific *S. aureus* V8 protease digest fragments of insulin analogues and animal derived insulins. Amino acid differences compared to Insulin Human are highlighted.

Table 2. Amino Acid Differences: Insulin Human, Insulin Analogues, and Animal Derived Insulins (Differences with Insulin Human Are Shown in Boldface)

Insulin	Amino Acid Differences Compared to Insulin Human ^a	
Insulin aspart	Fragment III	(B22) Arg-Gly-Phe-Phe-Tyr-Thr- Asp -Lys-Thr (B30)
Insulin glargine	Fragment II	(A18) Asn-Tyr-Cys- Gly (A21)
	Fragment III	(B22) Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr- Arg-Arg (B32)
Insulin glulisine	Fragment I	(B1) Phe-Val- Lys -Gln-His-Leu-Cys-Gly-Ser-His-Leu (B11)
	Fragment III	(B22) Arg-Gly-Phe-Phe-Tyr-Thr-Pro- Glu -Thr (B30)
Insulin lispro	Fragment III	(B22) Arg-Gly-Phe-Phe-Tyr-Thr- Lys-Pro -Thr (B30)
Insulin porcine	Fragment III	(B22) Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys- Ala (B30)

Table 2. Amino Acid Differences: Insulin Human, Insulin Analogues, and Animal Derived Insulins (Differences with Insulin Human Are Shown in Boldface) (continued)

Insulin	Amino Acid Differences Compared to Insulin Human ^a	
Insulin bovine	Fragment I	(A5) Gln-Cys-Cys- Ala -Ser- Val -Cys-Ser-Leu-Tyr-Gln-Leu-Glu (A17)
	Fragment III	(B22) Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys- Ala (B30)

^a A and B denote the A and B chains of insulin, respectively; numbers denote amino acid position in the chain.

• **PEPTIDE MAPPING PROCEDURE**

The following procedure is applicable for preparing peptide maps of Insulin Human, insulin analogues, and animal derived insulins.

HEPES buffer: Dissolve 2.38 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) in about 90 mL of water in a 100-mL volumetric flask. Adjust with 5 M sodium hydroxide to a pH of 7.5, dilute with water to volume, and mix.

Sulfate buffer: 2.0 M ammonium sulfate and 0.5 M sulfuric acid (1:1). Mix, and filter.

• **INSULIN DIGESTION**

The following procedure provides efficient cleavage of the glutamyl bonds of insulin. [NOTE—Volumes up to 20-fold higher can be used as long as the ratio of the solutions remains the same. If interfering autolysis byproducts are observed in the chromatogram when a digest of the enzyme alone is run, the enzyme-to-insulin ratio must be decreased and digestion time must be increased.]

Enzyme solution: Prepare a 1-mg/mL solution of *S. aureus* V8 protease in water (approximately 500 units/mg using casein as the substrate).

Sample solution: Prepare a 2.0-mg/mL solution of the insulin to be examined in 0.01 N hydrochloric acid. To a clean vial add 25 µL of the 2.0-mg/mL insulin solution, 100 µL of *HEPES buffer*, and 20 µL of *Enzyme solution* (final ratio is 25:100:20). Cap the vial, and incubate at 25° for 6 h. Stop the reaction by adding an equal volume of *Sulfate buffer*. Longer incubation times may be needed for analogues with poor solubility at pH 7.5.

Standard solution: Prepare at the same time and in the same manner a solution of the appropriate USP Insulin Reference Standard as directed in the *Sample solution*.

• **PEPTIDE FRAGMENT DETERMINATION**

Determine the peptide fragments using the following peptide mapping procedure (see *Biotechnology-Derived Articles—Peptide Mapping* (1055)).

Solution A: Acetonitrile, water, and *Sulfate buffer* (100:700:200). Filter, and degas.

Solution B: Acetonitrile, water, and *Sulfate buffer* (400:400:200). Filter, and degas.

Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	95	5
3	95	5
30	41	59
35	20	80
40	95	5
50	95	5

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 214 nm

Column: 4.6-mm × 10-cm; 5-µm packing L1

Column temperature: 40°

Flow rate: 1 mL/min

Injection volume: 50–100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram comparability: In the chromatogram obtained from the *Standard solution*, identify the peaks due to digest fragments I, II, III, and IV. The chromatogram of the *Standard solution* corresponds to that of the typical chromatogram provided with the appropriate USP Insulin Reference Standard.

Resolution: There should be complete separation of the peaks due to fragments II and III. The resolution is defined in the applicable insulin monograph.

Tailing factor: NMT 1.5 for digest fragments II and III

Analysis

Samples: *Sample solution* and *Standard solution*

Condition the *Chromatographic system* by running at initial conditions, $t = 0$ min, for at least 15 min. Carry out a blank gradient program before injecting the digests. Separately inject equal volumes of the *Standard solution* and the *Sample solution*, and record the responses of each peak.

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

LIMIT OF HIGH MOLECULAR WEIGHT PROTEINS

• PROCEDURE

Solution A: 1 mg/mL of L-arginine in water

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

Resolution solution: Store a suitable amount of insulin drug substance at room temperature for a sufficient period of time (5–10 days, or as needed) to obtain insulin with more than 0.4% high molecular weight proteins. Prepare a 4-mg/mL solution in 0.01 N hydrochloric acid. Store the solution in a refrigerator, and use within 7 days. Alternatively, dissolve about 4 mg of USP High Molecular Weight Insulin Human RS in 1 mL of 0.01 N hydrochloric acid.

Sample solution: In a small vial, prepare a 4-mg/mL solution of insulin in 0.01 N hydrochloric acid, and mix to dissolve. Store in a refrigerator, and use within 7 days.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 276 nm

Column: 7.8-mm × 30-cm; 5- to 10-μm packing L20

Temperatures

Autosampler: It is advisable to use a refrigerated autosampler.

Column: Ambient

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 with retention times greater than that of the insulin monomer.

Calculate the percentage of high molecular weight proteins in the portion of Insulin taken:

$$\text{Result} = \frac{\sum r_H}{(\sum r_H + r_M)} \times 100$$

$\sum r_H$ = sum of the responses of all peaks with retention times less than that of the insulin monomer

r_M = peak response of the insulin monomer

Acceptance criteria: As stated in the applicable insulin monograph

ADDITIONAL REQUIREMENTS

• USP REFERENCE STANDARDS <11>

USP High Molecular Weight Insulin Human RS
(alternative, optional)