Loss on drying   Not more than 10.0% (0.2 g, 105°C, 24 hours).

Bacterial endotoxins   Less than 10 EU/mg.

Assay   Perform this procedure quickly. Weigh accurately about 7.5 mg of Insulin Human (Genetical Recombination), dissolve in 0.01 mol/L hydrochloric acid TS to make exactly 5 mL, and use this solution as the sample solution. Separately, weigh accurately a suitable amount of Human Insulin Reference Standard, dissolve exactly in 0.01 mol/L hydrochloric acid TS to make a solution so that each mL contains about 40 Insulin Units, and use this solution as the standard solution. Perform the test with 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas of human insulin, A_{TI} and A_{SI}, and the peak areas of the desamide substance at the relative retention time of 1.3 to the human insulin, A_{TD} and A_{SD}, respectively, of these solutions.

Amount (Insulin Unit/mg) of human insulin

$$(C_{257}H_{383}N_{65}O_{77}S_6) = \frac{W_S \times F}{C} \times \frac{A_{TI} + A_{TD}}{A_{SI} + A_{SD}} \times \frac{5}{W_T}$$

F: Label unit (Insulin Unit/mg) of Human Insulin Reference Standard.

D: Volume (mL) of 0.01 mol/L hydrochloric acid TS used to dissolve the reference standard.

W_T: Amount (mg) of the sample calculated on the dried basis.

W_S: Amount (mg) of Human Insulin Reference Standard.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 214 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: A mixture of phosphoric acid-sodium sulfate buffer solution, pH 2.3 and acetonitrile for liquid chromatography (3:1). Adjust the mixing ratio of the component of the mobile phase so that the retention time of human insulin is between 10 minutes and 17 minutes.

Flow rate: 1.0 mL per minute.

System suitability—

System performance: When the procedure is run with 20 μL of human insulin desamide substance-containing TS under the above operating conditions, human insulin and human insulin desamide substance are eluted in this order with the resolution between these peaks being not less than 2.0, and the symmetry coefficient of the peak of human insulin is not more than 1.8.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak areas of human insulin is not more than 1.6%.

Containers and storage   Containers—Tight containers.
    Storage—Light-resistant, and at −20°C or below.

Insulin Injection

インスリン注射液

Insulin Injection is an aqueous solution for injection. It contains not less than 95% and not more than 105% of the labeled Insulin Units.

Method of preparation   Suspend Insulin in Water for Injection, dissolve by adding Hydrochloric Acid, and prepare as directed under Injections. It contains 0.10 to 0.25 g of Phenol or Cresol and 1.4 to 1.8 g of Concentrated Glycerin for each 100 mL of Insulin Injection. It should not contain sodium chloride.

Description   Insulin Injection is a clear, colorless or pale yellow liquid.

Identification   Adjust Insulin Injection to pH between 5.1 and 5.3 with a solution of sodium hydroxide (1 in 100): a precipitate is produced. Adjust the solution to a pH between 2.5 and 3.5 with dilute hydrochloric acid: the precipitate dissolves.

pH   2.5 – 3.5

Residue on ignition   Measure exactly a volume of Insulin Injection, equivalent to 500 to 1000 Units according to the labeled Units, in a tared platinum dish, and evaporate slowly by heating on a water bath to dryness. Add 2 drops of nitric acid to the residue, and heat at first very gently, then strongly to incinerate. Place in a muffle furnace, and heat at 600°C for 15 minutes, cool in a desiccator (silica gel), and weigh: the mass of the residue is not more than 1.0 mg for each labeled 1000 Units.

Nitrogen content   Perform the test as directed under the Nitrogen Determination: not less than 0.50 mg and not more than 0.64 mg of nitrogen (N: 14.01) is found for each labeled 100 Units.

Assay   (i)   Animals: Select healthy rabbits each weighing not less than 1.8 kg. Keep the rabbits in the laboratory not less than 1 week before use in the assay by feeding them with an appropriate uniform diet and water.

(ii)   Diluent for insulin: Dissolve 1.0 to 2.5 g of phenol or *m*-cresol in 500 mL of 0.01 mol/L hydrochloric acid VS, and add 14 to 18 g of glycerin and 0.01 mol/L hydrochloric acid VS to make 1000 mL.

(iii)   Standard stock solution: Weigh accurately about 0.02 g of Insulin Reference Standard, and dissolve it in the diluent for insulin to make a standard stock solution containing exactly 20.0 Units in each mL. Preserve this solution between 1°C and 15°C, and use within 6 months.

(iv)   Standard solution: Dilute two portions of the standard stock solution to make two standard solutions with the diluent for insulin, one to contain exactly 2.0 Units in each mL which is designated as the high-dose standard solution S_H, and the other to contain exactly 1.0 Unit in each mL which is designated as the low-dose standard solution S_L.

(v)   Sample solution: According to the labeled Units, dilute two portions of Insulin Injection to make two sample solutions with the diluent for insulin, one to contain exactly 2.0 Units in each mL which is designated as the high-dose sample solution T_H, and the other to contain exactly 1.0

Unit in each mL which is designated as the low-dose sample solution T_L.

(vi) Dose for injection: Select the dose for injection on the basis of trial or experience. Inject a fixed identical volume, usually 0.3 to 0.5 mL, of the standard solutions and the sample solutions throughout the whole run.

(vii) Procedure: Divide the animals into 4 equal groups of not less than 6 animals each, with least difference in body mass. Withhold all food, except water, for not less than 14 hours before the injections, and withhold water during the assay until the final blood sample is taken. Handle the animals with care in order to avoid undue excitement.

Inject into each of the animals subcutaneously the dose of the standard solutions and the sample solutions indicated in the following design.

| First group | S_H | Third group | T_H |
| Second group | S_L | Fourth group | T_L |

The second injection should be made on the day after the first injection or within 1 week, using the dose of the standard solutions and the sample solutions indicated in the following design.

| First group | T_L | Third group | S_L |
| Second group | T_H | Fourth group | S_H |

At 1 hour and 2.5 hours after the time of injection, obtain a sufficient blood sample to perform the test from a marginal ear vein of each animal, and determine the blood sugar content of the blood samples according to (viii).

(viii) Blood sugar determination: Place 5.0 mL of a solution of zinc sulfate heptahydrate (9 in 2000) in a test tube 18 mm in outside diameter and 165 mm in length, add 1.0 mL of a solution of sodium hydroxide (1 in 250), and add gently 0.10 mL of the blood sample to the mixture in the test tube using a blood sugar pipet. Suck up the supernatant liquid into the pipet, wash out the remaining blood in the inner wall of the pipet, and repeat this procedure. Shake thoroughly the contents in the test tube, and heat the test tube in a water bath for 3 minutes. Filter the mixture through a funnel 30 to 40 mm in diameter in which a pledget of absorbent cotton, previously washed with two 3-mL portions of warm water, has been placed, receive the filtrate into a test tube 30 mm in inside diameter and 90 mm in length, wash the test tube and the funnel with two 3-mL portions of water, and combine the washings with the filtrate. Add 2.0 mL of alkaline potassium hexacyanoferrate (III) TS, heat in a water bath for 15 minutes, cool immediately, add 3.0 mL of potassium iodide-zinc sulfate TS and 2.0 mL of diluted acetic acid (100) (3 in 100), and titrate the liberated iodine with 0.005 mol/L sodium thiosulfate VS (indicator: 2 to 4 drops of starch-sodium chloride TS). Perform a blank determination. From the consumed volume (mL) of 0.005 mol/L sodium thiosulfate VS, obtain the blood sugar content (%) according to the following table.

(ix) Calculation: Sum up the two blood sugar values of each animal after each injection. Subtract the blood sugar value effected by the first injection from that effected by the second injection of each animal in the first group and the third group. The differences are symbolized as y_1 and y_3, respectively. Subtract the blood sugar value effected by the second injection from that effected by the first injection of each animal in the second group and the fourth group. The differences are symbolized as y_2 and y_4, respectively. Sum up not less than 6 values of individual differences in the blood sugar values y_1, y_2, y_3, and y_4 to obtain Y_1, Y_2, Y_3, and Y_4,

Conversion Table for the Blood Sugar Content (%)

mL*	0	1	2	3	4	5	6	7	8	9
0.0	0.385	0.382	0.379	0.376	0.373	0.370	0.367	0.364	0.361	0.358
0.1	0.355	0.352	0.350	0.348	0.345	0.343	0.341	0.338	0.336	0.333
0.2	0.331	0.329	0.327	0.325	0.323	0.321	0.318	0.316	0.314	0.312
0.3	0.310	0.308	0.306	0.304	0.302	0.300	0.298	0.296	0.294	0.292
0.4	0.290	0.288	0.286	0.284	0.282	0.280	0.278	0.276	0.274	0.272
0.5	0.270	0.268	0.266	0.264	0.262	0.260	0.259	0.257	0.255	0.253
0.6	0.251	0.249	0.247	0.245	0.243	0.241	0.240	0.238	0.236	0.234
0.7	0.232	0.230	0.228	0.226	0.224	0.222	0.221	0.219	0.217	0.215
0.8	0.213	0.211	0.209	0.208	0.206	0.204	0.202	0.200	0.199	0.197
0.9	0.195	0.193	0.191	0.190	0.188	0.186	0.184	0.182	0.181	0.179
1.0	0.177	0.175	0.173	0.172	0.170	0.168	0.166	0.164	0.163	0.161
1.1	0.159	0.157	0.155	0.154	0.152	0.150	0.148	0.146	0.145	0.143
1.2	0.141	0.139	0.138	0.136	0.134	0.132	0.131	0.129	0.127	0.125
1.3	0.124	0.122	0.120	0.119	0.117	0.115	0.113	0.111	0.110	0.108
1.4	0.106	0.104	0.102	0.101	0.099	0.097	0.095	0.093	0.092	0.090
1.5	0.088	0.086	0.084	0.083	0.081	0.079	0.077	0.075	0.074	0.072
1.6	0.070	0.068	0.066	0.065	0.063	0.061	0.059	0.057	0.056	0.054
1.7	0.052	0.050	0.048	0.047	0.045	0.043	0.041	0.039	0.038	0.036
1.8	0.034	0.032	0.031	0.029	0.027	0.025	0.024	0.022	0.020	0.019
1.9	0.017	0.015	0.014	0.012	0.010	0.008	0.007	0.005	0.003	0.002

*Indicates the volume of 0.005 mol/L sodium thiosulfate VS required in titration. For example, if the amount was 1.28 mL, the blood sugar content would be 0.127% from the above table.

respectively.

Units in each mL of Insulin Injection
= antilog M × Units in each mL of
the high-dose standard solution

$$\times \frac{b}{a}$$

$$M = 0.301 \times \frac{Y_a}{Y_b}$$

$$Y_a = -Y_1 + Y_2 + Y_3 - Y_4$$

$$Y_b = Y_1 + Y_2 + Y_3 + Y_4$$

a: Volume (mL) of the sample.

b: Total volume (mL) of the high-dose sample solution prepared by diluting the volume of the sample with diluent for insulin.

Compute L (P = 0.95) by using the following equation: L should be not more than 0.1212. If L exceeds 0.1212, repeat the assay by increasing the number of animals or improving the assay conditions in a better way until L becomes not more than 0.1212.

$$L = 2\sqrt{(C - 1)(CM^2 + 0.09062)}$$

$$C = \frac{Y_b^2}{Y_b^2 - 4fs^2t^2}$$

f: Number of the animals of each group.

$$s^2 = \frac{\Sigma y^2 - \dfrac{Y}{f}}{n}$$

Σy^2: The sum of squares of y_1, y_2, y_3, and y_4 in each group.

$Y = Y_1^2 + Y_2^2 + Y_3^2 + Y_4^2$

$n = 4(f - 1)$

t^2: Value shown in the following table against n for which s^2 is calculated.

n	$t^2 = F_1$	n	$t^2 = F_1$	n	$t^2 = F_1$
1	161.45	13	4.667	25	4.242
2	18.51	14	4.600	26	4.225
3	10.129	15	4.543	27	4.210
4	7.709	16	4.494	28	4.196
5	6.608	17	4.451	29	4.183
6	5.987	18	4.414	30	4.171
7	5.591	19	4.381	40	4.085
8	5.318	20	4.351	60	4.001
9	5.117	21	4.325	120	3.920
10	4.965	22	4.301	∞	3.841
11	4.844	23	4.279		
12	4.747	24	4.260		

Containers and storage Containers—Hermetic containers. Storage—In a cold place, and avoid freezing.

Expiration date 24 months after preparation.

Isophane Insulin Injection (Aqueous Suspension)

イソフェンインスリン水性懸濁注射液

Isophane Insulin Injection (Aqueous Suspension) is an aqueous suspension for injection. It contains not less than 90% and not more than 110% of the labeled Insulin Units, and not less than 0.01 mg and not more than 0.04 mg of zinc (Zn: 65.39) for each labeled 100 Units.

When Sodium Chloride is used in the preparation of Isophane Insulin Injection (Aqueous Suspension), this should be stated on the label.

Method of preparation Prepare as directed under Injections, with Insulin and Protamine Sulfate. To each 100 mL of Isophane Insulin Injection (Aqueous Suspension) add either 0.38 to 0.63 g of Dibasic Sodium Phosphate, 1.4 to 1.8 g of Concentrated Glycerin, 0.15 to 0.17 g of Cresol, and 0.06 to 0.07 g of Phenol, or 0.38 to 0.63 g of Dibasic Sodium Phosphate, 0.42 to 0.45 g of Sodium Chloride, 0.7 to 0.9 g of Concentrated Glycerin, and 0.18 to 0.22 g of Cresol.

Description Isophane Insulin Injection (Aqueous Suspension) is a white aqueous suspension. When allowed to stand, it separates into a white precipitate and colorless supernatant liquid, and the precipitate returns easily to the suspension state on gentle shaking.

When examined microscopically, the precipitate mostly consists of fine, oblong crystals of 5 to 30 μm in major axis, and does not contain amorphous substances or large aggregates.

Identification Proceed as directed in the Identification under Insulin Zinc Protamine Injection (Aqueous Suspension).

pH 7.0 – 7.4

Purity (1) Protein—Perform the test as directed under the Nitrogen Determination: not exceeding 0.85 mg of nitrogen (N: 14.01) is found for each labeled 100 Units.

(2) Isophane ratio—(i) Buffer solution A: Dissolve 2.0 g of anhydrous disodium hydrogenphosphate, 16 g of glycerin, 1.6 g of *m*-cresol, and 0.65 g of phenol in water to make exactly 200 mL.

(ii) Buffer solution B: Dissolve 2.0 g of anhydrous disodium hydrogenphosphate, 4.35 g of sodium chloride, 8.0 g of glycerin, and 2.0 g of *m*-cresol in water to make exactly 200 mL.

(iii) Insulin solution: Weigh accurately 1000 Units of Insulin Reference Standard, dissolve in 1.5 mL of diluted hydrochloric acid (1 in 360), and add 5.0 mL of buffer solution A and water to make 20 mL. Adjust the pH to 7.2 with dilute hydrochloric acid or sodium hydroxide TS. The solution is clear. Dilute with water to make exactly 25 mL. The solution is clear, and the pH is between 7.1 and 7.4. When it is stated on the label that Sodium Chloride is used in the preparation, use 5.0 mL of buffer solution B instead of buffer solution A in the above procedure.

(iv) Protamine solution: Weigh accurately 50 mg of Protamine Sulfate Reference Standard, and dissolve in 2 mL of buffer solution A and water to make 8 mL. Adjust the pH to 7.2 with dilute hydrochloric acid or sodium hydroxide TS, and dilute with water to exactly 10 mL. The solution is clear, and the pH is between 7.1 and 7.4. When it is stated on the label that Sodium Chloride is used in the preparation, use 2 mL of buffer solution B instead of buffer solution A in the above procedure.

(v) Procedure: When Isophane Insulin Injection (Aqueous Suspension) contains 40 Units per ml, centrifuge a portion of the suspension, measure exactly two 10-mL portions of the supernatant liquid in two tubes A and B, respectively, add exactly 1 mL of the insulin solution to tube A, and 1 mL of the protamine solution to tube B, mix the contents of each tube, allow to stand for 10 minutes, and determine the turbidity of each mixture by using a photometer or a nephelometer: the turbidity of the mixture in tube B is not greater than that in tube A. When Isophane Insulin Injection (Aqueous Suspension) contains 80 Units per ml, measure exactly 5 mL of the supernatant liquid, and proceed in the same manner.

Assay (1) Insulin—To Isophane Insulin Injection (Aqueous Suspension) add diluted hydrochloric acid (1 in 100) to adjust pH to about 2.5, and proceed with the clear solution as directed in the Assay under Insulin Injection.

(2) Zinc—Pipet a volume of Isophane Insulin Injection (Aqueous Suspension), equivalent to about 400 Units according to the labeled Units, add 1 mL of 0.1 mol/L hydrochloric acid TS and water to make exactly 100 mL, dilute, if necessary, with water to contain 0.6 to 10 μg of zinc (Zn: 65.39) per mL, and use this solution as the sample solution. Separately, pipet a volume of Standard Zinc Solution for the Atomic Absorption Spectrophotometry, dilute with water to contain 0.4 to 1.2 μg of zinc (Zn: 65.39) per mL, and use this solution as the standard solution. Perform the test with the sample solution and the standard solution according to the Atomic Absorption Spectrophotometry under the following conditions, and determine the amount of zinc in the sample solution using the analytical curve obtained from the absorbance of the standard solution.

  Gas: Combustible gas—Acetylene gas
     Supporting gas—Air
  Lamp: Zinc hollow-cathode lamp
  Wavelength: 213.9 nm