Insulin Assays <121>

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Posting Date	29–Mar–2019
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Expert Committee	Biologics Monographs 1–Peptides
Reason for Revision	Omission of USP Insulin Beef RS and requirements related to bovine insulin due to the absence of commercial bovine insulin products in the United States

In accordance with the Rules and Procedures of the 2015–2020 Council of Experts, the Biologics Monographs 1–Peptides Expert Committee has revised the Insulin Assays <121> general chapter. The purpose for the revision is to remove the USP Insulin Beef RS and requirements related to bovine insulin because there are no approved manufacturers of therapeutic bovine insulin in the United States and suitable reference materials are not available.

Although USP Insulin Beef RS is used as a Reference Standard for the *Assay* in Insulin Assays <121> for insulins of bovine origin or of mixed bovine and porcine origin, there are no uses of this Reference Standard for non-bovine insulins. Therefore, references to bovine insulin and to the USP Insulin Beef RS have been removed from the chapter.

The Insulin Assays <121> Revision Bulletin supersedes the currently official general chapter.

Should you have any questions, please contact Diane McCarthy, Senior Manager (301-692-3637 or diane.mccarthy@usp.org).

⟨121⟩ INSULIN ASSAYS

INTRODUCTION

The most prominent manifestation of insulin activity, an abrupt decrease in blood glucose, was the basis for biologic assay from the time of its first clinical use. The assay, although relatively cumbersome, has the great merit of accurately reflecting the effect on the diabetic patient. The advent of practical yet sophisticated physicochemical methods (e.g., liquid chromatography) to measure insulin potency quantitatively has resulted in a more accurate and precise compendial test for insulin and insulin products. However, the bioidentity of insulin and insulin products cannot be assessed by these methods. Thus, a bioidentity test in rabbits is included in this chapter, and its use is called for in the appropriate monographs.

The *Rabbit Blood Sugar Method—Quantitative* is used to determine the potency of Insulin Reference Standards, for the validation of the stability of new insulin preparations, and to determine the specific activities of insulin analogs.

ASSAY

Change to read:

• **Rabbit Blood Sugar Method—Quantitative**

Diluent: Prepare an aqueous solution containing 0.1%–0.25% (w/v) of either cresol or phenol, 1.4%–1.8% (w/v) of glycerin, and sufficient hydrochloric acid to produce a pH between 2.5 and 3.5, unless otherwise directed in the individual monograph.

Standard stock solution: Prepare a solution containing 40 USP Insulin Units/mL of USP Insulin RS of the appropriate species in *Diluent* and having a pH between 2.5 and 3.5, unless otherwise directed in the individual monograph. ▲▲ (RB 1-May-2019) Store in a cold place, protected from freezing, and use within 6 months.

Standard solutions: Dilute portions of the *Standard stock solution* with *Diluent* to make two solutions, one to contain 1.0 USP Insulin Unit/mL (*Standard solution 1*), and the other to contain 2.0 USP Insulin Units/mL (*Standard solution 2*).

Sample stock solution: Proceed as directed in the *Standard stock solution*, except to use a suitable quantity of the preparation under test in place of USP Insulin RS of the appropriate species. The *Sample stock solution* contains about 40 USP Insulin Units/mL.

Sample solutions: Dilute portions of the *Sample stock solution* with *Diluent* to make two dilutions of the preparation under test, one of which may be expected, on the basis of the assumed potency, to contain 1.0 USP Insulin Unit/mL (*Sample solution 1*), and the other to contain 2.0 USP Insulin Units/mL (*Sample solution 2*). In the case of neutral insulin injection, adjust to a pH of 2.5–3.5 before making the dilutions.

Doses of the solutions to be injected: Select, on the basis of trial or experience, the dose of the dilutions to be injected, the volume of which usually will be between 0.30 and 0.50 mL. For each animal, the volume of the *Standard solution* is the same as that of the *Sample solution*.

Preparation of animal: Select suitable, healthy rabbits, each weighing NLT 1.8 kg. Keep the rabbits in the laboratory for NLT 1 week before use in the assay, maintaining them on an adequate uniform diet, with water available at all times.

Analysis: Divide the rabbits into four equal groups of preferably NLT six rabbits each. On the preceding day, approximately 20 h before the assay, provide each rabbit with an amount of food that will be consumed within 6 h. Follow the same feeding schedule before each test day. During the assay, withhold all food until after the final blood specimen is taken. Handle the rabbits with care to avoid undue excitement, and inject subcutaneously the doses indicated in the following design (see *Table 1*), the second injection being made on the day after the first injection, or NMT 1 week later. The time between the first and second injections is the same for all rabbits.

Table 1

Group	First Injection	Second Injection
1	*Standard solution 2*	*Sample solution 1*
2	*Standard solution 1*	*Sample solution 2*
3	*Sample solution 2*	*Standard solution 1*
4	*Sample solution 1*	*Standard solution 2*

Blood samples: At 1 h ± 5 min and 2.5 h ± 5 min after the time of injection, obtain from each rabbit a suitable blood specimen from a marginal ear vein. Blood can also be collected effectively from the central auricular artery.

Dextrose determination: Determine the dextrose content of the blood specimens by a suitable procedure that is adapted to automated analysis. The following procedure may be used.

Anticoagulant solution: Dissolve 1 g of edetate sodium and 200 mg of sodium fluoride in 1 L of water, and mix.

Dextrose standard preparations: Transfer known concentrations of USP Dextrose RS to suitable vessels, and dilute quantitatively and stepwise with *Anticoagulant solution* (1:9) to obtain a range of *Dextrose standard preparations* containing between 20 and 100 mg per 100 mL, having known concentrations similar to the concentrations in the rabbit blood samples.

Sample preparations: Pipet into separate, suitable vessels 0.1 mL of each *Blood sample* and 0.9 mL of *Anticoagulant solution*.

Analysis: Subject the *Sample preparations* to dialysis across a semipermeable membrane for a sufficient time so that the dextrose passes through the membrane into a saline TS solution containing glucose oxidase, horseradish peroxidase, 3-methyl-2-benzothiazolinone hydrazone hydrochloride TS, and *N,N*-dimethylaniline. The absorbances of the *Sample*

preparations are determined at 600 nm in a recording colorimeter. The absorbances of the *Dextrose standard preparations* are similarly determined at the start and the end of each run.

Calculation: Calculate the response of each rabbit to each injection from the sum of the two blood sugar values, and subtract its response, disregarding the chronological order in which the responses were observed, to obtain the individual differences, *y*, as shown in *Table 2*.

When the data for one or more rabbits are missing in an assay, do not use the confidence interval formulas given here, but seek statistical help. The data can still be analyzed with proper analysis of variance.

When the number of rabbits, *f*, carried through the assay is the same in each group, total the *y*'s in each group and compute:

$$T_a = -T_1 + T_2 + T_3 - T_4$$

and

$$T_b = T_1 + T_2 + T_3 + T_4$$

The logarithm of the relative potency of the test dilutions is $M' = 0.301\,T_a/T_b$. The potency of the injection in USP Units/mg equals the antilog (log $R + M'$), where:

$$R = v_S/v_U$$

v_S = number of USP Units/mL of the *Standard solution*
v_U = number of mg/mL of insulin of the corresponding *Sample solution*

Determine the 95% confidence interval for the log-relative potency using Fieller's Theorem (see *Appendix* and *Design and Analysis of Biological Assays* ⟨111⟩). If the confidence interval is more than 0.082, which corresponds at $P = 0.95$ to confidence limits of about ±10% of the computed potency, repeat the assay until the combined data of the two or more assays, redetermined as described in *Combination of Independent Assays* in ⟨111⟩, meet this acceptable limit.

Table 2

Group	Differences	Individual Response (*y*)	Total Response (*T*)	Standard Deviations of Differences (*S*)
1	*Standard solution 2 – Sample solution 1*	y_1	T_1	S_1
2	*Sample solution 2 – Standard solution 1*	y_2	T_2	S_2
3	*Sample solution 2 – Standard solution 1*	y_3	T_3	S_3
4	*Standard solution 2 – Sample solution 1*	y_4	T_4	S_4

Appendix: Fieller's Theorem for Determining the Confidence Interval for a Ratio

This version of Fieller's Theorem is for the case where the numerator and denominator are uncorrelated. The equation assumes that the numerator and denominator are normally distributed and that the groups of rabbits are of equal sizes.

Then, the 95% confidence interval for the ratio is:

$$(L,U) = \frac{M' \pm \frac{t}{T_b}\sqrt{\left(1-g\right)S_N^2 + (M')^2 S_D^2}}{1-g}$$

where *f* (degrees of freedom in the standard errors) = $4(k-1)$, where *k* is the number of rabbits in a group, *t* is the upper 97.5 percentile of the *t*-distribution with *f* degrees of freedom, and

$$g = \frac{t^2 S_D^2}{T_b^2}$$

If $g \geq 1$, the denominator is not significantly different from 0 and the formula does not work.

$$S_N = 0.301\sqrt{k}\sqrt{S_1^2 + S_2^2 + S_3^2 + S_4^2}$$
$$S_D = \sqrt{k}\sqrt{S_1^2 + S_2^2 + S_3^2 + S_4^2}$$

• **BIOIDENTITY TEST**

Proceed as directed in *Rabbit Blood Sugar Method—Quantitative* with the following modifications.

Procedure: Divide the rabbits into four equal groups of two rabbits each.

Calculation: Proceed as directed for *Calculation* in *Rabbit Blood Sugar Method—Quantitative*, but do not determine the confidence interval of the log-relative potency, *M'*.

Interpretation: If the potency value obtained is NLT 15 USP Units/mg, the *Bioidentity Test* requirement is met. If the potency value is less than 15 USP Units/mg, repeat the test using eight more rabbits. If the average potency of the two sets of tests is NLT 15 USP Units/mg, the requirement of the test is met.

ADDITIONAL REQUIREMENTS

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Dextrose RS
USP Insulin Aspart RS
▲▲ (RB 1-May-2019)
USP Insulin Glargine RS
USP Insulin Human RS
USP Insulin Lispro RS
USP Insulin Pork RS