Comparison of *in vitro* and *in vivo* insulin bioidentity assays to monitor the quality of insulin products

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**Abstract**

Over 100 million individuals manage their diabetes with insulin products daily and rely on the pharmacological quality of these products to regulate their blood glucose. Since March 23rd, 2020, insulin products are regulated as biologics. Under the regulations for Biologics License Application Review, it is expected that the potency of the insulin products and their associated biomarkers will be assessed quantitatively in a cell-based assay or bioassay that, ideally, represent the product mechanism of action. Currently, the USP is recommending an in vivo rabbit bioidentity test for the assessment of the biological activity, or bioidentity, of insulins (USP <121>). However, reduction in animal experiments is a worldwide goal for many and it is likely that in vitro bioassays will be submitted for assessing the bioidentity of insulin products. Our goal is to evaluate how to best transition testing of insulin products from in vivo assays to in vitro bioassays. With this study, we are planning to compare the potency and kinetics of stressed and unstressed insulin products using both the USP Rabbit Blood Sugar Method and an in-cell western in vitro bioassay previously publicly described. Data that will be collected include: effect on glucose metabolism (monitoring of the full activation of the insulin receptor), dose linearity, percentage coefficient of variation, mean value, maximum effect, minimum effect, standard deviation, as well as practicability, cost and time. Comparison of these parameters will facilitate the transition of insulin bioassays from in vivo to in vitro and will support the assessment of prospective in vitro bioassays to monitor the quality of insulin products.

**Introduction**

- Approximately 34.2 million people in the US have diabetes
- Since March 23rd, 2020, insulin products are regulated as biologics. Under the regulations for Biologics License Application Review, it is expected that the potency of the insulin products and their associated biomarkers will be assessed quantitatively in a cell-based assay or bioassay that, ideally, represents the product mechanism of action. Currently, the USP is recommending an in vivo rabbit bioidentity test for the assessment of the biological activity, or bioidentity, of insulins (USP <121>). However, reduction in animal experiments is a worldwide goal for many and it is likely that in vitro bioassays will be submitted for assessing the bioidentity of insulin products.
- Since February 2021, USP added an in vitro bioidentity test for the kinetics of stressed and unstressed insulin products using both the USP Rabbit Blood Sugar Method and an in-cell western in vitro bioassay previously publicly described. Data that will be collected include: effect on glucose metabolism (monitoring of the full activation of the insulin receptor), dose linearity, percentage coefficient of variation, mean value, maximum effect, minimum effect, standard deviation, as well as practicability, cost and time.
- With this study, we will compare the potency and kinetics of stressed and unstressed insulin products using both the USP Rabbit Blood Sugar Method and an in-cell western in vitro bioassay previously publicly described. Data that will be collected include: effect on glucose metabolism (monitoring of the full activation of the insulin receptor), dose linearity, percentage coefficient of variation, mean value, maximum effect, minimum effect, standard deviation, as well as practicability, cost and time.

**Materials and Methods**

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- **The Rabbit Blood Sugar Method (based on USP <121>):**
  - 2 samples solutions can be tested against USP standard solutions
  - 2-4 animals are required per batch test
  - USP insulin standards will be subjected to various stressors. The stressed samples will be tested against unstressed standards for comparison of the effect of stress on the potency and kinetic of insulins. Stressors will consist of shaking or temperature variations (brene-thaw).

**In vitro in-cell western assay (based on USP <121>):**

- Binding of human insulin or insulin analogs to the human insulin receptor (hIR) induces the auto-phosphorylation of its kinase domain which is necessary for kinase and receptor activation. Quantification of insulin-induced auto-phosphorylation of the hIR is an appropriate downstream read-out for insulin and insulin analogs biological activity. A Chinese hamster ovary (CHO) cell line over-expressing the hIR forms the basis of this cellular in vitro assay. Auto-phosphorylation of the hIR is visualized with specific primary antibodies in combination with fluorescent labeled secondary antibodies. A simultaneous cell DNA staining (with a different wavelength than the secondary antibody) enables normalization of the results according to the cell number per microwell plate. The final detection is performed with a fluorescent compatible plate-reader (c.f. Figure 3).

**Conclusion**

- To best transition testing of insulin products from *in vivo* assays to *in vitro* bioassays, we will assess the robustness of the USP Rabbit Blood Sugar Method, the USP in-cell western in vitro bioassay and the real-time mouse glucose monitoring model.
- Potency of unstressed and stressed insulin products will be assessed in parallel using the three different models.
- Data will be analyzed following USP <111> and USP <121> recommendations.
- Additional orthogonal *in vitro* approaches representative of insulin mechanism of action will be also developed to further support the transition of insulin bioassays from *in vivo* to *in vitro*.
- This project will support the assessment of prospective *in vitro* bioassays to monitor the quality of insulin products.

**Figure 3.** Brief illustration of the *in vitro* in-cell western assay to monitor insulin products bioidentity.

For each well of each assay plate, we will:
- Normalize the assay signals detected from the secondary antibody that is bound to the primary antibody with the signals from the fluorescent dye for the cell DNA staining.
- Generate a dose-response curve by plotting the processed signal responses from each set of Diluted standard solutions or Diluted sample solutions from one assay plate against their concentrations on a logarithmic scale using a four-parameter logistic (4-PL) constrained model.

**Figure 4.** Illustration of the real-time mouse blood sugar method to monitor insulin products bioidentity.

A chemically induced hyperglycemic mouse model will be developed to assess the potency and kinetics of stressed and unstressed insulin with real-time telemetry.
- An implantable transmitter is surgically placed in the carotid of the mice.
- Hyperglycemia induced with streptozocin.
- Transmitter signals are converted into physiological parameters: blood pressure, temperature, heart rate, and blood glucose level.
- Administration of stressed and unstressed insulin are administered, and real-time physiologic changes are continuously recorded.

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