**Online Supplemental Information**

**Diabetes Care, Novel Communication**

**Commercially available human insulin products demonstrate stability throughout the cold supply chain across the United States**

**Procedures**

**Study design and setting**

Insulin activity was quantitatively assessed from rapid-, short-, intermediate-, long-acting, and pre-mixed formulations produced by three major domestic manufacturers (i.e., Eli Lilly, Novo Nordisk, and Sanofi). Nine insulin products (Supplementary Table 1) were acquired from four pharmacy or grocery chain retail providers within five United States geographical regions (Seattle, WA – Northwest; Houston, TX – Southwest; Gainesville, FL – Southeast; Boston, MA – Northwest; Ann Arbor, MI - Midwest) for a total of 20 replicates of each product and 174 total samples in November-December, 2018. Immediately following purchase, the insulins were placed into thermostable coolers with temperature recording devices and transported to the University of Florida for quantitative analysis.

**Insulin analysis**

For LC-UV analysis (a USP method), insulin samples from each site/region were prepared for detection as described in their respective USP Official Monographs (Supplemental Table 2). The Insulin Glargine Injection Monograph utilized both an ionic strength and solvent gradient. However, using these conditions, we did not observe a Glargine elution peak, even when modifying the gradient profile. Consequently, the chromatographic conditions from the Insulin Human Injection Monograph were used for Glargine analysis. Chromatographic separation and detection was achieved using a Vanquish UHPLC Variable Wavelength Detector (ThermoScientific, San Jose, CA) and the data analyzed using Chromeleon 7 software (ThermoScientific).

Preparation of human insulin samples for SPE-LC-MS was performed as follows. For the human insulin formulations, we replicated the methodology utilized in the recent report by Carter and Heinemann (1). Specifically, insulin samples were mixed with bovine insulin (Product number I0516; Sigma-Aldrich, St. Louis, MO) as the internal standard and dissolved in 30/10/60 methanol/acetic acid/water with 0·05% bovine serum albumin (BSA), and diluted to 375 ng/mL. The diluted samples were mixed with an equal volume of 10 mM Tris base and extracted using an Oasis HLB µElution Plate (Waters Corp., Milford, MA, USA) using negative pressure. After conditioning with 200 µL methanol followed by 200 µL water, each well was loaded with 500 µL sample. Wells were washed with 200 µL water containing 1% acetic acid and 5% methanol, and the samples eluted twice with 25 µL of 60/10/30 methanol/acetic acid/water. The eluate was combined and diluted with 50 µL water before LC-MS analysis. To evaluate the impact of sample preparation, a second method was used: rather than diluting in organic diluent, samples were diluted 1 in 50 with 10 mmol/L HCl containing 0.05% BSA before diluting to a final concentration of 375 ng/mL with water containing 0.05% BSA. The samples were then extracted by SPE, as described above, before LC-MS analysis.

For LC-MS analysis, insulin standards and samples were analyzed using a Thermo Q-Exactive Orbitrap mass spectrometer with Dionex UHPLC and autosampler. Separation was achieved on a Cortecs UPLC C18+ 1.6 µm, 2.1 x 50 mm column (Waters Corp) with an injection volume of 10 µL. The flow rate was 500 µL/min and the gradient consisted of 0.1% formic acid in water (mobile phase A) and 0·1% formic acid in acetonitrile (mobile phase B); mobile phase B increased linearly from 20% to 26% over 4 min, where it was held for 0·5 min before linearly returning to baseline in 0.1 min, it was allowed re-equilibrate for 1.4 min before the end of the run. Samples were analyzed by both MS and MS/MS within the same run using positive heated electrospray ionization and a resolution of 17,500. For MS/MS the collision energy was 35V with a first mass of 190 *m/z*. Other mass spectrometry parameters are provided in Supplemental Table 5.

For direct analysis, insulin standards and samples were prepared according to their respective USP Official Monographs (Supplemental Table 2) and mixing with an equal part bovine insulin (1 mg/mL). The human insulin samples and standards were then diluted 20-fold with 10 mmol/L HCl containing 0·5% BSA, while the other species were diluted 50-fold in the same buffer. Samples were analyzed by LC-MS as described above.

**Statistics**

The variables for statistical analysis were as follows: 1) insulin product, 2) pharmacy, 3) region, 4) and U/mL results. In addition, we compared analysis methods in the reporting of U/mL. The analysis focused on regional differences in insulin stability and differences in product formulation to expected insulin units across the methods used. Quantitative results from each method were compared against the same method and between different methods employed to assess the quality of the measurement of insulin and the activity of insulin in each formulation. Accuracy and precision to specified U/mL were calculated. Specially, accuracy was defined as how similar the measured value is to the actual value and was calculated based on the labelled concentration of 100 U/mL, while precision was calculated based on the mean of the replicates divided by the standard deviation, represented as relative standard deviation (%RSD).

**Data and Resource Availability**

The datasets generated during and analyzed during the current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

**Supplemental Table 1**

**Insulin products tested covers human and analogues as well as pens and vials.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Insulin Product** | **Format** | **Source** | **Sample Quantity** |
| **Humalog (U-100 lispro, rapid-acting insulin analogue)** | Vial | 5 regions, 4 pharmacies | 20 |
| **Novolog (U-100 aspart, rapid-acting insulin analogue)** | Vial | 5 regions, 4 pharmacies | 20 |
| **Humulin R (U-100 regular, recombinant human insulin)** | Vial | 5 regions, 4 pharmacies | 19\* |
| **Novolin R (U-100 regular, recombinant human insulin)** | Vial | 5 regions, 4 pharmacies | 20 |
| **Humulin N (U-100 NPH, recombinant human insulin)** | Vial | 5 regions, 4 pharmacies | 20 |
| **Novolin N (U-100 NPH, recombinant human insulin)** | Vial | 5 regions, 4 pharmacies | 20 |
| **Humulin 70/30 (mix of U-100 NPH and U-100 regular, recombinant human insulin)** | Pen | 5 regions, 4 pharmacies | 17\* |
| **Lantus (U-100 glargine, long-acting insulin analogue)** | Vial | 5 regions, 4 pharmacies | 19\* |
| **Basaglar (U-100 glargine, long-acting, insulin analogue)** | Pen | 5 regions, 4 pharmacies | 19\* |
|  |  | Total | 174 |

\*Logistical problems at the pharmacies mean that we were unable to purchase 3 Humulin 70/30 samples, and HumulinR, Lantis and Baslagar samples.

**Supplemental Table 2. USP Monographs and LC columns used for insulin analysis.**

|  |  |  |
| --- | --- | --- |
| **Insulin Type** | **USP Official Monograph (USP 41)** | **LC Column** |
| **Human** | Insulin Human Injection | LiChrosorb RP-18 5µm, 4.6 x 150mm |
| **Aspart** | Insulin Aspart Injection | LiChrosorb RP-18 5µm, 4.0 x 250mm |
| **Lispro** | Insulin Lispro Injection | Spherisorb ODS2 5µm, 4.6 x 100mm |
| **Glargine** | Insulin Glargine Injection\* | LiChrosorb RP-18 5µm, 4.6 x 150mm |

\*Glargine was chromatographically analysed using the conditions from the Insulin Human Injection Monograph.

**Supplemental Table 3**

**Insulin analogue concentrations measured by USP and LC-MS methods.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Product (analogues) | Region | USP (U/mL mean) | %RSD\* | LC-MS (U/mL mean) | %RSD\* |
| **Basaglar** | SW | 98.3 | 0.7% | 101.5 | 1.7% |
| **Basaglar** | SE | 98.2 | 0.8% | 101.0 | 0.3% |
| **Basaglar** | NW | 96.7 | 0.2% | 100.8 | 2.6% |
| **Basaglar** | NE | 96.6 | 0.5% | 102.0 | 3.9% |
| **Basaglar** | MW | 96.6 | 0.5% | 99.9 | 4.1% |
| **Humalog** | SW | 99.3 | 0.5% | 92.9 | 1.0% |
| **Humalog** | SE | 99.8 | 2.1% | 93.7 | 2.1% |
| **Humalog** | NW | 97.5 | 0.7% | 98.8 | 1.0% |
| **Humalog** | NE | 98.2 | 0.3% | 98.1 | 2.2% |
| **Humalog** | MW | 98.4 | 0.5% | 98.3 | 2.6% |
| **Lantus** | SW | 100.8 | 0.3% | 103.8 | 2.6% |
| **Lantus** | SE | 101.8 | 0.6% | 103.5 | 0.6% |
| **Lantus** | NW | 98.8 | 0.4% | 102.9 | 2.7% |
| **Lantus** | NE | 99.4 | 0.8% | 99.4 | 1.5% |
| **Lantus** | MW | 99.7 | 1.1% | 102.5 | 2.8% |
| **Novolog** | SW | 100.7 | 0.3% | 108.0 | 0.8% |
| **Novolog** | SE | 101.0 | 0.3% | 97.4 | 4.5% |
| **Novolog** | NW | 102.3 | 0.7% | 100.2 | 4.0% |
| **Novolog** | NE | 103.5 | 0.5% | 98.4 | 3.4% |
| **Novolog** | MW | 103.5 | 0.7% | 99.8 | 7.6% |

\*RSD: relative standard deviation

**Supplemental Table 4**

**LC-MS analysis of human insulin purchased from the Northwest region of the United States without SPE, with SPE from the published report (1), and with SPE modified with dilute acid.** All values are reported as mean U/mL ± SD.

|  |  |  |  |
| --- | --- | --- | --- |
| **Insulin Type** | **No SPE** | **SPE (organic diluent)** | **SPE (aqueous diluent)** |
| **Novolin N** | 98.4 ± 0.6 | 72.7 ± 1.0 | 101.8 ± 1.1 |
| **Novolin R** | 95.7 ± 0.7 | 73.6 ± 3.7 | 97.6 ± 1.9 |
| **Humulin N** | 96.2 ± 1.6 | 72.6 ± 1.0 | 99.2 ± 1.0 |
| **Humulin R** | 96.0 ± 1.6 | 71.0 ± 1.0 | 97.7 ± 2.5 |
| **Humulin 70/30** | 96.0 ± 0.5 | 70.7 ± 0.4 | 98.2 ± 1.4 |

**Supplemental Table 5. Parameters for LC-MS analysis.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | | **Human** | **Aspart** | **Lispro** | **Glargine** | **Bovine** |
| **Full Scan Mass Spectrometry** | | | | | |  |
|  | **Scan Range (m/z)** | 950 – 1180 | 950 – 1180 | 950 – 1180 | 862 – 1152 | same as analyte |
|  | **Isolation Window (m/z)** | 6 | | | | |
| **Parallel Reaction Monitoring (MS/MS)** | | | | | |  |
|  | **Isolation Window (m/z)** | 20 | 25 | 20 | 4 | same as analyte |
| **Charge State** | | **Center Mass** | | | |  |
|  | **+5** | 1154.5 | 1157.0 | 1155.0 | NA | 1147.5 |
|  | **+6** | 962.5 | 964.0 | 963.0 | 1011.3 | 956.5 |
|  | **+7** | NA | NA | NA | 867 | NA |

1. Carter AW, Heinemann L. Insulin Concentration in Vials Randomly Purchased in Pharmacies in the United States: Considerable Loss in the Cold Supply Chain. J Diabetes Sci Technol 2017:1932296817747292