Comparison of bioanalytical methods for the quantitation of PEGylated human insulin

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Abstract

Purpose: The quality of bioanalytical data is dependent upon selective, sensitive, and reproducible analytical methods. With evolving technologies available, bioanalytical scientists must assess which is most appropriate for their molecule through proper method validation. For an early stage PEGylated insulin program, the characteristics of four platforms, ELISA, ECLA, Gyrolab, and LC–MS/MS, were evaluated using fit-for-purpose method development and validation, while also evaluating costs.

Method: Methods selected for validation required acceptable performance based on satisfaction of a priori criteria prior to proceeding to subsequent stages of validation. LBA pre-validation included reagent selection, evaluation of matrix interference, and range determination. LC–MS/MS pre-validation included selection of a signature peptide; optimization of sample preparation, HPLC, and LC–MS/MS conditions; and calibration range determination. Pre-study validation tested accuracy and precision (mean bias criteria ± 30%; precision ≤ 30%). Pharmacokinetic (PK) parameters were estimated for an in vivo study with WinNonlin noncompartmental analysis. Statistics were performed with JMP using ANOVA and Tukey–Kramer post hoc analysis. A cost analysis was performed for a 200-sample PK study using the methods from this study.

Results: All platforms, except Gyrolab, were taken through validation. However, a typical Gyrolab method was included for the cost analysis. Ranges for the ELISA, ECLA, and LC–MS/MS were 8.52–75, 2.09–125, and 100–1000 ng/mL, respectively, and accuracy and precision fell within a priori criteria. PK samples were analyzed in the 3 validated methods. PK profiles and parameters are similar for all methods, except LC–MS/MS, which differed at t = 24 h and with AUC0-24. Further investigation into this difference is warranted. The cost analysis identified the Gyrolab platform as the most expensive and ELISA as the least expensive, with method specific consumables attributing significantly to costs.

Conclusions: ECLA had a larger dynamic range and sensitivity, allowing accurate assessment of PK parameters. Although this method was more expensive than the ELISA, it was the most appropriate for the early stage PEGylated insulin program. While this case study is specific to PEGylated human insulin, it highlights the importance of evaluating and selecting the most appropriate platform for bioanalysis during drug development.

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Keywords: ELISA, ECLA, Gyrolab, LC–MS/MS, Bioanalysis, Ligand-binding assay

Abbreviations: ACN, acetonitrile; AUC, area under the curve; Cmax, maximal plasma concentration; %C.V., percent coefficient of variation; DTT, dithiothreitol; ECL, electrochemiluminescence; ECLA, electrochemiluminescence assay; ELISA, enzyme-linked immunosorbent assay; LC–MS/MS, liquid chromatography mass spectrometry; FA, formic acid; HPLC, high performance liquid chromatography; HQC, high quality control; IPA, isopropyl alcohol; IS, internal standard; LBA, ligand-binding assay; LLOQ, lower limit of quantitation; LQC, low quality control; mAb, monoclonal antibody; MeOH, methanol; MQC, mid quality control; PEG, polyethylene glycol; PK, pharmacokinetic; QC, quality control; %R.E., percent recovery efficiencies; RLU, relative light units; SD, Sprague-Dawley; STZ, streptozotocin; %T.E., percent total error; TFA, tri-fluoro acetic acid; ULOQ, upper limit of quantitation.

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1. Introduction

The quality of bioanalytical data derived from preclinical and clinical studies is completely dependent upon analytical methods that are selective, sensitive, and reproducible (FDA, 2001). With evolving technologies and platforms available for bioanalysis, and the increasing number of macromolecules in development, bioanalytical scientists must accurately assess which method is most appropriate for their molecule. The various methods and platforms available should be evaluated through proper method development and validation, each informed by guidance documents.

Several techniques are available for the bioanalysis of macromolecules, but ligand binding assays (LBAs) are at the forefront. The most common LBA platform is the ELISA (Myler et al., 2011), requiring two binding reagents, one as a capture and the other as a detection reagent. The ELISA is a simple, relatively inexpensive, well-understood and established method. However, it is plagued with inadequate sensitivity and dynamic range, as well as large sample and reagent volume requirements. The electrochemiluminescent assay (ECLA) platform provides several advantages over ELISA, including a wider dynamic range, improved sensitivity, and reduced volume requirements. The disadvantages of ECLA include the cost of plates and the problem of a single source supplier (Kahn and Findlay, 2010). A nanoscale LBA, the Gyrolab, utilizes microfluidic technology to perform an immunoassay on a compact disc (CD) with micro-columns and structures containing an inlet, volume-definition chambers, and an overflow channel. Roman et al. (2011) and Mora et al. (2010) found that this platform offers sensitivity and dynamic range equivalent to the ECLA while reducing analyst hands-on time. But they also noted that key issues with this platform included the potential for carryover, the inability to perform runs in parallel, and the cost of the instrument and consumables.

Liquid chromatography (LC) used in tandem with mass spectrometry (MS/MS) was predominantly used for the bioanalysis of small molecules due to well-defined analyte structure and metabolites (Viswanathan et al., 2007). Recent advances in instrumentation and techniques have expanded the use of LC–MS/MS to macromolecule analysis (Ezan et al., 2009; Heudi et al., 2008). LC–MS/MS methods may be able to achieve similar sensitivity to that of an immunoassay with improved specificity and reduced method development time. In comparison to immunoassays, LC–MS/MS methods for large molecule quantitation require specialized equipment and extensive sample manipulations, and they are highly complex and fairly expensive, thus hampering their widespread application (Ezan et al., 2009).

Several publications have compared bioanalytical platforms with regard to assay performance (Ellis et al., 2012; Guglielmo-Viret et al., 2005; Heudi et al., 2008; Mora et al., 2010; Roman et al., 2011) including pharmacokinetic (PK) analysis (Mora et al., 2010; Roman et al., 2011), but a comprehensive evaluation of multiple platforms has not yet been published. We evaluated the characteristics of four platforms, ELISA, ECL, Gyrolab, and LC–MS/MS, using fit-for-purpose method development and validation, while also evaluating the costs associated with each platform for an early stage PEGylated insulin (PEG-insulin) program.

2. Materials and methods

2.1. Common reagents

Site-specifically PEGylated recombinant human insulin (PEG-insulin) was prepared as previously described (Miao et
2.2. Validation of immunoassays

The validation of each method was based on fit-for-purpose adaptations to the Findlay et al. paper (2000) and is detailed below. Development and validation were viewed as a continuum, requiring acceptable performance based on the satisfaction of a priori criteria, prior to proceeding to subsequent stages of validation.

2.2.1. Pre-validation

Pre-validation included selection of antibody pair, evaluation of matrix interference, and definition of calibration range for LBAs. For LC–MS/MS, fit-for-purpose adaptations to the Wal et al. paper (2010) were included with the selection of a signature peptide; optimization of sample preparation, HPLC, and LC–MS/MS conditions; and definition of the calibration range.

2.2.2. Pre-study validation

At least six or 75% of standards must have a mean bias (% R.E.) within ±30% and precision (% C.V.) ≤30%. Validation required at least two runs testing accuracy and precision with samples spiked at five levels, including lower level of quantitation (LLOQ), low quality control (LQC), mid quality control (MQC), high quality control (HQC), and upper limit of quantitation (ULOQ), required to be within ±30% and ≤30%, respectively, for each method. Percent total error (% T.E.) was required to fall within ≤40%.

2.2.3. In-study validation

Standards and two sets of quality controls (QCs) (LQC, MQC, and HQC) were required per run. For QCs, run acceptance was modified from the 4–6–20 (Viswanathan et al., 2007) rule for early stage methods to 4–6–30 for all platforms. Dilution of samples was done in plasma matrix, if dilution was performed.

2.3. ECLA

High-bind plates (catalog no. L15-XB; Meso-Scale Discovery, MD), Rockville, MD) were coated overnight at 4 °C with anti-PEG IgM monoclonal antibody (mAb) (catalog no. AGP4-PABM-A; Academia Sinica, Taiwan) in PBS (catalog no. 20012043; Gibco, Grand Island, NY). After a blocking step with I-Block (catalog no. T2015; Applied Biosystems, Grand Island, NY) supplemented with 0.1% Tween-20 (Thermo Fisher Scientific, Inc.), calibrators, QCs, and samples were applied at a 10× dilution in General Sample Diluent (SD) (catalog no. 520; Immunochemistry Technologies, Bloomington, MN) overnight at 4 °C. Bound analyte was detected using Insulin R/CD220 (catalog no. 1544-IR-050/CF; R&D Systems, Inc., Minneapolis, MN) conjugated to biotin, which then bound to streptavidin–horseradish peroxidase (catalog no. 21130; Thermo Fisher Scientific, Inc.) and quenched with 0.3 M sulfuric acid. Optical density was measured at 450 nm–650 nm on a Molecular Devices (Sunnyvale, CA) SpectraMax Plus 384 reader and analyzed with a 4PL curve fit on SoftMax Pro Software. All steps were separated by three washes with imidazole buffered saline supplemented with 0.02% Tween-20.

2.4. ELISA

Nunc Maxisorp flat-bottom plates (catalog no. 456537; Thermo Fisher Scientific, Inc.) were coated overnight at 4 °C with anti-PEG IgM mAb in BupH Carbonate–Bicarbonate Buffer (catalog no. 28382; Thermo Fisher Scientific, Inc.). After a blocking step in Blocker Casein in PBS (catalog no. 37528; Thermo Fisher Scientific, Inc.), calibrators, QCs, and samples were applied at a 50× dilution in Blocker Casein overnight at 4 °C. Subsequently, bound PEG-insulin was detected using Insulin R/CD220 conjugated to biotin, which then bound to streptavidin–horseradish peroxidase (catalog no. 21130; Thermo Fisher Scientific, Inc.). The assay was developed with 3,3’,5,5’-tetramethylbenzidine (catalog no. N301; Thermo Fisher Scientific, Inc.) and quenched with 0.3 M sulfuric acid. Optical density was measured at 450 nm–650 nm on a Molecular Devices (Sunnyvale, CA) SpectraMax Plus 384 reader and analyzed with a 4PL curve fit on SoftMax Pro Software. All steps were separated by three washes with imidazole buffered saline supplemented with 0.02% Tween-20.

2.5. Gyrolab

Pre-validation of the Gyrolab platform was not acceptable; therefore, a final method has not been included. Gyrolab xP workstation, Gyrolab Control v 5.4, Gyrolab Evaluator v3.3, and Biaffy and ADA CDs were used in pre-validation (Uppsala, Sweden). Conjugation was performed with DyLight 650 (catalog no. 53051, Thermo Fisher Scientific, Inc.).

2.6. LC–MS/MS

Bovine insulin (catalog no. 128–100; Cell Applications, Inc., San Diego, CA), the internal standard (IS), was spiked into standards, QCs, and unknowns at a constant concentration. Precipitation was performed on 50 μL of sample with IPA/0.1% TFA. Following centrifugation, 150 μL of supernatant was concentrated under vacuum and reconstituted with 50 μL 10% MeOH/90% 50 mM ammonium bicarbonate. Reduction and alkylation were performed with 3 μL 1 M dithiothreitol (DTT) (catalog no. 20291; Thermo Fisher Scientific, Inc.), followed by 6 μL 1 M iodoacetamide (catalog no. 90034; Thermo Fisher Scientific, Inc.) and quenched with 6 μL 1 M DTT. 1 μL 10% FA was added to samples. 40 μL of sample was injected into an Agilent Technologies, Inc. 1200 Series HPLC with a Zorbax Eclipse XDB-C18 1.8 μm, 4.6 × 50 mm column (catalog no. 927975-902; Agilent, Santa Clara, CA) with a C18 guard column (catalog no. 821125-936, Agilent). Gradient started with 20% B (0.1%FA/2%ACN) to 65%B at 5 min with a flow rate of 0.4 mL/m. MS instrumentation was Agilent Technologies, Inc. 6510 Q-TOF. For SIM ions, the following conditions were applied: Bovine m/z = 293.09 (m/z 1284.53; z = 2, ret. time 3.6 m, Δ ret. time 1 m, collision energy: 35 eV); human m/z = 293.09 (m/z 1306.56; z = 2, ret. time 3.8 m, Δ ret. time 1 m, collision energy: 35 eV).
2.7. In vivo study

Male Sprague-Dawley rats received a single IV injection of streptozotocin to induce hyperglycemia. Animals that were hypertglycemic (blood glucose levels >400 mg/dL) received a single subcutaneous dose of test article. Serial serum samples were collected at different time-points post-dose to quantitate the PEG-insulin by immunoassay. All animal procedures were in accordance with established guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

2.8. Pharmacokinetics

Pharmacokinetic parameters were estimated using the modeling program WinNonlin (Pharsight, version 5.1). Noncompartmental analysis for individual animal data was used, and concentration data was uniformly weighted. Statistical evaluations were performed with JMP software using ANOVA and Tukey–Kramer post-hoc analysis.

2.9. Cost analysis

A cost analysis was performed to determine the cost of running a single PK study of approximately 200 samples with the validated methods. Gyrolab methods found in literature were used for this analysis (Mora et al., 2010; Roman et al., 2011). Analyst time assumed total analyst operating time — sample dilution, calibration and QC preparation, plating, plate washes, reagent addition, and plate reading. The employee per hour cost was $28.89 (Bureau of Labor Statistics, 2013). The cost of method specific consumables and critical capture and detection reagents was the overall costs divided by the amount or volume required per assay. A useful life of ten years was assumed for instrumentation with a straight-line method of depreciation. The daily depreciation rate was added to the study cost. Catalog prices were used for cost estimates.

3. Results and discussion

3.1. Pre-validation

3.1.1. LBAs

An early stage ECLA and colorimetric ELISA were developed with an anti-PEG IgM mAb capture and biotinylated Insulin R/CD220 detection reagent. This pair provided sufficient signal to background ratios for both LBAs. To reduce matrix effects, SD1 with 10% matrix was identified as the optimal condition for the ECLA. Optimal conditions for the ELISA were Blocker Casein in PBS with 2% matrix. Matrix interference assessments are shown in Fig. 1A and B, respectively. Tentative range for the ECLA was 2–125 ng/mL and 9–75 ng/mL for the ELISA. A suitable Gyrolab method could not be developed. Multiple antibody pairs, buffers, and Gyrolab methods and CDs were tested, but we observed little to no signal responses. This may have been due to the presence of the PEG moiety in our PEG-insulin, but more studies need to be undertaken to determine whether the Gyrolab platform is incompatible with PEGylated compounds. In order to properly assess Gyrolab, an in depth comparison like the one presented here needs to be done with a non-PEGylated compound.

3.1.2. LC–MS/MS

Although tryptic peptides were initially evaluated, the nondigested A-chain of human insulin was chosen for quantitation: GIVEQCSTSICSLYQLENVYN (2611.94 Da). The bovine insulin A-chain MW is 2567.88 Da and rat insulin MW is 2597.91 Da, and respective masses reflect alkylation of the cysteine residues. The A-chain of the different species had unique +2 charge states that could be isolated for MS/MS fragmentation, followed by quantitation of the daughter ion (Fig. 1D). The developed method sufficiently resolved and enriched for the human insulin A-chain from 0.1 to 1 μg/mL, as well as the bovine insulin IS in rat plasma matrix (Fig. 1C). The tentative range for this method was 100–1000 ng/mL, which is not as sensitive as historically published methods using nanoflow HPLC with quantitation-specific triple quadrupole mass spectrometry. For this work, the method was developed on a standard Agilent 1200 HPLC in tandem with a Q-TOF used for broad applications.

3.2. Pre-study validation

Accuracy and precision for sample performance are summarized in Table 1. For the ECLA, the LLOQ was 2.09 ng/mL and ULOQ was 125 ng/mL. Observed precision ranges for QCs at 125, 94.9, 22.0, 6.06 and 2.09 ng/mL (n = 8) had %C.V.s of 3.07 to 9.61%. Observed accuracy ranges were −18.6 to −1.57%. For the ELISA, the LLOQ was 8.52 ng/mL and ULOQ was 75 ng/mL. Observed precision ranges for QCs at 75.0, 56.3, 32.3, 18.8 and 8.52 ng/mL (n = 6) were 5.67 to 22.1%. Observed accuracy ranges were −5.03 to −0.224%. We had a smaller N for the LC–MS/MS method due to the lengthy run time and limited availability of the instrument. Observed precision ranges for QCs at 1000, 750, 300, 149 and 100 ng/mL ranged from 7.94 to 29.2%. Observed accuracy ranges were −4.33 to 13.8%. %T.E. for all platforms was <30%. Each method was satisfactory based upon a priori criteria set for early stage methods.

3.3. In vivo study

All runs for each platform passed in-study validation a priori criteria, and were comparable to pre-study validation results. PK samples were analyzed in all 3 validated platforms. Data could not be obtained for the elimination phase with the ELISA and LC–MS/MS due to inadequate sensitivity and sparse sampling. The PK profiles appear similar in shape (Fig. 2A), but plasma concentration obtained from LC–MS/MS at t = 24 h was higher than both ELISA and ECLA. Only a limited amount of PK parameters could be assessed without an accurate half life (Fig. 2B). The calculated Cmax was similar for all platforms. Assessment of AUC from t = 0–24 h found a higher AUC0-24 with LC–MS/MS in comparison to that determined with ELISA. The differences observed with LC–MS/MS may be due to analytical variability in the validated methods due to the early stage of development and validation, but further investigation is warranted.

3.4. Cost analysis

The cost analysis (Table 2) for a 200-sample PK study identified the standard Gyrolab method as the most expensive at $11.62/sample, followed by the validated LC–MS/MS.
Fig. 1. Pre-validation. Plot of ECLA Buffer, SD1, and STZ rat plasma at 1:10 MRD (A); plot of ELISA Buffer, Blocker Casein, and STZ rat plasma at 1:50 MRD (B); LC–MS/MS daughter ion chromatogram PEG-insulin (0.1, 0.5, & 1 μg/mL) (C) and MS fragmentation pattern (D).
The cost of reagents and platform-specific consumables had the most influence on overall costs. For the validated LC–MS/MS method, instrumentation costs increased approximately 10-fold in comparison to both ECLA and Gyrolab and almost 50-fold in comparison to ELISA. This was due to the lengthy run time required for the LC–MS/MS method. The Gyrolab consumables, or CDs, were 3× more costly than consumables for both ECLA and LC–MS and 120× more expensive than ELISA consumables.

LBA-specific critical reagent costs in the ELISA were 1.5× and 11× more expensive than ECLA and Gyrolab, respectively. ELISA required more of each reagent in comparison to the ECLA and Gyrolab, but the Gyrolab only required nL volumes, significantly reducing costs. Direct analyst time was equivalent for ELISA and ECLA, and reduced 1.5-fold with LC–MS and 3-fold with Gyrolab due to the automation of sample and reagent addition on these platforms.

4. Conclusion

The ECLA, ELISA, and LC–MS/MS platforms were successfully developed and validated, but a suitable Gyrolab method could not be developed. Similar accuracy and precision were observed for each validated method. However, the ECLA had a larger dynamic range and improved sensitivity. This was an advantage during analysis of the PK study, as it allowed an accurate estimation of the half life and reduced the number of sample dilutions. This was the only method which provided an accurate assessment of PK parameters. Throughput was equivalent to that of the ELISA, but was an improvement over the validated LC–MS/MS method. It would have been interesting to compare a method on the Gyrolab to the ECLA, but it did not appear to be feasible with this PEGylated compound. Although the ECLA method was slightly more expensive than the ELISA, the additional advantages provided by the ECLA in this study supported the use of this method in future development.
Table 2
Cost analysis. Comparison of costs associated with each validated method. Gyrolab costs were associated with a standard Gyrolab method.

<table>
<thead>
<tr>
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<th>ELISA</th>
<th>ECLA</th>
<th>Gyrolab</th>
<th>LC–MS/MS</th>
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<tr>
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<tr>
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</tbody>
</table>

- Daily depreciation rate × number of assay days = 2 days for ELISA and ECLA as compared to 1 day for Gyrolab and 5 days for LC–MS/MS (15 minute run per sample). Assumes useful life of 10 years with straight-line depreciation and no salvage value. Instrument costs used were $25K, $150K, $265.7K, and $480K, respectively.
- Analyst time includes sample dilutions, standard and QC prep, reagent preparation and addition times, and plate reading and analysis times. For ELISA and ECLA, analyst time equals approximately 4 h, 4 h, 2.5 h, and 5 h for ELISA, ECLA, Gyrolab and LC–MS, respectively.
- Assumes 3–4 plate runs per day.
- Assumes 2 separate runs in 1 day.
- Assuming 1 column and guard will support the entire study.

While this case study is specific to PEG-insulin, it highlights the importance of evaluating and selecting the most appropriate platform for bioanalysis during drug development.

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