Altasciences CLINICAL RESEARCH ALGORITHME PHARMA

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# Quantitation of Insulin Glargine and Major Metabolites in Human Plasma Using Hybrid LBA-LC/MS with Automated Magnetic Particle Processing

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## OVERVIEW

### PURPOSE

To develop a high-throughput, sensitive and specific method for the quantitation of Insulin Glargine and major metabolites in human plasma.

### METHOD

The method is based on immunoaffinity purification of intact Insulin Glargine, M1 and M2 using mouse anti-insulin monoclonal antibody-coated magnetic beads, followed by LC-MS/MS analysis on a Sciex API 5000. Porcine insulin was used as an internal standard.

### RESULTS

The assay was adapted to 96-well plate and magnetic particle processing was automated using a Thermo KingFisher Flex™. An LLOQ of 50 pg/mL was achieved for Glargine and M1/M2. The assay was fully validated and can be adapted for the analysis of other insulin analogs.

## INTRODUCTION

Insulin Glargine is a bio-engineered long-acting insulin analogue used to regulate sugar levels in type 1 and type 2 diabetes. Following subcutaneous injection, Glargine is enzymatically cleaved to generate two active metabolites, M1 and M2 (Figure 1). While highly sensitive assays have historically been developed for insulin by immunoassay, a general lack of specificity in the technique has spurred implementation of LC-MS/MS. Closely related insulins may be distinguished with the additional advantages of shorter development time, multiplexing capability, and enhanced precision and accuracy. Most recently, hybrid LBA-LC/MS approaches have leveraged the best of both techniques, as illustrated in the current research, via development of a highly sensitive and specific method for the determination of Glargine and M1/M2 metabolites extracted from human plasma.

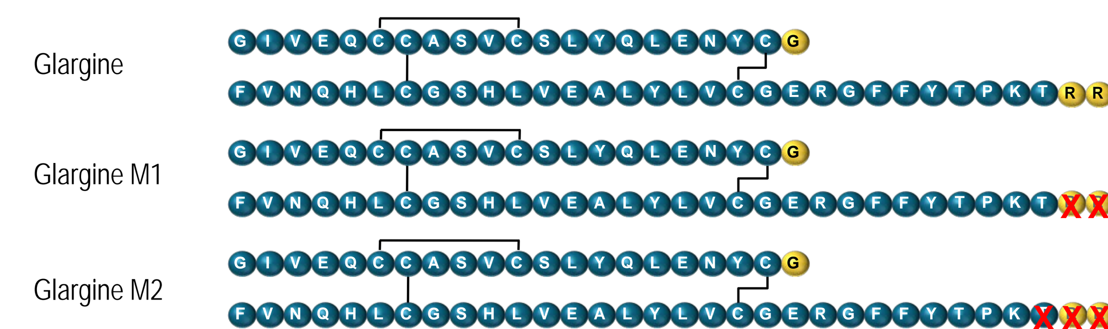


Figure 1. Amino Acid Sequences for Insulin Glargine, M1 and M2

## METHODS

### SAMPLE PROCESSING

**Immunoaffinity Purification**
- Plasma (0.5 mL) + Porcine insulin (IS) + mAb-coated beads (0.1 mL)
- Rotate gently for 1 hour at RT

**Automated Particle Processing (KingFisher Flex™)**
- Wash 1: PBST (0.5 mL)
- Wash 2: PBS (0.5 mL)
- Elution: 1% formic acid in MeOH:$H_2O$ (1:1, 0.1 mL)

**LC-MS/MS**
- Inject 20 µl

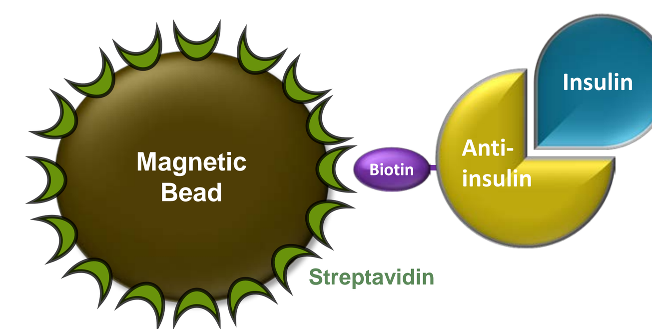


Figure 2. Immunoaffinity Capture of Insulin Glargine and Metabolites

### CHROMATOGRAPHY

- Agilent Technologies Series 1100 pumps and autosampler
- XBridge Protein BEH C4 column (50 x 2.1mm, 3.5 µm)
- Gradient elution with 0.1% HCOOH + 2% TFE in $H_2O$ and ACN

### DETECTION

- Characterization: Sciex TripleTOF 5600™
- Quantitation: Sciex API 5000 operated in ESI(+) MRM mode
- MRM transitions highlighted in Table 1

Table 1. MRM Transitions for Glargine, M1, M2 and Porcine Insulin (IS)

| Analyte | Parent Ion (*m/z*) | Fragment (*m/z*) | CE |
|---|---|---|---|
| Glargine | 867.3 (7+) | 136.1 | 45 |
| M1 | 959.6 (5+) | 136.1 | 45 |
| M2 | 942.7 (5+) | 136.1 | 45 |
| Porcine (IS) | 964.0 (6+) | 226.2 | 65 |

## RESULTS

### CHALLENGES IN METHOD DEVELOPMENT BY LC-MS/MS

Major challenges associated with LC-MS/MS analysis of Insulin Glargine include assay specificity against matrix components and interferences, high non-specific binding, formation of multiple precursor ions (Figure 3) and poor fragmentation (Figure 4), which prevent the achievement of sensitivity levels comparable to LBA assays.

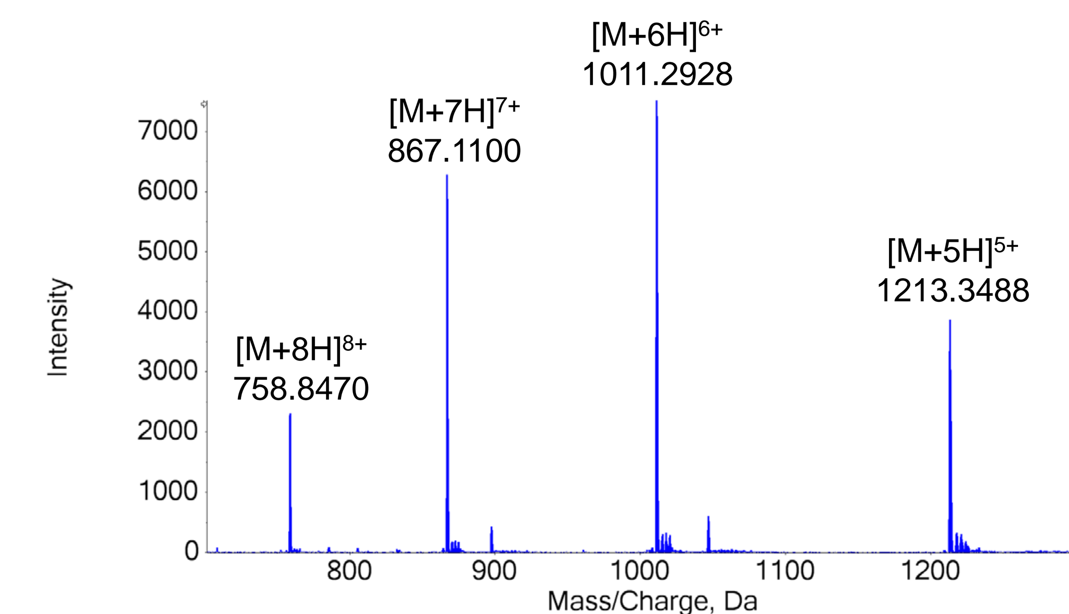


Figure 3. Charge Distribution of Insulin Glargine Analyzed by TOF-MS

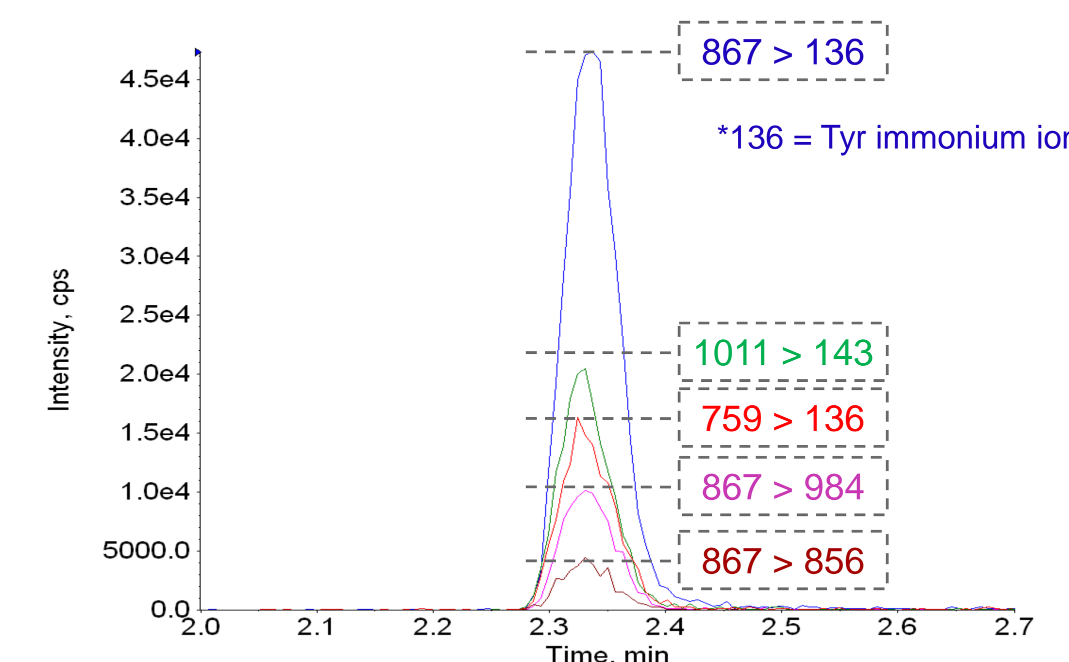


Figure 4. Extracted Ion Chromatograms (XIC) of Different Precursor Ions of Insulin Glargine

### HYBRID LBA/LC-MS ASSAY: SPECIFICITY, SENSITIVITY AND MULTIPLEXING CAPABILITY

Unlike solid-phase extraction (SPE), the immunoaffinity purification (IAP) approach was the most selective and amenable to enrichment (Figure 5). The hybrid approach allowed the development of a specific, sensitive and multiplexable method as highlighted in Figures 6 and 7.

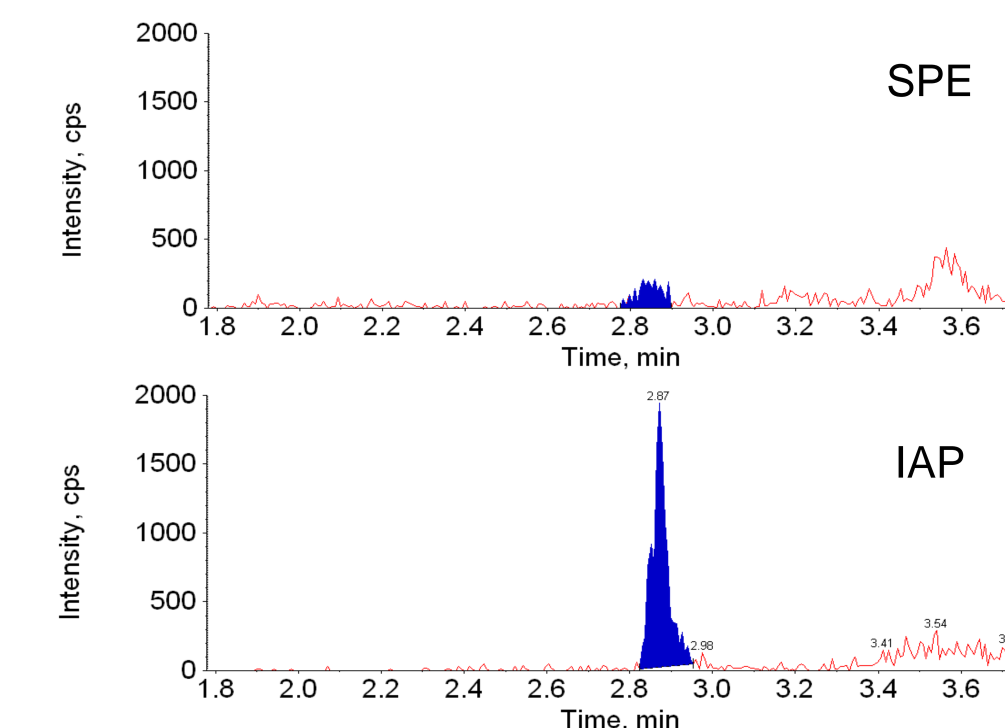


Figure 5. XIC of Insulin Glargine (300 pg/mL) in Human Plasma Using the SPE (Top) Versus IAP (Bottom) Approach

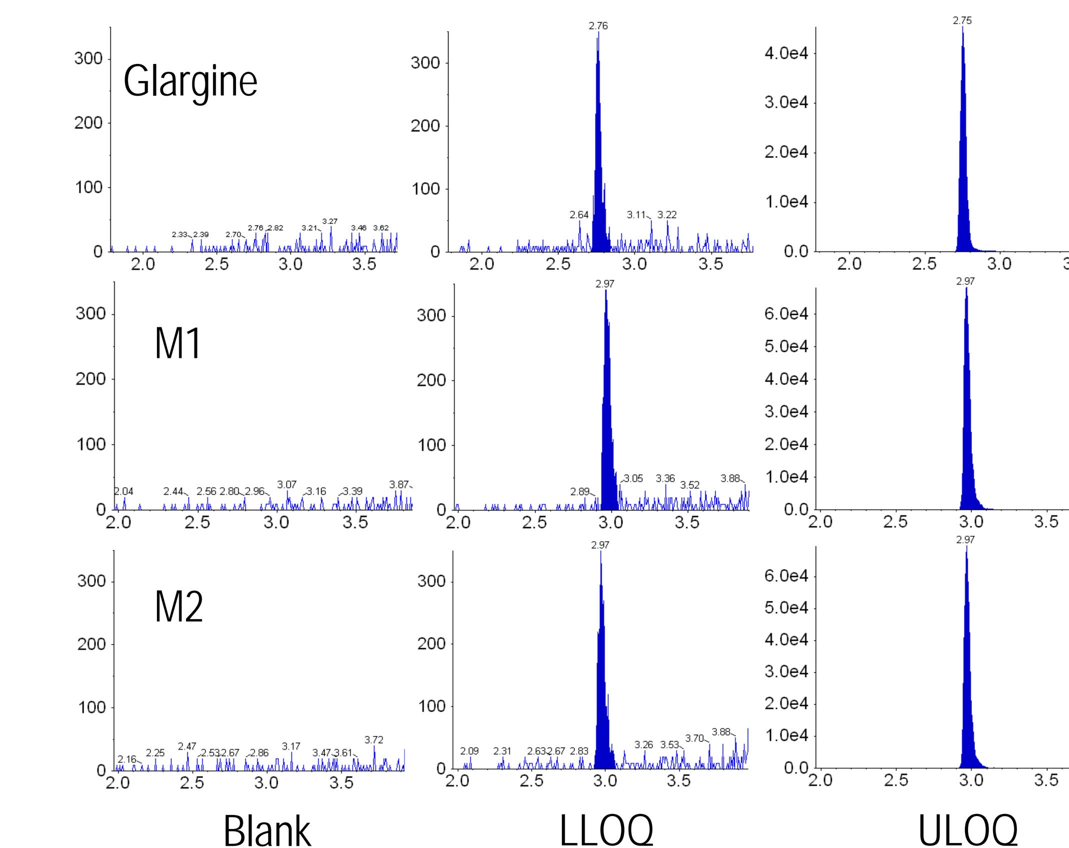


Figure 6. Chromatograms of Extracted Blank, LLOQ (50 pg/mL) and ULOQ (10,000 pg/mL) for Insulin Glargine, M1 and M2

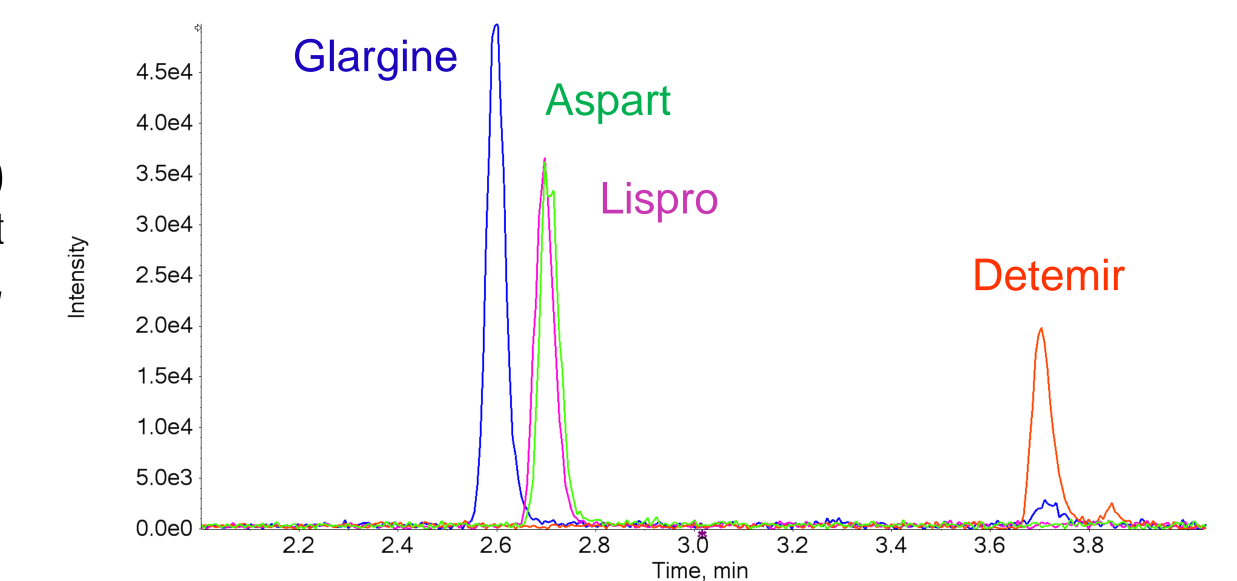


Figure 7. XICs of Insulin Analogs Aspart, Lispro and Detemir Extracted Using the Glargine Hybrid LBA-LC/MS method

Table 3. Regression Curve Range and Linearity of Insulin Analogs

| Insulin Analog | Range (pg/mL) | Linearity ($R^2$) | MRM Transition |
|---|---|---|---|
| Glargine | 50 – 10,000 | 0.999 | 867/136 |
| Lispro | 50 – 10,000 | 0.998 | 968/217 |
| Aspart | 50 – 10,000 | 0.995 | 971/136 |
| Detemir | 50 – 10,000 | 0.995 | 1184/454 |

### METHOD VALIDATION

Table 4. Summary of Method Validation

| Evaluation | Results for Insulin Glargine* |
|---|---|
| Precision and Accuracy (inter-day) | LLOQ QC: 98.5%, CV = 9.0%<br>All QC levels: 96.0% - 103.3%, CV = 3.8% - 9.0% |
| Percent Extraction Yield | 67.3% to 73.1% through all QC levels |
| Matrix Factor | Acceptable for 8 lots including lipemic and hemolyzed |
| Selectivity | Acceptable for 8 lots including lipemic and hemolyzed |
| Autosampler Storage Stability | 115.8 hours at 4°C nominal |
| Short-Term Stability | 24.8 hours at 4°C nominal |
| Long-Term Stability | 71 days at -80°C |
| Freeze Thaw | 3 cycles |
| Whole Blood Stability | 3.0 hours in an Ice/Water bath |

*M1 and M2 results also within acceptance criteria (data not shown)

## CONCLUSION

A specific, sensitive and high-throughput method was developed and fully validated for Insulin Glargine and metabolites in human plasma, highlighting the advantages of hybrid LBA-LC/MS methods in leveraging both sensitivity and specificity. With minor modifications, this method can also be applied to the analysis of other insulin analogs.