Quantitation of Insulin Analogue Glargine and its two Metabolites M1 and M2 in Human Plasma Using a Hybrid IP-LC-MS/MS Approach

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Introduction

Insulin Glargine is a recombinant long-acting human insulin analogue marketed as Lantus®, which is used in the treatment of insulin-dependent diabetes mellitus. After subcutaneous injection, Glargine precipitates partially at the injection site, being slowly released and enzymatically cleaved to generate two active metabolites, M1 and M2.

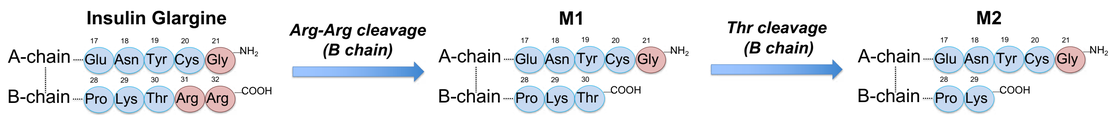

Figure 1. Insulin Glargine and its metabolites M1 and M2

Concentrations of these three analytes need to be determined to understand the pharmacokinetics, pharmacodynamics and toxicology of Glargine and its metabolites. Historically, ELISA or RIA assays have been developed for quantitation of insulin analogues, but their lack of specificity pushed the implementation of LC-MS/MS approaches, well known for their selectivity. Here we present a hybrid immunoaffinity purification (IP) procedure taking advantage of MSIA insulin tips (Thermo Fisher Scientific), combined with high sensitive LC-MS/MS methodology for quantitation of insulin Glargine, M1 and M2 in human plasma in the range of 50.0 – 10000 pg/mL, covering the LLOQ of 70.0 pg/mL required for clinical use.

Method

Sample extraction

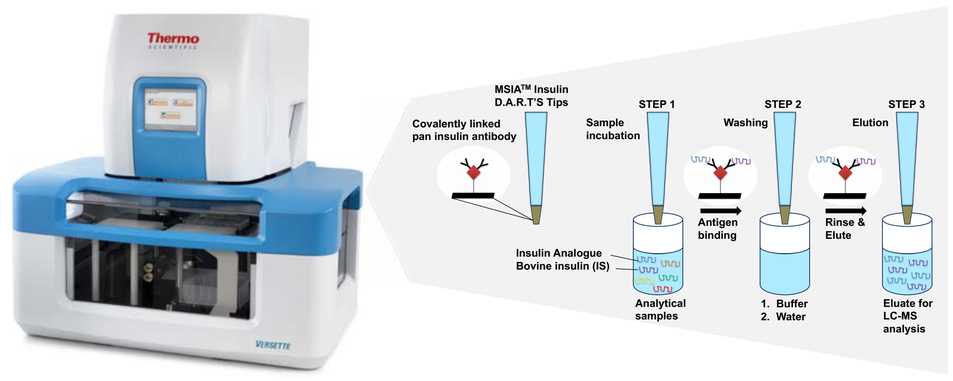

Figure 2. Immunoaffinity purification procedure with the 6 stages Versette™ Automated Liquid Handler (Thermo Fisher Scientific).

- 400 µL sample (human EDTA plasma)
- 250 µL internal standard (bovine insulin)
- Sample incubation (**STEP 1**)
- Washing (**STEP 2**)
- Elution in H_2O/ACN/TFA 67:33:0.4 (v/v/v) (**STEP 3**)
- Sample dilution with H_2O/Formic acid 100:0.1 (v/v)
- Storage at 5 °C until injection

LC-MS/MS conditions

- Conditioning effects were observed with several C_{18} columns tested during method development.
- Summing-up multiple times the same transition for each analyte was crucial to achieve the required sensitivity.

Table 1. Chromatographic conditions and MS/MS parameters.

Chromatographic conditions	
UPLC	Waters ACQUITY UPLC I-Class
Analytical column	Waters CORTECS C_{18}+, 50 × 2.1 mm, 2.7 µm
Mobile phase A **Mobile phase B**	Water/Formic acid 100:0.1 (v/v) Acetonitrile/Formic acid 100:0.1 (v/v)
Flow rate / Column temperature / Injection volume	0.5 mL/min / 50 °C / 60 µL
Total run time	5 min
MS/MS conditions	
Mass spectrometer	SCIEX TQ 6500+
Source / Polarity	ESI / Positive
Followed MRM transitions	Insulin Glargine: m/z 867.1 → m/z 984.2 M1: m/z 959.4 → m/z 1131.2 M2: m/z 942.3 → m/z 1098.0 Bovine insulin (IS): m/z 956.5 → m/z 1121.1

Results

Blanks

LLOQ: 50.0 pg/mL

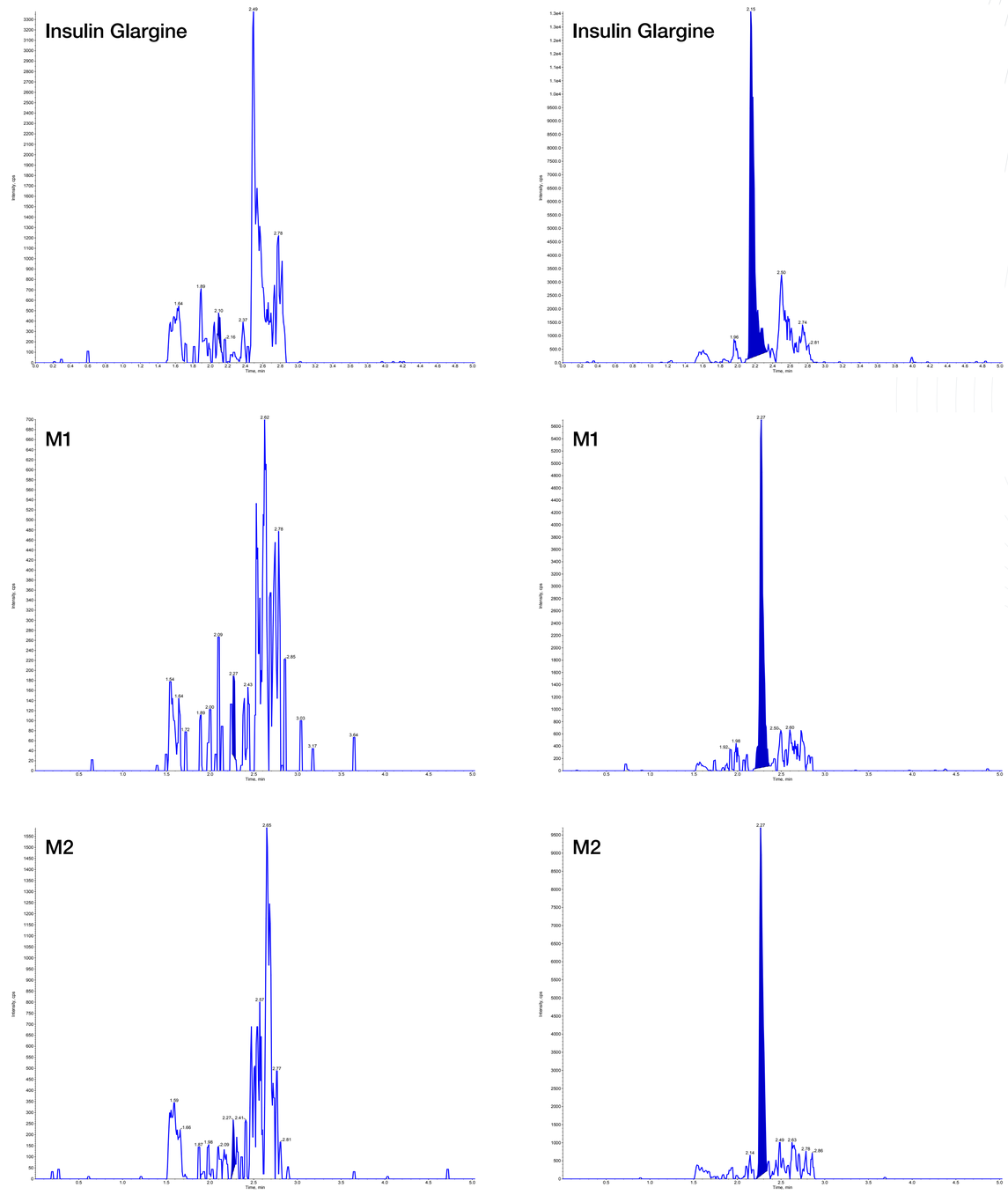

Figure 3. Typical chromatograms of the blanks (left) and LLOQ samples (right) for Insulin Glargine (top), M1 (middle) and M2 (bottom).

Method qualification summary

Table 2. Inter-run precision and accuracy of three independent runs for insulin Glargine (top), M1 (middle) and M2 (bottom). For quantitation, a linear regression model with $1/x^2$ weighting factor was used.

Inter-batch precision and accuracy results: Insulin Glargine, M1 and M2		QC LLOQ 50.0 pg/mL	QC Low 150 pg/mL	QC Mid 3000 pg/mL	QC High 7500 pg/mL
Insulin Glargine	Accuracy (%)	95.6	98.0	96.0	91.6
	CV (%)	8.5	9.4	7.9	4.3
	N	18	18	18	18
M1	Accuracy (%)	94.4	100.0	95.7	92.4
	CV (%)	13.1	7.5	7.6	4.1
	N	18	18	18	18
M2	Accuracy (%)	98.2	99.3	95.3	91.1
	CV (%)	18.6	7.9	7.0	4.2
	N	18	18	18	18

- The 4-6-20 acceptance criteria was applied.
- Column carry-over was observed and minimized.
- No selectivity issues were observed in multiple individual matrices.
- Recovery was around 40%, consistent between Low, Mid and High QCs.
- Matrix effect was assessed in multiple individual matrices and the overall precisions and accuracies were within acceptance criteria.
- Stabilities (combined freeze/thaw + bench-top, long-term and processed samples) were shown.

Conclusions

To the best of our knowledge, this is the first IP-LC-MS/MS method allowing for selective quantitation of insulin Glargine, M1 and M2 in human plasma within the defined range and using 400 µL sample volume. After its validation, this hybrid IP-LC-MS/MS method could be employed to monitor Glargine and its metabolites in biosimilars clinical trials.