W5208

Development and Validation of a Specific ELISA Suitable for the Pharmacokinetic Measurement of Insulin Glargine and its Metabolites M1 and M2

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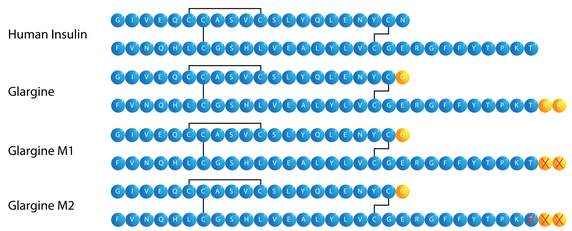
Background

Glargine insulin is a peakless, long-acting insulin analog that provides 24 hr basal insulin replacement and achieves target HbA1C level with less hypoglycemia in most patients[1]. Glargine provides 24 hr insulin replacement by a unique mode. The protracted action is related to a shift in the isoelectric properties upon subcutaneous injection, which brings about formation of micro-precipitates from which small amounts are slowly released[2].

Insulin glargine is a peptide containing 53 amino acids (MW = 6063 Da). It is highly homologous to native human insulin, with only minor differences in the amino acid sequence. It differs from insulin by a substitution at A21 (Asp to Gly), and the addition of 2 Arg residues on the B chain (B31 and B32) (Figure 1). Therefore, specific measurement of glargine and native insulin in the same sample is a great challenge. In addition, glargine is enzymatically transformed to metabolites M1 and M2 with the loss of two arginines and a threonine on the B-chain (Figure 1). This biotransformation and the cross-reactivity of both glargine and its metabolites in insulin assays make the measurement of glargine a complex issue.

Specific measurement of glargine and its metabolites is of great importance in many studies, including research related to the development of novel analogs and biosimilars. To meet the need for specific detection of glargine and its metabolites, methods were developed for measurement of glargine, and its metabolites without cross-reactivity to human endogenous insulin and other insulin analogs.

Figure 1. Amino Acid Sequences for Human Insulin, Glargine and its' Metabolites (M1 & M2).



Methods

Multiple antibodies were generated in rabbit, mouse and chicken using various peptide sequences derived from glargine sequence. After exhaustive characterization and evaluation, a pair of antibodies, (mouse monoclonal and chicken polyclonal) were selected for the development of the immunoassay method.

The assay utilized a homogenous, solution-phase incubation to generate a complex of two antibodies bound to glargine present in calibrator/test samples. The complex was captured by using an anti-species antibody coated to the ELISA plate. The wells were washed to remove unbound material and an enzyme-labeled anti-species antibody added. An unbound labeled antibody was then removed and a chromogenic substrate added to the bound labeled antibody. The development of the colored reaction product was directly proportional to the concentration of glargine present in the sample and was detected using a spectrophotometric plate reader.

In an attempt to evaluate the suitability of the method for the measurement of glargine metabolites, glargine was incubated in serum at 37°C to generate M1 and M2 metabolites in-vitro. The assay was used for the qualititative evaluation of cross-reactivity for glargine metabolites.

Results

The following parameters were evaluated during the validation of the method: precision, accuracy, matrix effects, sensitivity, dilutional linearity, and cross-reactivity to endogenous insulin and insulin analogs.

Figure 2. Representative Calibration Curve.

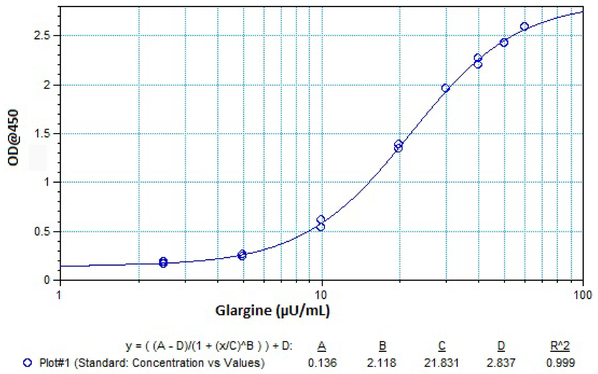


Table 1. Inter-Batch Accuracy Precision of Validation Samples.

Inter-Batch	Concentration of Insulin Glargine (µU/mL)				
	50	40	25	12	5
Run 1	43.48	36.38	21.94	11.24	*
Run 2	50.1	41.94	25.61	11.06	3.99
Run 3	46.61	40.81	22.1	10.32	5.05
Run 4	46.01	38.78	26.22	12.36	4.23
Run 5	47.00	35.39	22.32	11.73	4.91
Run 6	49.62	43.41	22.93	10.68	5.31
Mean	47.14	39.45	23.52	11.23	4.70
SD	2.23	2.89	1.73	0.67	0.50
%CV	5	7	7	6	11
%Bias	-6	-1	-6	-6	-6
n	6	6	6	6	5

*Removed from Calculation (%CV > 20%)

Table 2. Intra-Batch Accuracy Precision of Validation Samples (Representative Data).

Intra-Batch	Concentration of Insulin Glargine (µU/mL)				
	50	40	25	12	5
Replicate # 1	49.50	42.00	25.22	11.41	3.62
Replicate # 2	49.81	37.56	27.13	11.34	4.59
Replicate # 3	49.14	41.58	26.28	11.85	3.25
Replicate # 4	51.95	39.14	23.67	9.97	4.41
Replicate # 5	52.81	42.44	26.31	11.41	4.02
Replicate # 6	49.39	48.90	25.1	10.40	4.05
Mean	50.43	41.94	25.62	11.06	3.99
SD	1.41	3.56	1.11	0.65	0.45
%CV	3	8	4	6	11
%Bias	1	5	2	-8	-20
n	7	7	6	6	6

Table 3. Selectivity Data.

Lot #	Spiked Concentration (µU/mL)	Observed Concentration (µU/mL)	Percent Recovery
1	10	8.32	83
	0	BLQ	NA
2	10	8.74	87
	0	BLQ	NA
3	10	9.86	99
	0	BLQ	NA
4	10	9.96	100
	0	BLQ	NA
5	10	11.55	116
	0	BLQ	NA
6	10	13.33	133
	0	BLQ	NA
7	10	9.82	98
	0	BLQ	NA
8	10	7.79	78
	0	BLQ	NA
9	10	10.32	103
	0	BLQ	NA
10	10	7.52	75
	0	BLQ	NA

BLQ = Below the Limit of Quantitation

Table 4. Validation Summary Data.

Validation Parameter	Description/Results
Method Description	Homogenous solution phase sandwich ELISA with colorimetric detection
Calibrator Concentrations (µU/mL)	2.5 (anchor point), 5, 10, 20, 30, 40, 50 and 60 (anchor point)
Assay Volume Required (mL)	0.050
Regression Type	4PL
QC Concentrations (µU/mL)	LLOQ (5), Low (12), Mid (25), High(40), ULOQ (50)
Limit of Detection (µU/mL)	2.9
Limit of Quantitation (µU/mL)	5 µU/mL
QC Intra-Batch Precision Range (% CV)	3% to 11%
QC Intra-Batch Accuracy Range (% Bias)	-20% to 5%
QC Inter-Batch Precision Range (% CV)	5% to 11%
QC Inter-Batch Accuracy Range (% Bias)	-6% to -1%
Dilutional Integrity	Samples diluted up to 5-fold can be quantified accurately within assay acceptance criteria. Maximum quantifiable concentration is 200 µU/mL
Hook Effect	No hook effect was observed up to 200 µU/mL
Matrix Effect	No significant matrix effect was observed. Nine out of 10 lots have acceptable recovery (75-125%)
Cross-Reactivity against Human Insulin	No cross-reactivity was observed up to 100 µU /mL; less than 5% at 500 µU/mL
Cross-Reactivity against Detemir, Lispro, Aspart, and Glulisine	Less than 1% at 200 µU /mL
Cross-Reactivity to Glargine Metabolites (M1 & M2)	Antibodies are cross-reactive to M1 and M2 metabolites generated in-vitro. Additional quantitative evaluation of cross-reactivity is on-going.

Conclusions and Future Work

The ELISA was found to be suitable for the pharmacokinetic measurement of insulin glargine and its' biosimilars. Initial data shows that the assay is capable of measuring glargine metabolites. Additional cross-validation using an orthogonal method (i.e. liquid chromatography-tandem mass spectrometry) to confirm the validity of the assay for the quantitative measurement of glargine metabolites is ongoing.

References

1. Ciaraldi, T. P., & Sasaoka, T. (2011). Review on the in vitro interaction of insulin glargine with the insulin/insulin-like growth factor system: Potential implications for metabolic and mitogenic activities. Hormone and metabolism research, 1 - 10.
2. Heinemann, L., Linkeschova, R., Rave, K., Hompesch, B., Sedlak, M., & Heise, T. (2000). Time-action profile of the long acting insulin analog insulin glargine (HOE901) in comparison with those of NPH insulin and placebo. Diabetes Care, 644 - 649.

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