Determination of glargine and its metabolites M1 and M2 by using a combination of insulin assays of different specificity

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CONCLUSION

We conclude that measurement of insulin, glargine and its metabolites in human samples is possible by using two different insulin ELISAs with different specificity. The method described may be a valuable tool to determine glargine or its metabolites in patient samples.

OBJECTIVE

The objective of this study was to find a method for determination of glargine and its metabolites along with endogenous insulin in human samples.

BACKGROUND

Insulin and insulin analogs are standard therapies for treatment of both type 1 and type 2 diabetes. Specific measurement of each analog is of great importance in many studies, including research related to the development of novel analogs and biosimilars. Most insulin analogs are highly homologous to native human insulin, with only minor differences in the amino acid sequence. Therefore, specific measurement of an analog and native insulin in the same sample is a great challenge. In the case of glargine, there is a general problem with cross-reactivity in human insulin assays. After subcutaneous injection, glargine is enzymatically transformed to metabolites M1 and M2 with the loss of two arginines and a threonine on the B-chain. This biotransformation and the cross-reactivity of both glargine and its metabolites in insulin assays makes measurement of glargine a complex issue. To meet the need for specific detection of glargine and its metabolites, we developed methods for measurement of glargine, its metabolites and native human insulin in the same sample.

METHODS

The Mercodia Insulin ELISA is a simultaneous assay with a sample volume of 25 µL. The incubation time is 60 minutes plus a 15 minute substrate incubation. The cross-reactivity of glargine in the simultaneous assay was determined to be 8.1 – 31%. Based on our knowledge of the assay specificity, we hypothesized that changing the protocol to a sequence assay would reduce the cross-reactivity to glargine. For the sequence protocol see Protocol Summary. Performance of the Mercodia Insulin ELISA sequence application was compared to that of the simultaneous Mercodia Insulin ELISA (i.e., the reference method), using the methodology described in Miller *et al.* (2009) *Clin Chem* 55:1011-18. The cross-reactivity of glargine and glargine metabolites M1 and M2 (kindly provided by R&D DSAR/Biomarkers, Bioim.&Biol.Ass. FF, Sanofi-Aventis Deutschland GmbH, Frankfurt) was evaluated in the sequence assay.

The Mercodia Iso-Insulin ELISA is a simultaneous assay with different specificity than the Mercodia Insulin ELISA. The Mercodia Iso-Insulin ELISA measures insulin from a variety of species, and most commercially available insulin analogs (see specificity table). The cross-reactivity of glargine and glargine M1 and M2 was evaluated in the Iso-Insulin assay.

Recovery studies were performed using four human serum samples. 10 µL of glargine, M1 and M2 at a concentration of 300 mU/L were added to 100 µL of each sample and incubated for 12 minutes. Recovery of endogenous insulin, glargine, M1 and M2 was determined using the Mercodia Insulin ELISA (sequence) application and the Mercodia Iso-Insulin ELISA in parallell.

RESULTS

The Mercodia Insulin ELISA sequence application specifically measures endogenous insulin, with no cross-reactivity to glargine, M1 or M2 (see table 1).

Endogenous insulin, glargine, M1 and M2 in the same sample could be measured by using the Mercodia Insulin ELISA sequence application and the Mercodia Iso-Insulin ELISA in parallell (see tables 2-4).

The performance of the Mercodia Insulin ELISA sequence application was similar to the reference method (results not shown).

Measurement of glargine in a sample:

After subcutanous injection of a therapeutic dose, glargine is minimally detectable in blood, whereas its metabolite M1 accounts for most (~90%) of the plasma insulin concentration up to 31 hours [Lucidi *et al.* (2011), *Meeting abstract 1056, 47th EASD meeting, Lisbon, PT].* To measure M1 in a sample with unknown concentration:

1. Measure endogenous insulin in the sample using Mercodia Insulin ELISA sequence application = x
2. Measure endogenous insulin + M1 in the sample using Mercodia Iso-Insulin ELISA = y
3. M1 concentration is calculated by (y – x) / 0.41, based on 41% cross-reactivity of M1 in the Mercodia Iso-Insulin ELISA.

Recovery studies

	Total insulin *Mercodia Iso-Insulin ELISA*			Endogenus insulin *Mercodia Insulin ELISA Sequence Application*		
Sample ID	Neat sample (mU/L)	Sample + 27mU (GI) (mU/L)	Recovery (GI)	Neat sample (mU/L)	Sample + 27mU (GI) (mU/L)	Recovery insulin
S-7514	5.5	16.5	100%	5.7	4.7	83%
S-7058	22.4	35.3	106%	20.6	20.7	101%
S-5883	28.0	40.9	105%	25.3	25.8	102%
S-5271	53.4	66.5	103%	48.8	49.9	102%

Table 2 Recovery of glargine (GI) and endogenous insulin in samples. The recovery of glargine in samples was 100–106% (mean 104%). The recovery of endogenous insulin was 83–102% (mean 97%).

	Total insulin *Mercodia Iso-Insulin ELISA*			Endogenus insulin *Mercodia Insulin ELISA Sequence Application*		
Sample ID	Neat sample (mU/L)	Sample + 27mU M1 (mU/L)	Recovery M1	Neat sample (mU/L)	Sample + 27mU M1 (mU/L)	Recovery insulin
S-7514	5.5	15.1	91%	5.7	4.6	80%
S-7058	22.4	34.3	102%	20.6	19.4	94%
S-5883	28.0	39.2	100%	25.3	24.7	98%
S-5271	53.4	64.9	100%	48.8	48.3	99%

Table 3 Recovery of M1 and endogenous insulin in samples. The recovery of M1 in samples was 91–102% (mean 98%). The recovery of endogenous insulin was 80–99% (mean 93%).

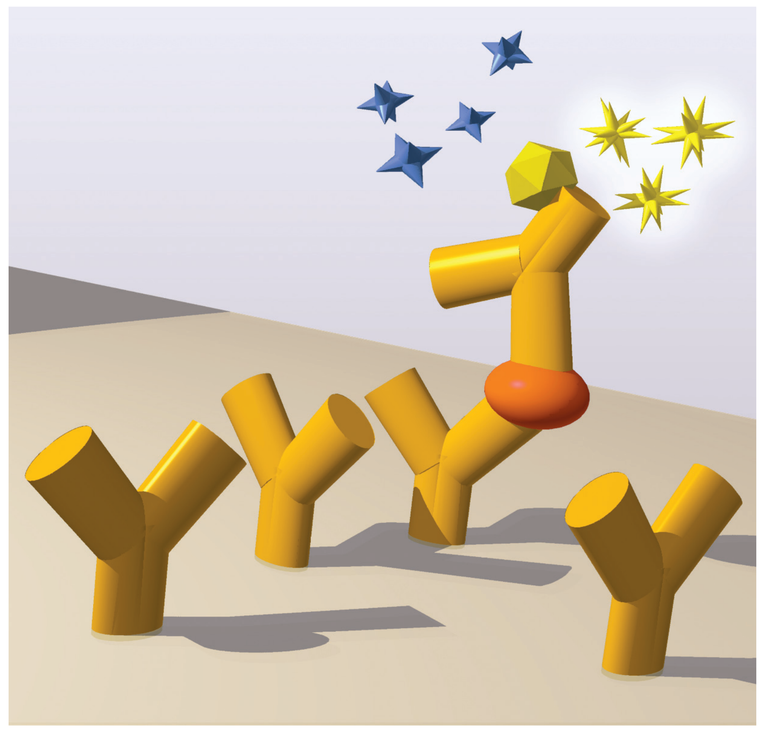
	Total insulin *Mercodia Iso-Insulin ELISA*			Endogenus insulin *Mercodia Insulin ELISA Sequence Application*		
Sample ID	Neat sample (mU/L)	Sample + 27mU M2 (mU/L)	Recovery M2	Neat sample (mU/L)	Sample + 27mU M2 (mU/L)	Recovery insulin
S-7514	5.5	11.6	90%	5.7	5.0	87%
S-7058	22.4	30.4	102%	20.6	20.3	99%
S-5883	28.0	36.0	101%	25.3	25.6	101%
S-5271	53.4	61.0	100%	48.8	49.9	102%

Table 4 Recovery of M2 and endogenous insulin in samples. The recovery of M2 in samples was 90–102% (mean 98%). The recovery of endogenous insulin was 87–102% (mean 97%).

Specificity

	Mercodia Iso-Insulin ELISA		Mercodia Insulin ELISA Sequence Application
	Mean	Range	
Human	100%	-	100%
Glargine	44%	37-56%	Not detected
Glargine M1	41%	32-51%	Not detected
Glargine M2	28%	21-46%	Not detected

Table 1 Cross-reactivities to insulin, insulin glargine, M1 and M2.
Glargine, M1 and M2 were tested in the concentration range of 3 – 500 mU/L.



The detection antibody binds to a second epitope (immunogenic part) on the antigen.

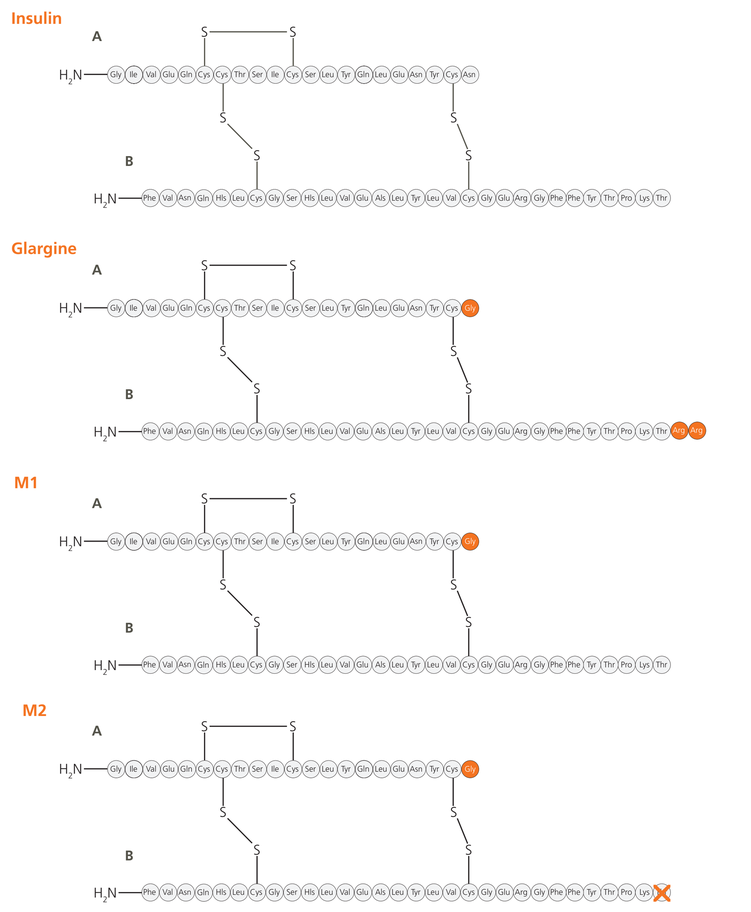
Protocol Summary

Mercodia Insulin ELISA Modified to a Sequence Assay*,**

Add Calibrators, controls and samples	25 µL
Add Assay Buffer***	50 µL
Incubate	1 hour at 18-25°C on a plate shaker
Wash plate with wash buffer 1X solution	6 times
Add enzyme conjugate 1X solution	100 µL
Incubate	1 hour at 18-25°C on a plate shaker
Wash plate with wash buffer 1X solution	6 times
Add Substrate TMB	200 µL
Incubate	20 minutes at 18-25°C
Add Stop Solution	50 µL Shake for 5 seconds to ensure mixing
Measure A_{450}	Evaluate results

*Technical Note TN34-0143
**For research use only
****Note!* Assay Buffer not included in kit - must be ordered separately.

Structure of Human Insulin, Glargine and its Metabolites



Structure of human insulin, glargine and its metabolites.
Glargine: substitution of asparagine by glycine on position A21 and addition of two residues of arginine on position B31 and B32. M1: glargine metabolite, A21-Gly-insulin. M2: glargine metabolite, A21-Gly-des-30B-Thr-insulin.