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Case Study

Biomarker Qualification and Validation Challenges: The Insulin Case Study

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Introduction

Biomarkers follow varied paths into clinical trials for pharmaceutical development. Before implementation in clinical studies, candidate biomarkers are subjected to qualification and validation. Analytical validation ensures adequate assay performance in relation to the questions addressed in the clinical study (fit-for-purpose). Biomarker qualification entails collecting sufficient evidence of the relationship between a biomarker with the relevant biological processes and clinical end points.

Biomarkers used in pharmaceutical research had not been under clear regulatory guidance until recently. The first FDA paper where biomarkers were mentioned was the PK guidance from 2013. In this document, it was acknowledged that "biomarkers can be used for a wide variety of purposes during drug development; therefore, a fit-for-purpose approach should be used when evaluating the extent of method validation that is appropriate." It is stated that biomarker method validation should aim to address the same performance characteristics as required in method validation for PK assays, and that the approach used for PK assays should be the starting point for validation of biomarker assays—although the FDA acknowledges that direct application of PK guidelines to biomarker assays is probably not possible. The FDA also suggested that the level of analytical validation of an assay should depend on the purpose of the biomarker data. For instance, an assay should be fully validated when the data are used for go/no go decisions. It should be up to the sponsor to decide what level of validation needs to be applied on a fit-for-purpose approach.

For the biomarker insulin, assay development results had turned out to be widely desperate (1) and in consequence no conclusive recommendations on insulin assay development exist up till today. The most common limitations for successful insulin bioassay development lay in technical difficulties posed by endogenous insulin levels, cross-reactivity with insulin analogues etc.

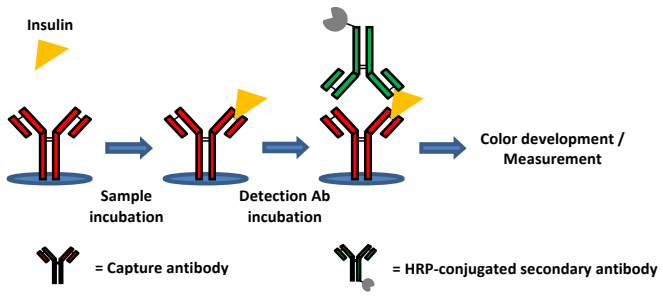
In this study we demonstrate that the introduction of a specific surrogate matrix in an ELISA based bioassay for measuring human insulin, significantly suppressed the variability arising from endogenous insulin, typically observed in insulin depleted sera. Furthermore, a specially formulated dilution buffer included in the sample incubation step abrogates cross-reactivity with insulin analogues.

This novel insulin bioassay has been successfully validated where cross reactivity with a wide range of insulin analogues was completely abrogated. These results demonstrate the superiority of the novel bioassay, when compared with insulin depleted sera bioassays. Moreover, the assay has been successfully automated for future high through-put insulin measurements.

Assay Development

The developed insulin-specific assay used to quantify insulin in human serum samples is based on a commercial sandwich anti-human insulin colorimetric ELISA kit (Figure 1). The kit is based on two monoclonal antibodies that recognize distinct epitopes in human insulin: one for capture and a second for detection (HRP labeled).

Figure 1. Sandwich ELISA format

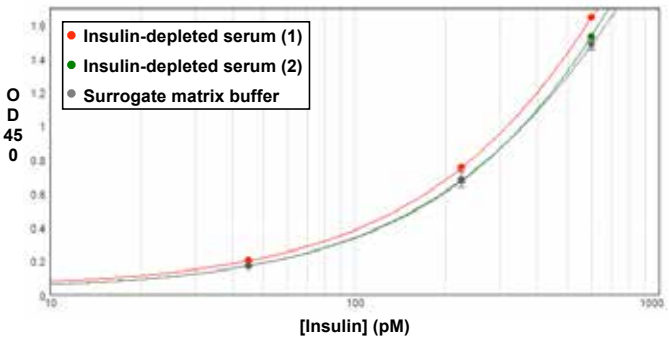


The assay is fast, simple, and robust, which allowed for the introduction of modifications aimed at circumventing technical difficulties commonly encountered during bioanalytical method development for insulin (i.e., endogenous insulin, cross-reactivity with insulin analogues).

Endogenous levels of insulin-ranging in normal individuals from 10 to 400 pM - represent a major challenge during bioanalytical assay development. Insulin-depleted sera employed as matrix for standard curve preparation show lot-to-lot variation in their response curves affecting long term studies.

To overcome this limitation, a buffer of defined composition was developed to replace insulin-depleted serum as the standard curve matrix (surrogate matrix buffer). To this end, dose-response curves of recombinant insulin prepared in different buffers were compared to a curve prepared in a validated insulin-depleted serum: the buffer with the resulting lowest mean deviation was selected as the surrogate matrix (Figure 2).

Figure 2. Comparison of calibration curves of human insulin prepared with two different insulin-depleted sera and with the surrogate matrix buffer.



An additional challenge in the development of insulin bioanalytical methods are the insulin analogues, as they may interfere with the accurate measurement of insulin. Initial tests showed that the assay cross-reacts particularly with two insulin analogues, Aspart and Glargine, while does not with Degludec, Determir, Glulisine or Lispro.

Different strategies were explored to abrogate this interference, including using specific blocking anti-analogue antibodies, different washing buffers and sample dilution buffers. To test the different approaches, insulin-depleted serum samples were spiked with high concentrations of Aspart and Glargine insulin analogues. Best results were obtained with the inclusion of a dilution buffer during the sample incubation step (Table 1).

Table 1. Cross-reactivity of Aspart and Glargine insulin analogues with the insulin assay in the absence (W/O) or presence (W) of a sample dilution buffer.

	% of Cross-Reactivity	
Insulin Analogue	**W/O dilution buffer**	**W dilution buffer**
Aspart (10000 pM)	4.15	< 0.15
Glargine (10000 pM)	16.74	< 0.15

These two modifications allowed for the development of a highly specific bioanalytical insulin assay with an analytical range of 20 pM to 600 pM. Method validation confirmed the assay performance with the included modifications (see Assay Validation).

Assay Validation

The human insulin assay performance was validated following international standards. The standard curve was prepared in surrogate matrix buffer with an analytical range of 20 pM to 600 pM and quality control (QC) samples were prepared in low insulin level pool ([insulin] ≈ 20 pM). The following concentrations were selected: 20.9 pM (LLOQ-QC, corresponding to the un-spiked pool), 55 pM (LQC), 225 pM (MQC), 420 pM (HQC) and 600 pM (ULOQ-QC).

The following assay parameters were evaluated: precision and accuracy (standards and QC samples), parallelism, dilution linearity, selectivity, cross-reactivity with insulin analogues and stability. Additionally, the method precision and accuracy performance was validated on an automated system.

Precision and Accuracy

Precision and accuracy was evaluated in 27 (twenty-seven) experiments for standards, and in 6 (six) independent experiments (5 replicates of each control level / experiment) for quality controls.

Inter-run precision for standards

	STD 1 (20 pM)	STD 2 (35 pM)	STD 3 (60 pM)	STD 4 (100 pM)	STD 5 (160 pM)	STD 6 (250 pM)	STD 7 (450 pM)	STD 8 (600 pM)
Mean	19.5	35.1	60.1	98.7	163	248	450	600
%CV	7.3	3.6	2.4	2.2	2.2	2.0	1.4	0.6
%Dev	-2.5	0.3	0.2	-1.3	1.9	-0.8	0.0	0.0
n	26	26	27	27	27	27	27	26

Inter-run precision for QC samples

	LLOQ-QC (20.9 pM)	LQC (55.0 pM)	MQC (225 pM)	HQC (420 pM)	ULOQ-QC (600 pM)
Mean	21.2	55.2	222	421	594
%CV	5.5	4.9	4.1	4.4	5.0
%Dev	1.4	0.4	-1.3	0.2	-1.0
n	30	30	30	30	30

Parallelism

Parallelism was evaluated by diluting three individual samples with high insulin levels with surrogate matrix buffer (four dilutions within the analytical range were evaluated).

	Individual 1			Individual 2			Individual 3		
Dilution Factor	**[Insulin] (pM)**	**%CV**	**%Dev**	**[Insulin] (pM)**	**%CV**	**%Dev**	**[Insulin] (pM)**	**%CV**	**%Dev**
undiluted	158	1.4		172	0.6		212	0.5	
2	182	0.5	15.2	188	0	9.3	218	1.1	2.8
3	189	2.5	19.6	192	0.9	11.6	224	0.2	5.7
4	186	0.7	17.7	201	0.5	16.9	225	0	6.1
6	188	0.8	19.0	203	3	18.0	220	1	3.8
Mean	**180.6**			**191.2**			**219.8**		
SD	**12.9**			**12.4**			**5.2**		
%CV	**7.2**			**6.5**			**2.4**		

Dilution linearity

Dilution linearity was evaluated with low insulin level pool sample spiked with 20000 pM of recombinant insulin. Serial dilutions were then performed with surrogate matrix buffer and the samples analyzed.

Dilution factor	0	10	40	100	400
Nominal concentration (pM)	20000	2000	500	200	50
Measured concentration (pM)	>ULOQ	>ULOQ	533	227	55.8
SD			11.7	5.12	1.50
%CV	N/AP	N/AP	2.2	2.3	2.7
%Dev			6.6	13.5	11.6
n	5	5	5	5	5

Selectivity

Twelve samples were evaluated for selectivity, including a lipemic sample. Selected samples were spiked with an additional 30 pM of recombinant insulin and analysed.

Sample ID	Condition	Basal Level	Spike (+30.0 pM)		
		Measured Conc. (pM)	Theoretical Conc. (pM)	Measured Conc. (pM)	% Recovery
CM/17-0373		39.8	69.8	64.9	93.0
CM/17-0379		55.2	85.2	80.8	94.8
CM/17-0382		41.8	71.8	69.1	96.2
CM/17-0389		26.9	56.9	57.0	100.2
CM/17-0394		31.9	61.9	62.0	100.2
CM/17-0402		30.3	60.3	55.1	91.4
CM/17-0408		30.3	60.3	58.9	97.7
CM/17-0414		20.7	50.7	49.8	98.2
CM/17-0417		29.1	59.1	58.7	99.3
CM/17-0969		24.8	54.8	51.5	94.0
CM/17-0974		40.2	70.2	66.3	94.4
CM/17-1429	lipemic	86.9	117	110	94.1
Number of serum samples assessed					12
Number of serum samples meeting acceptance criteria					12
% of serum samples meeting acceptance criteria					100

Cross-reactivity

Cross-reactivity of the assay with insulin analogues was evaluated during validation as the effect of the analogues on the measurement of both, the LLOQ-QC and ULOQ-QC.

Samples containing insulin at the LLOQ and ULOQ levels were spiked with different insulin analogues and the samples analysed. The deviation in insulin concentration was then evaluated.

The concentrations of analogues were selected based on previous PK studies analysed at Celerion. They comprise in all cases the Cmax values expected during therapeutics treatment with the corresponding analogues.

	Aspart (2000 pM)	Degludec (10000 pM)	Detemir (10000 pM)	Glargine (800 pM)	Glulisine (2000 pM)	Lispro (2000 pM)
Nominal conc. (pM)	20.9					
Measured conc. (pM)	22.6	22.4	21.2	21.8	21.5	19.7
SD	0.666	0.460	0.678	0.559	0.371	0.865
%Dev	8.1	7.2	1.4	4.3	2.9	-5.7
n	5	5	5	5	5	5
Nominal conc. (pM)	600					
Measured conc. (pM)	573	553	576	567	559	559
SD	7.85	12.9	4.47	6.02	14.2	8.53
%Dev	-4.5	-7.8	-4.0	-5.5	-6.8	-6.8
n	5	5	5	5	5	5

Stability

Stability studies of biopharmaceuticals are routinely performed during method validation at Celerion. Short-term, long-term, bench-top and freeze/thaw cycles (-20 °C and -80 °) of quality control samples are evaluated, as well as specific conditions such as stability in hemolytic samples. These studies are drug-specific, and therefore, they are not reported for this method.

Stability of standards in surrogate matrix buffer was also investigated in this case, as the newly developed buffer is a novel introduction for the assay. Standards were subjected to 4 freeze/thaw cycles at -20 °C or -80 °C and analysed with a freshly prepared standard curve (only standards 1 and 8 are shown).

Condition	STD 1 (20 pM)		STD 8 (600 pM)	
	Conc. (pM)	%Dev	Conc. (pM)	%Dev
Freeze/thaw:	19.6	-2.0	597	-0.5
4 cycles at -20°C	19.9	-0.5	599	-0.2
	20.8	4.0	593	-1.2
	20.1	0.5	604	0.7
	21.1	5.5	597	-0.5
	20.3	1.5	581	-3.2
Mean	20.3		595	
%CV	2.8		1.3	
%Dev	1.5		0-8	
n	6		6	
Freeze/thaw:	21.1	5.5	625	4.2
4 cycles at -80°C	20.7	3.5	587	-2.2
	20.1	0.5	600	0.0
	20.6	3.0	604	0.7
	20.2	1.0	602	0.3
	20.2	1.0	604	0.7
Mean	20.5		604	
%CV	1.9		2.0	
%Dev	2.5		0.7	
n	6		6	

Automation

The assay precision and accuracy performance was also validated on an automated system. Each automated batch analysis comprises 8 plates; for validation purposes, plates 1 and 8 were used as test plates, while plates 2 to 7 were used as dummy plates (only inter-run precision and accuracy evaluation of quality control samples are shown).

	LLOQ-QC (20.9 pM)	LQC (55.0 pM)	MQC (225 pM)	HQC (420 pM)	ULOQ-QC (600 pM)
Mean	19.2	54.7	206	362	495
%CV	9.0	3.9	4.3	5.9	3.8
%Dev	-8.1	-0.5	-8.4	-13.8	-17.5
n	10	14	19	19	15

The automated method allows the analysis of ≈500 samples per day.

Conclusion

Bioanalytical assays for the biomarker insulin are negatively impacted by the presence of endogenous insulin, as well as by the interference of insulin analogues.

In the present work, a bioanalytical assay to measure the concentration of human insulin in human serum samples was developed and validated. The assay is based on a commercial kit and has an analytical range from 20 pM to 600 pM.

The two novel features introduced in this assay, i.e. use of surrogate matrix buffer and sample dilution buffer, significantly reduced the variability due to lot-to-lot variation of insulin-depleted sera and abrogated the cross-reactivity with insulin analogues.

Bioanalytical assays developed at Celerion Switzerland AG offer important tools for early clinical development of biopharmaceutical, as well as for the evaluation of biomarkers

References

1. Robbins DC et al; Report of the American Diabetes Association's task force on standardization of the insulin assay. Diabetes 1996;45:424-56