Challenges in FGF-21 Metabolic Biomarker Assay Development

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Introduction

Fibroblast growth factor 21 (FGF-21) is a key peptide hormone involved in energy homeostasis. It has various important functions such as:

- Regulating fatty acid oxidation
- Displaying anti-inflammatory properties in pancreatitis
- Improving glycemia

In consequence FGF-21 has been proposed as a novel therapeutic biomarker for metabolic diseases, including diabetes. In this study we discuss the bioanalytical assay development challenges in order to detect FGF-21.

Analytical Method

In this study we have developed a highly sensitive, quantitative sandwich ELISA for the detection of the metabolic biomarker FGF-21. In details 100µl of sample are incubated on capture antibody coated plates, washed and incubated with a detection antibody. Next, residual detection antibodies are discarded by another washing step, followed by streptavidin-HRP incubation and detection upon TMB administration (Fig. 1).

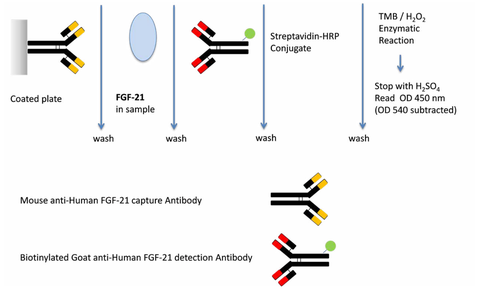

Figure 1: Principle of the assay

The following adaptations were done starting from a commercially available kit:

- Purchase of reference item showing an extended stability
- Preparation of Quality controls (QCs) using endogenous FGF-21 or recombinant form spiked on endogenous level for ULOQ
- Adaptation of the analytical range
- Optimization of the assay buffer for measurement in human serum

Challenges Encountered and Solutions

The main challenges encountered during method development and validation, were:

- Parallelism assessment
- Lot-to-lot variability of key reagents

Parallelism is defined as an established parallel relationship between a dose response curve from a study sample dilution series and a curve from a calibration standard series, with no difference among back-calculated concentrations for multiple dilutions of a study sample.

Parallelism is the hallmark parameter within biomarker analysis and was therefore tested early during method development.. High biases were initially observed when sera were serially diluted in bovine serum albumin (BSA) containing buffers (Fig. 2, diluents A and B). BSA replaced by Fetal Bovine Serum (FBS) for standard curve preparation as well as for sample dilution allowed significant lowered biases confirmed later on in validation (Fig. 2, diluent C and Fig. 8).

	% Bias		
DF	Diluent A	Diluent B	Diluent C
1	N/AP	N/AP	N/AP
2	29	29	-0.3
4	46	52	-3.4
8	53	77	-10

Figure 2: Parallelism assessment before and after method optimization

A significant change in terms of OD values was observed between different lots of detection antibody (Fig. 3). In order to solve this issue HRP conjugate was titrated to define an optimized dilution which compensate the observed variation of signals arising from the different antibody detection lots.

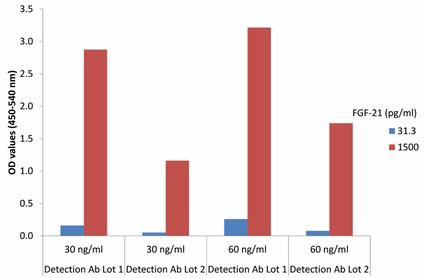

Figure 3: Titration of detection antibody lots

Validation

Following the fit-for-purpose principle, biomarker assays are developed and validated according to different standards often categorized as Gold, Silver and Bronze (M. S. Fjording, European Bioanalysis Forum, 2014)

The here presented work aimed at developing and validating a Gold standard FGF-21 comprising the key parameters:

- Precision & Accuracy
- Selectivity
- Dilution Linearity
- Hook effect
- Parallelism
- Stability

Precision and Accuracy (P&A) Selectivity/Recovery

All QC (Quality Control) samples (LLOQ, LQC, MQC HQC, and ULOQ) were analysed against the calibration curve and used to assess accuracy, precision and total error (Fig. 4). All QC levels fulfilled acceptance criterion, following the FDA and EMA guidances.

	LLOQ-QC 62.6 pg/mL	LQC 151 pg/mL	MQC 635 pg/mL	HQC 1150 pg/mL
Inter-run Mean	53.1	136	624	1090
Inter-run SD	9.49	16.0	51.8	71.3
Inter-run %CV	17.9	11.8	8.3	6.5
Inter-run %Bias	-15.2	-9.9	-1.7	-5.2
%Total Error	33.0	21.7	10.0	11.8
n	35	36	36	36

Figure 4: Quality control sample data from P&A runs

Selectivity/Recovery

Selectivity is the ability to measure the analyte of interest in the presence of unrelated compounds in the matrix. Due to the endogenous levels of FGF-21 in human serum, no blank samples were available for this assessment. Recovery of the analyte was determined by add-on spiking of ten individual human serum lots, including two lipemic and two hemolytic lots. Whenever possible, lots with low (≤ LQC level) endogenous FGF-21 levels were selected.

All matrices were analysed unspiked and spiked with 100 pg/mL of human FGF-21. The nominal concentration of the spiked samples was calculated as the sum of the determined endogenous concentration and the spiked concentration (Fig. 5). The bias of all samples was within ±20.0% of the expected concentration, fulfilling the acceptance criteria of FDA/EMA.

	Endogenous FGF-21 [pg/mL]	Expected recovery (endogenous +100 pg/mL) [pg/mL]	Determined recovery [pg/mL]	% Bias
Serum-1	125	225	208	-7.6
Serum-2	128	228	195	-14.5
Serum-3	112	212	198	-6.6
Serum-4	138	238	213	-10.5
Serum-5	126	226	223	-1.3
Serum-6	217	317	257	-18.9
Serum-7 [a]	137	237	256	8.0
Serum-8 [b]	155	255	224	-12.2
Serum-9 [b]	148	248	216	-12.9
Serum-10 [a]	221	321	307	-4.4

[a] lipemic lot
[b] hemolytic lot

Figure 5: Selectivity/Recovery

Dilution Linearity and Hook Effect

The purpose of this test was to investigate if concentrations of analyte higher than the ULOQ can be accurately determined after a dilution into the analytical range. To test these effects, a high concentration sample (12000 pg/mL) was prepared and diluted in five independent dilution series as follows

Each concentration level was analysed in 5 duplicates (one duplicate per dilution series prepared). Samples with concentrattions above the analytical range were measured as >ULOQ as expected, indicating the absence of hook effect for FGF-21 concentrations up to 12000 pg/mL (Fig. 6). All samples of concentration levels within the analytical range gave valid results. For the dilution factors 6 and 18 and 90, the bias of the back-calculated concentration was within ±20.0% of the nominal concentration, fulfilling the set acceptance criteria (Fig. 7). Hence it can be concluded that study samples at up to 12000 pg/mL of human FGF-21 can accurately be diluted into range.

Dilution factor (total, before MRD)	Dilution factor (from previous step)	Final FGF-21 Conc. (pg/mL, before MRD)	Comments
(no dilution)	N/AP	12000	>ULOQ, testing of hook effect
3	3	4000	>ULOQ, testing of hook effect
6	2	2000	within range
18	3	667	within range
90	5	133	within range

Figure 6: Dilution Linearity

	DQC 12000 DF 6 2000 pg/mL	% Bias	DQC 12000 DF 18 667 pg/mL	% Bias	DQC 12000 DF 90 133 pg/mL	% Bias
	2000	0.0	628	-5.8	138	3.8
	2050	2.5	632	-5.2	146	9.8
	1970	-1.5	642	-3.7	140	5.3
	1950	-2.5	659	-1.2	135	1.5
	2000	0.0	721	8.1	147	10.5
Mean	1990		656		141	
SD	37.8		38.0		5.17	
%CV	1.9		5.8		3.7	
%Bias	-0.5		-1.6		6.0	
n	5		5		5	

Figure 7: Dilution Linearity (repeat)

Parallelism

Parallelism testing was performed in order to evaluate matrix effects and different affinities to metabolites when diluting study samples. For testing purposes six individual matrices with high endogenous FGF-21 levels were diluted in FBS: 1:2, 1:3, 1:4 and 1:5. Undiluted and diluted samples were measured in one duplicate each. Precision and accuracy were calculated (Fig. 8), as well as the overall precision was evaluated.

All measured samples were within the analytical range. The overall precision (%CV) was 4.6% and all acceptance criteria were met.

DF	Serum-1	% Bias	Serum-2	% Bias	Serum-3	% Bias
1	526	N/AP	583	N/AP	606	N/AP
2	530	0.8	596	2.2	646	6.6
3	545	3.6	581	-0.3	630	4.0
4	491	-6.7	645	10.6	602	-0.7
5	569	8.2	597	2.4	593	-2.1
Mean	532		600		615	
SD	28.5		26.0		21.9	
%CV	5.4		4.3		3.6	
%Bias	1.1		2.9		1.5	
n	5		5		5	

Figure 8: Parallelism (representative data)

Stability in Matrix

The stability of human FGF-21 was evaluated in human serum as well as in FBS. For all assessments, six duplicates (using three aliquots) at a low and a high concentration level were analysed at time zero (after one freeze/thaw cycle). Stability samples were then exposed to different conditions and were analysed against freshly spiked calibration standards. The stability of LQC and HQC samples in polypropylene tubes was assessed after 6 freeze (20°C and -80°C)/thaw cycles. This assessment was combined with the short-term stability experiment. For the freezing temperature at -20°C, samples were left at room temperature for 13 hours in one of the thaw periods, yielding a total thaw time of 29 hours and a total freezing time of 17 days. For the freezing temperature at -80°C, samples were left at room temperature for 17 hours in one of the thaw periods, yielding a total thaw time of 31 hours and a total freezing time of 30 days. Stability could be demonstrated for both temperatures.

Conclusions and Discussion

In recent years FGF-21 gained more and more attention as a key metabolic biomarker for fat and glucose metabolism as well as an inflammation regulator in pancreatitis.

In the present study we show the successful development and validation of a highly sensitive bioanalytical method in order to detect endogenous FGF-21 levels. Bioanalytical assay development challenges in order to properly assess the key biomarker parameter parallelism, were successfully overcome by the introduction of the stabilizer FBS. Furthermore detection antibody lot differences were overcome by proper titration of HRP-conjugate.

All together we successfully developed and validated a highly sensitive FGF-21 biomarker assay, with a large analytical range, meeting all FDA/EMA criterion for the key biomarker parameters precision and accuracy, selectivity, dilution linearity, hook effect, stability and last but not least parallelism.