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Development of a Sensitive Sandwich ELISA Method for the Determination of Aggrecan Fragments in Human Serum for Biomarker Analysis

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Introduction

In the context of arthritis and joint injury, aggrecan is primarily proteolyzed by matrix metalloproteases and aggrecanases (specifically ADAMTS-4 and ADAMTS-5, which are disintegrins and metalloproteinases with thrombospondin motifs). The release of aggrecan fragments from cartilage can be quantified in synovial fluid, serum, and urine, as well as in cartilage explant cultures, serving as a diagnostic and prognostic biomarker for osteoarthritis. An enzyme-linked immunosorbent assay (ELISA) has been developed for the quantification of specific aggrecan fragments generated by ADAMTS-4-mediated cleavage at the 373Glu-374Ala bond within the aggrecan interglobular domain.

Methods

The ELISA employs a commercially available ARGSV1-G2 monoclonal antibody (aggrecan fragments, recognizing the G2 domain of aggrecan) to capture ARGSV1-G2. Standard stocks of ARGSV1-G2 (aggrecan fragments) were prepared by digesting aggrecan with ADAMTS-4 enzyme. A biotin-labeled anti-ARGSV mAb was used as the detection antibody to recognize the N-terminal 'ARGSVIL' sequence of ARGSV1-G2 (ARGS).

As the presence of glycosylated side chains in natural ARGS in human serum samples affects the precision and accuracy of the assay, serum samples were subjected to a pre-treatment process involving deglycosylation. This was performed by using a saline dilution buffer containing 0.03 mU chondroitinase ABC (C2905, Sigma) and protease (K0069, TCI) per milliliter at a ratio of 1:1.5 for a period of 3 hours at 37 ° C.

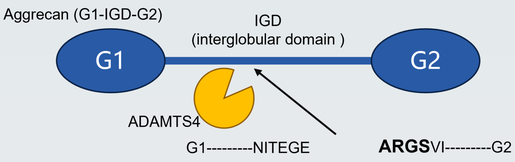

Figure 1. structure of aggrecan G1-G2

Results

Standard Curve, Accuracy and Precision

The hydrolyzed ARGS-Aggrecan standards can be stored at -20° C for a minimum of 2 months and subjected to at least 3 freeze-thaw cycles (data not shown). The quantitative range of this method was from 50 pM/L to 800 pM/L, with satisfactory linearity and reproducibility (Table 1). The assay demonstrated satisfactory precision, with a range of 5.5% to 15.5% (Table 2). Accuracy, expressed as a percentage bias, ranged from -18.6% to 5.4%.

Table 1. The Standard Curves Summary of Analytical Runs

Run Number	Test Result for STD of ARGS (pM/L)						
	800.0	650.0	400.0	300.0	150.0	100.0	50.0
1	834.2	588.2	447.5	320.2	137.5	87.0	59.8
2	817.6	651.1	371.5	332.9	151.1	86.6	58.6
3	776.8	668.1	400.8	297.7	147.8	107.8	59.8
Inter-run Mean	809.5	635.8	406.6	316.9	145.5	93.8	59.4
Inter-run SD	29.5	42.1	38.3	17.8	7.1	12.1	0.7
Inter-run %CV	3.6	6.6	9.4	5.6	4.9	12.9	1.2
Inter-run %Bias	1.2	-2.2	1.7	5.6	-3.0	-6.2	18.8
n	3	3	3	3	3	3	3

Table 2. Accuracy and Precision Results for QCs

Item	ULOQ (800.0 pM/L)	HQC (620.0 pM/L)	MQC (250.0 pM/L)	LQC (75.0 pM/L)	LLOQ (50.0 pM/L)
Concentration	548.1	640.2	243.3	66.4	50.7
	656.5	582.8	192.0	60.0	56.1
	749.3	644.6	246.0	66.7	51.3
Intra-run Mean	651.3	622.5	227.1	64.4	52.7
Intra-run SD	100.7	34.5	30.4	3.8	3.0
Intra-run %CV	15.5	5.5	13.4	5.9	5.6
Intra-run %Bias	-18.6	0.4	-9.2	-14.1	5.4
n	3	3	3	3	3

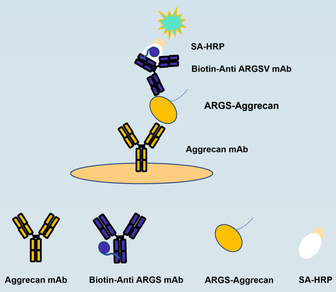

Figure 2. Biomarker assay for detect ARGS

Parallelism

Parallelism is the primary reference for determining the minimum required dilution (MRD) of the assay. Four individual serum samples were diluted with sample diluent at different factors. The MRD of the assay was determined to be 4.5 (Table 3).

Table 3. Summary for Parallelism

Sample	Dilution Factor	Result (pM/L)	Recovery%	Recovery%	Recovery%
Individual 1	1.5	353.9	100.0%	NA	NA
	3.0	333.4	94.2%	100.0	NA
	4.5	379.3	107.2%	113.8	100.0
	6.1	455.9	128.8%	136.7	120.2
Individual 2	1.5	535.4	100.0%	NA	NA
	3.0	530.9	99.2%	100.0	NA
	4.5	602.6	112.6%	113.5	100.0
	6.1	687.0	128.3%	129.4	114.0
Individual 3	1.5	204.9	100.0%	NA	NA
	3.0	314.3	133.7%	100.0	NA
	4.5	420.5	178.9%	122.8	100.0
	6.1	531.5	226.1%	155.3	125.6
	9.0	490.2	208.6%	143.2	115.9
Individual 4	1.5	235.1	100.0%	NA	NA
	3.0	342.3	145.6%	100.0	NA
	4.5	423.1	180.0%	123.6	100.0
	6.1	527.2	224.3%	154.0	124.6
	9.0	493.5	210.0%	144.2	116.6

Specificity

The specificity of the ARGS assay was evaluated by testing the effect of varying concentrations of full-length aggrecan on the assay at the HQC and LQC levels. The tolerable concentration of full-length aggrecan was determined to be 300 pM/L at the HQC level , and 400 pM/L at the LQC level (Table 4).

Sample	ARGS Concentration (pM/L)	Aggrecan Conc. (pM/L)	Result (pM/L)	Recovery %
SPE-L-01	75.0	1000.0	NA	NA
SPE-L-02	75.0	800.0	NA	NA
SPE-L-03	75.0	400.0	72.7	97.0
SPE-L-04	75.0	300.0	82.2	109.6
SPE-H-01	620.0	1000.0	285.2	46.0*
SPE-H-02	620.0	800.0	270.7	43.7*
SPE-H-03	620.0	400.0	391.2	63.1*
SPE-H-04	620.0	300.0	449.6	72.5

Note: *, Failure of bias for HQC at this Aggrecan concentration.

Stability

Standard curves and QCs were prepared freshly on the day of analysis for all stability sample assessments.

Serum with a high endogenous ARGS concentration was used as the stability sample. Stability results showed that %recovery was within the range of 70.0-130.0% when samples were stored at room temperature for up to 4 h, at -80° C and 2-8° C for up to 24 h, and in the context of freeze/thaw cycles at -80° C up to 3 times.

Sample	Storage Condition	Dilution Factor	Detection Result (pM/L)	Recovery%	Results
Individual 1	-80℃-24h	3.9	314.1	100.0%	Pass
	RT-4h	3.9	313.0	99.6%	
	4℃-24h	3.9	288.7	91.9%	
	-80℃-3F/T	3.9	284.2	90.5%	
Individual 2	-80℃-24h	3.9	253.8	100.0%	Pass
	RT-4h	3.9	267.9	105.6%	
	4℃-24h	3.9	259.1	102.1%	
	-80℃-3F/T	3.9	270.6	106.6%	
Individual 3	-80℃-24h	3.9	402.8	100.0%	Pass
	RT-4h	3.9	332.1	82.4%	
	4℃-24h	3.9	313.8	77.9%	
	-80℃-3F/T	3.9	313.4	77.8%	
Individual 4	-80℃-24h	3.9	791.2	100.0%	Pass
	RT-4h	3.9	745.9	94.3%	
	4℃-24h	3.9	761.2	96.2%	
	-80℃-3F/T	3.9	720.6	91.1%	

Note: The -80° C 24h stability sample results were used as a baseline for calculations.

Conclusion

We have successfully developed an ELISA-based assay for the quantification of specific aggrecan fragments generated by aggrecanase-mediated cleavage. The assay met the established criteria for accuracy and precision, linear range, parallelism, specificity, and short-term stability.