Day1-P22

Development and Validation of a Novel ECL Method for the Quantitation of Complement C3a/C3a desArg in Human Plasma

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NOVEL ASPECT

A novel ECL method was successfully developed and validated to quantitatively measure complement C3a/C3a desArg level in human K2 EDTA plasma.

INTRODUCTION

Complement system contains more than 30 proteins in plasma and on cell surfaces at high endogenous level. It plays crucial roles in both innate and adaptive immune responses (Dunkelberger JR, Song WC., 2010). Complements act through proteolytic cascade pathways and produce enzymatically active molecules and biologic effectors, including anaphylatoxin C3a (Bajic G, et al. 2013). The activity of C3a is inactivated rapidly in plasma within a few minutes after formation through removal of the C-terminal arginine by carboxypeptidases (Law S.K.A. and Reid K.B.M., 1995). Accurate measurement of C3a/C3a desArg in plasma can be challenging due to their high abundancy and the heat sensitive nature of complements, such as heat induced conversion of C3 to C3a and C3a desArg (Seya T, Nagasawa S., 1988). A novel electrochemiluminescence (ECL) immunoassay method was developed to overcome the technique hurdles and successfully validated for quantitatively measuring C3a/C3a desArg in human plasma samples.

METHOD

ECL platform was chosen to develop a sandwich immunoassay for quantitative measurement of C3a/C3a desArg in human K2 EDTA plasma (Figure 1A). A biotinylated anti-human C3a/C3a desArg capture antibody binds to a streptavidin coated assay plate. C3a/C3a desArg is captured from human K2 EDTA plasma samples and detected by a SULFO-TAG conjugated anti-human C3a/C3a desArg detection antibody. A native human C3a desArg protein, purified from human serum after C3 convertase activation to convert C3 to C3a and followed by natural carboxypeptidase N treatment to remove the C-terminal arginine, is used as reference standard. Due to the high endogenous level of C3a/C3a desArg in human plasma, K2 EDTA plasma samples are diluted extensively in assay buffer before analysis. Therefore, assay buffer was used as surrogate matrix. Reference standard is spiked into surrogate matrix to generate the standard curve and quality controls, including Upper Limit of Quantification (ULOQ), high/middle/low level QCs (HQC, MQC and LQC) and Lower Limit of Quantification (LLOQ). Gender balanced normal human K2 EDTA plasma samples are mixed to produce a normal base pool (NBP) and used as dilution quality control (DQC) to monitor assay performance when testing human K2 EDTA plasma samples.

RESULT(S)

A novel ECL method was successfully developed and validated to quantitatively determine C3a/C3a desArg level in human K2 EDTA plasma samples. Figure 1B shows a representative standard curve with broad linear dynamic range. The validated quantitative range of the curve is from 16.38 pg/mL (LLOQ) to 4000 pg/mL (ULOQ). The specificity of the assay was demonstrated with the potential interference molecule, native human complement C3 protein, at reported native ratio of C3 vs C3a/C3a desArg at 2000:1 (Hubens WHG, et al. 2021) or our observed ratio at 100:1. C3 protein is measured below LLOQ when spiked at 2000-fold or 100-fold of MQC level in surrogate matrix. Additionally, co-existence of C3 protein doesn't impact the accuracy of C3a/C3a desArg measurement (Table 1). Therefore, the assay is highly specific for the detection of C3a/C3a desArg. The assay is selective when assaying normal K2 EDTA plasma samples. Among 10 tested samples, 100% samples spiked at LLOQ level and 80% spiked at HQC level are within ±20% Relative Error (RE) (Table 2). Furthermore, hemolytic (Table 3) and lipemic (Table 4) conditions don't interfere with assay performance. 80% Hemolytic samples, normal K2 EDTA plasma samples spiked in 4 mg/mL Hemoglobin, are within ±30 %RE criteria from unspiked nominal samples. And 100% 1 mg/mL Intralipid spiked K2 EDTA plasma samples are within ±15 %RE from unspiked nominal samples. The matrix effect to the assay was further investigated through parallelism assessment. All six K2 EDTA normal plasma samples that generate reportable results within validated quantitative range show parallelism to standard curve with less than 15% RE (Table 5). The sample dilution factor 1:5,000 is within parallelism range of all samples. Lastly, the quantitation of C3a/C3a desArg can tolerate Cmax levels of three different therapeutic drugs (Table 6). From above assessments, the assay is demonstrated to specifically and selectively detect C3a/C3a desArg in K2 EDTA human plasma samples. Accuracy and precision of the assay were determined with quality controls at five different levels (LLOQ, LQC, MQC, HQC and ULOQ) and with DQC (Table 7). All assessed QC samples show within 20% inter-assay precision (%CV) and within ±15% inter-assay accuracy (%RE) to nominal concentrations. The total error (%TE) of all samples are within 20%, except for LLOQ shows 27.1 %TE. As shown in Table 8, stability samples are stable at 2-8 °C for up to 16 hours at LQC level and for up to 24 hours with DQC and at HQC level. LQC and DQC samples can be stored at ambient room temperature (15 – 30 °C) for up to 2 hours, and HQC sample can be stored for up to 4 hours. All samples (LQC, HQC and DQC) tolerate 5 times freeze/thaw cycles. The long-term stability (LTS) assessment is ongoing. All assessed LTS samples are stable for at least 6 months (Data not shown).

Table 1A. Specificity Results (Reference Samples) at MQC Level

Sample	Nominal Concentration (pg/mL)	Mean Response (ECLU)	Concentration (pg/mL)	Accuracy (%RE)
C3a desArg	250.00	2,096	224.19	-10.3
C3a	250.00	2,867	305.79	22.3

Table 1B. Specificity Results (C3:C3a/desArg)=2000:1) at MQC Level

Sample	Nominal Concentration (pg/mL)	Mean Response (ECLU)	Concentration (pg/mL)	Accuracy (%RE)
C3a desArg + C3	250.00	2,322	248.19	-0.7
C3a + C3	305.79	3,489	371.14	21.4
C3a desArg+ C3a + C3	555.79	6,361	668.56	20.3
Sample ID	**LLOQ (pg/mL)**	**Mean Response (ECLU)**	**Concentration (pg/mL)**	**Below LLOQ**
C3	16.38	98	5.23	YES

Table 1C. Specificity Results (C3:C3a/desArg)=100:1) at MQC Level

Sample	Nominal Concentration (pg/mL)	Mean Response (ECLU)	Concentration (pg/mL)	Accuracy (%RE)
C3a desArg + C3	250.00	2,104	225.04	-10.0
C3a + C3	305.79	2,866	305.73	0.0
C3a desArg+ C3a + C3	555.79	5,990	630.39	13.4
Sample ID	**LLOQ (pg/mL)**	**Mean Response (ECLU)**	**Concentration (pg/mL)**	**Below LLOQ**
C3	16.38	64	1.12	YES

Table 2A. Normal Human Plasma Selectivity Results

Sample	Unspiked Samples			LLOQ Spiked Samples		
	Mean Response (ECLU)	PreMRD Concentration (pg/mL)	Corrected Nominal Concentration (pg/mL)	Mean Response (ECLU)	PreMRD Concentration (pg/mL)	Accuracy (%RE)
Individual 1	964	140.58	156.96	1,283	187.02	19.2
Individual 2	2,374	338.93	355.31	2,489	354.59	-0.2
Individual 3	4,732	654.90	671.28	4,384	608.63	-9.3
Individual 4	5,524	760.21	776.59	6,304	864.15	11.3
Individual 5	3,088	408.98	425.36	3,410	449.94	5.8
Individual 6	1,527	205.57	221.95	1,473	198.34	-10.6
Individual 7	15,686	1,913.24	1,929.62	15,133	1,849.74	-4.1
Individual 8	33,673	3,922.05	3,938.43	34,154	3,974.54	0.9
Individual 9	7,067	901.31	917.69	7,222	920.03	0.3
Individual 10	716	94.52	110.90	825	109.82	-1.0

Table 2B. Normal Human Plasma Selectivity Results

Sample	Unspiked Samples			HQC Spiked Samples		
	Mean Response (ECLU)	PreMRD Concentration (pg/mL)	Corrected Nominal Concentration (pg/mL)	Mean Response (ECLU)	PreMRD Concentration (pg/mL)	Accuracy (%RE)
Individual 1	1,295	147.14	3,147.14	*	*	*
Individual 2	4,710	513.87	3,513.87	37,822	3,632.69	3.4
Individual 3	6,837	731.11	3,731.11	*	*	*
Individual 4	8,351	882.79	3,882.79	40,252	3,850.17	-0.8
Individual 5	3,088	408.98	3,408.98	26,405	3,121.61	-8.4
Individual 6	1,527	205.57	3,205.57	21,439	2,566.78	-19.9
Individual 7	15,686	1,913.24	4,913.24	38,956	4,496.75	-8.5
Individual 8	33,673	3,922.05	6,922.05	59,389	6,677.91	-3.5
Individual 9	7,067	901.31	3,901.31	27,951	3,292.99	-15.6
Individual 10	716	94.52	3,094.52	21,095	2,528.06	-18.3

* Sample has been tested in three runs, only one run passed and considered fail.

Table 3. Normal Hemolytic Human Plasma Selectivity Results

Sample	Unspiked Samples		Spiked Samples		
	Mean Response (ECLU)	PreMRD Concentration (pg/mL)	Mean Response (ECLU)	PreMRD Concentration (pg/mL)	Accuracy (%RE)
Individual 1	1,031	911,183.16	941	828,742.29	-9.0
Individual 2	2,011	1,780,126.82	1,359	1,207,170.90	***-32.2***
Individual 3	2,856	2,504,337.25	2,465	2,171,160.27	-13.3
Individual 4	12,333	10,137,512.82	9,053	7,545,712.64	-25.6
Individual 5	4,108	3,551,529.88	3,707	3,218,264.61	-9.4

High %RE (not within ±30 %RE criteria)

Table 4. Normal Lipemic Human Plasma Selectivity Results

Sample	Unspiked Samples		Spiked Samples		
	Mean Response (ECLU)	PreMRD Concentration (pg/mL)	Mean Response (ECLU)	PreMRD Concentration (pg/mL)	Accuracy (%RE)
Individual 1	1,031	911,183.16	1,101	974,903.59	7.0
Individual 2	2,011	1,780,126.82	2,015	1,784,031.27	0.2
Individual 3	2,856	2,504,337.25	2,822	2,475,107.14	-1.2
Individual 4	12,333	10,137,512.82	10,594	8,766,777.51	-13.5
Individual 5	4,108	3,551,529.88	4,025	3,482,392.19	-1.9

Table 5. Normal Human Plasma Parallelism Results Summary

Sample	%RE ± 30%	
	Min Dilution	Max Dilution
Sample 1	1:5,000	1:100,000
Sample 2	1:500	1:50,000
Sample 3	1:500	1:50,000
Sample 4	1:5,000	1:100,000
Sample 5	1:5,000	1:100,000
Sample 6	1:5,000	1:100,000

Table 6. Drug Tolerance in Human Plasma Results

Drug	Sample	Norminal Concentration (pg/mL)	PreMRD Concentration (pg/mL)	Accuracy (%RE)
Drug 1	Drug 1 Cmax	7,844,210.06	7,370,784.51	-6.0
	Drug 1 1/2 Cmax		8,340,270.54	6.3
	Drug 1 1/4 Cmax		8,830,971.63	12.6
	Drug 1 1/8 Cmax		7,289,818.82	-7.1
	Drug 1 1/16 Cmax		7,492,869.19	-4.5
Drug 2	Drug 2 Cmax	7,147,027.21	6,926,103.54	-3.1
	Drug 2 1/2 Cmax		7,245,901.78	1.4
	Drug 2 1/4 Cmax		6,799,237.83	-4.9
	Drug 2 1/8 Cmax		6,406,370.11	-10.4
	Drug 2 1/16 Cmax		6,563,574.28	-8.2
Drug 3	Drug 3 Cmax	8,509,616.01	6,917,174.14	-18.7
	Drug 3 1/2 Cmax		7,617,787.93	-10.5
	Drug 3 1/4 Cmax		8,840,937.95	3.9
	Drug 3 1/8 Cmax		6,721,436.95	-21.0
	Drug 3 1/16 Cmax		7,645,004.29	-10.2

Table 7. Accuracy and Precision Summary

Control	Nominal Concentration (pg/mL)	n	Mean Concentration (pg/mL)	StdDev (pg/mL)	Inter-Assay Precision (%CV)	Inter-Assay Accuracy [(a)] (%RE)	Total Error [(b)] (%TE)
LLOQ	16.38	18	18.22	2.60	15.9	11.2	27.1
LQC	40.00	18	44.09	3.15	7.9	10.2	18.1
MQC	250.00	18	251.54	25.91	10.4	0.6	11.0
HQC	3,000.00	18	2,936.10	230.95	7.7	-2.1	9.8
ULOQ	4,000.00	18	3,999.37	426.60	10.7	0.0	10.7
DQC	5,798,800.00	18	5,798,795.77	764,375.17	13.2	0.0	13.2

[(a)] %RE: Bias compared to nominal concentration.

[(b)] Total Error = |%RE| + %CV.

Table 8. Stability Results Summary

Short-term Stability Results at 2 to 8°C in MSD Diluent 58 (LQC & HQC) or Human Plasma (DQC)

Sample	Nominal Concentration (pg/mL)	Condition	Observed Concentration (pg/mL)			n	Mean (pg/mL)	Standard Deviation (pg/mL)	Precision (%CV)	Accuracy Result 1 (%RE)	Accuracy Result 2 (%RE)	Accuracy Result 3 (%RE)
			Result 1	Result 2	Result 3							
LQC	40.00	16 hours	38.74	40.31	41.56	3	40.20	1.41	3.5	-3.2	0.8	3.9
HQC	3,000.00	24 hours	2,926.11	3,255.69	2,835.49	3	3,005.76	221.13	7.4	-2.5	8.5	-5.5
DQC	5,798,800.00	24 hours	8,755,721.56	7,519,448.54	7,330,372.06	3	7,868,514.05	774,138.46	9.8	51.0	29.7	26.4

Short-term Stability Results at 15 to 30°C in MSD Diluent 58 (LQC & HQC) or Human Plasma (DQC)

Sample	Nominal Concentration (pg/mL)	Condition	Observed Concentration (pg/mL)			n	Mean (pg/mL)	Standard Deviation (pg/mL)	Precision (%CV)	Accuracy Result 1 (%RE)	Accuracy Result 2 (%RE)	Accuracy Result 3 (%RE)
			Result 1	Result 2	Result 3							
LQC	40.00	2 hours	38.74	34.65	40.70	3	38.03	3.09	8.1	-3.2	-13.4	1.8
HQC	3,000.00	4 hours	3,193.75	3,173.51	3,203.18	3	3,190.15	15.16	0.5	6.5	5.8	6.8
DQC	5,798,800.00	2 hours	5,551,669.41	5,522,362.89	5,027,909.61	3	5,367,313.97	294,297.82	5.5	-4.3	-4.8	-13.3

Freeze-thaw Stability Results in MSD Diluent 58 (LQC & HQC) or Human Plasma (DQC)

Sample	Nominal Concentration (pg/mL)	Condition	Observed Concentration (pg/mL)			n	Mean (pg/mL)	Standard Deviation (pg/mL)	Precision (%CV)	Accuracy Result 1 (%RE)	Accuracy Result 2 (%RE)	Accuracy Result 3 (%RE)
			Result 1	Result 2	Result 3							
LQC	40.00	5X F/T	37.31	45.83	42.18	3	41.77	4.27	10.2	-14.6	-12.3	-5.1
HQC	3,000.00	5X F/T	3,022.74	3,258.29	3,255.40	3	3,178.81	135.17	4.3	-9.9	-4.4	-1.7
DQC	5,798,800.00	5X F/T	6,836,276.49	6,749,162.07	6,888,161.30	3	6,824,533.29	70,239.76	1.0	-4.4	-6.4	-11.8

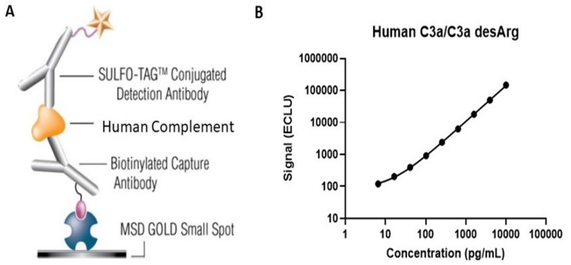

Figure 1: (A) Schematic of the sandwich ECL immunoassay. (B) A representative reference standard curve was fitted with a 4-parameter logistic regression model with a weighting factor of $1/Y^2$.

CONCLUSION

A Novel ECL method was successfully developed and validated for specifically and accurately quantify C3a/C3a desArg level in human K2 EDTA plasma samples. The method is highly sensitive and demonstrates consistent performance within a wide dynamic range. The method can be reliably applied for complement studies, with the potential to be adapted for quantitatively measuring C3a/C3a desArg in other human body fluids.

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