Fit-for-Purpose Validation of Alpha-1-Acid Glycoprotein ELISA for Quantification In Human Plasma: Substitution of Kit Calibrators May Be Challenging

Deborah Martin, Rachel Williams, Michael Paton, and Masood Khan - KCAS, Shawnee, Kansas 66216 USA

OBJECTIVE

Alpha-1-Acid Glycoprotein (AGP) is an acute phase protein. It is a potential biomarker of systemic tissue injury and inflammatory disorders. AGP also non-specifically binds with a variety of circulating drug molecules, affecting their PK/PD patterns. While working with an AGP ELISA kit we observed that contrary to popular belief, substitution of kit calibrators with bulk material for the sake of long term consistency may not necessarily be useful.

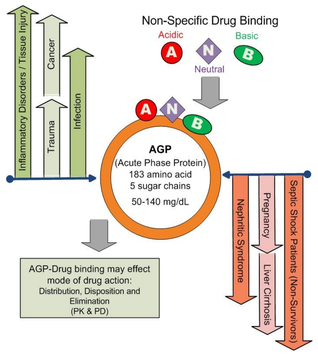
INTRODUCTION

AGP is synthesized in the liver and released into the blood during inflammatory events. Increased plasma and urine levels of AGP can predict an increased risk of heart disease and chronic kidney disease, along with being a biomarker for alcoholism and acute inflammation. Decreased levels of plasma AGP in severe septic shock patients can be used as a prognostic indicator of patient mortality. In view of the association of AGP levels with various disease states, AGP can be used as an easy to measure biomarker in clinical settings and drug development programs.

METHOD

- **Method A**: A commercial ELISA kit for quantification of AGP in human plasma (K_2EDTA) was run with substituted calibrators. Five levels of qualification samples (QS) spiked in PBST-BSA, and 3 levels of endogenous AGP in normal human plasma were used to establish and validate the assay performance characteristics.
- **Method B**: The same commercial ELISA kit for the quantification of AGP was run with kit calibrators. Five levels of qualification samples (QS) spiked in assay diluents were used to establish dynamic range of quantification and precision and accuracy of measurements.

Fig. 1: AGP : Drug Binding Protein & Potential Biomarker



RESULTS

Fig. 2: Precision Profile and Curve Fit Algorithm (Method A)

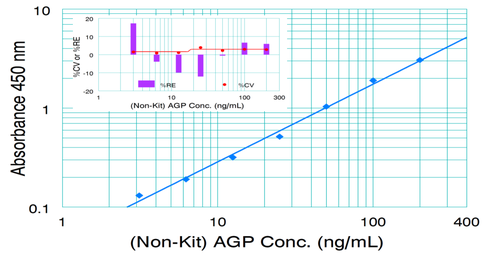


Table 1: Precision & Accuracy (Method A)

Parameters	Statistics	Nominal Concentrations (ng/mL)				
		3.5	7	28	70	200
		LLOQ	Low	Medium	High	ULOQ
# of Observations	# Results	12	12	12	12	12
	# Runs	4	4	4	4	4
Accuracy	Mean	3.9	6.9	30.0	76.8	224.4
	Mean Bias (%RE)	11.3	-1.8	7.3	9.7	12.2
Precision	Intra-Assay (%CV)	≤ 4.8	≤ 3.7	≤ 10.3	≤ 4.9	≤ 3.7
	Inter-Assay (%CV)	11.3	7.3	8.6	7.3	4.7
Total Error	%CV+%RE	22.6	9.1	15.9	17.0	16.9

All five levels of QS spiked with AGP were within the acceptance criteria of ≤ 20% CV and %RE for all levels except the LLOQ, which had an acceptance criteria of ≤ 25% CV and %RE.

Table 2: Method Selectivity

Plasma Sample	Measured Conc. (µg/mL)			%AR
	Unspiked	Spiked		
	Measured	Theoretical	Measured	
1	1,028.7	1,313.1	1,321.1	100.6
2	1,027.0	1,311.5	1,501.7	114.5
3	296.2	580.7	1,701.9	293.1
4	482.7	767.1	948.8	123.7
5	451.1	735.6	893.3	121.4
6	621.7	906.1	922.4	101.8
7	516.4	800.9	1,009.1	126.0
8	369.4	717.3	913.9	127.4
9	767.0	1,114.9	1,288.6	115.6
10	656.3	1,004.2	1,255.1	125.0
Mean Conc. in Buffer	925.1			

$$\%AR = \frac{\text{Spiked Measured Concentration}}{\text{Spiked Theoritical Concentration}} \times 100$$

% AR in 70% of samples ranged from 100.6 to 125.0%

RESULTS (CONT.)

Table 3: Dilutional Linearity

Dilution Factor	AGP Conc. (µg/mL)		%AR
	Measured	Dilution Adjusted	(% Nominal)
5000	75.8	378.8	87.9
10000	51.4	514.1	119.3
20000	22.6	452.5	105.0
40000	8.6	344.4	79.9
80000	3.5	279.9	64.9
Mean		393.9	91.4
n		5	5
SD		91.58	21.25
CV%		23.2	23.2

Nominal Conc. (µg/mL) = 431.0

Samples diluted between 5000 to 40000-fold dilutions ranged from 79.9 to 119.3 %AR.

Table 4: AGP Stability in Human Plasma

Stability	Control-1 (Low)			Control-2 (High)			Result
	% Nom.	n	%CV	% Nom.	n	%CV	
Bench-Top (15.5 hrs)	96.4	3	9.4	98.0	3	3.0	Pass
Refrigerator (50 hrs)	103.4	3	7.9	97.0	3	1.2	Pass
Freeze/Thaw (3 X)	96.2	3	4.6	90.2	3	5.8	Pass

Short-term stability of AGP in Human Plasma was established.

Fig 3: Precision Profile and Curve Fit Algorithm (Method B)

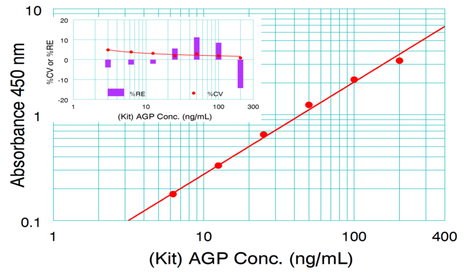


Table 5: Precision & Accuracy (Method B)

Parameters	Statistics	Nominal Concentrations (ng/mL)				
		3.5	7	28	70	200
		LLOQ	Low	Medium	High	ULOQ
# of Observations	# Results	9	9	9	9	9
	# Runs	3	3	3	3	3
Accuracy	Mean	3.2	6.6	29.4	77.4	176.5
	Mean Bias (%RE)	-5.7	-6.3	4.8	10.6	-11.7
Precision	Intra-Assay (%CV)	≤ 4.1	≤ 2.2	≤ 4.7	≤ 6.1	≤ 3.9
	Inter-Assay (%CV)	3.7	3.3	4.5	5.7	4.5
Total Error	%CV+%RE	9.5	9.6	9.3	16.3	16.2

Like the Method A, all five levels of QS spiked with AGP met the acceptance criteria described in Table 1.

RESULTS (CONT.)

Table 6: Precision of Measurements in Plasma Controls (Method B)

Plasma Controls	No. of Runs	No. of Results	Conc. (µg/mL)	Precision (%CV)	
				Intra-Assay	Inter-Assay
Control-1	3	9	231.2	≤ 3.8	3.6
Control-2	3	9	568.7	≤ 4.5	4.3
Control-3	3	9	855.1	≤ 3.2	3.6

- Plasma control samples showed excellent Inter- and Intra-assay precision of measurements.
- Concentrations determined in plasma control samples can be used as nominal concentration when these samples are used for stability evaluation experiments.

Table 7: Pros and Cons of Using Non-Kit and Kit Reference Material

	Non-Kit Reference Material	Kit Reference Material
Preparation	Lyophilized bulk material provided with approximate amount per vial	Pre-weighed lyophilized material is provided
	Reconstitute, dilute an aliquot and measure OD at 280 nm (UV)	Add appropriate volume of diluents to reconstitute
	Compute Concentration using Extinction Coefficient	Easy to compute concentration
	Prepare calibrators and freeze small aliquots	Fresh calibrators are prepared just before use
Lot-to-Lot Variability	One lot can be used for a long period of time	Over time the lot of reference standard may change
		Lot-to-lot variability needs to be monitored using a defined process
CoA	CoA with stability provided	Available on request
Availability	Manufacturer may discontinue the material or change specifications	Material would be available as long as kit is offered

SUMMARY and CONCLUSION

An ELISA kit for quantification of AGP was initially qualified using bulk reference material from a non-kit manufacturer (Method A). Later, consistent preparation of calibrators using this bulk material became difficult (material was discontinued). Consequently, method performance was successfully re-qualified using the AGP provided in the kits (Method B).

Based on our experience the following is recommended:

- Preferably secure the bulk reference material from the kit manufacturer. This will assure availability of material for the life cycle of kit.
- If non-kit material is selected it should only be obtained from a well established manufacturer that can provide assurances of product availability.

REFERENCES

- Khan, M., Adaptation of fit-for-purpose biomarker assay validation using commercial kits: A CRO perspective. Drug Research, 11/12: 33-34, (2010).
- Barroso-Sousa R *et.al.* Decreased levels of alpha-1-acid glycoprotein are related to the mortality of septic patients in the emergency department. Clinics 68 (8):1134-1139, (2013).
- Huang Z, Ung T. Effect of Alpha-1-Acid Glycoprotein Binding on Pharmacokinetics and Pharmacodynamic. *In* Current Drug Metabolism (Bentham Science Publishers) 14 (2), pp. 226-238 (2013).