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A Simple, Effective and Inexpensive Method To Estimate IgA Content In Human Immunoglobulin For Intravenous Use Preparations Based On Elisa Technique

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ABSTRACT
Quality Control of plasma derived products is utmost priority these days due to their increased use for therapeutic purpose. Among those, Human Immunoglobulins for Intravenous Use is one and its use has expanded significantly during the pandemic times. Every test parameter described for quality control evaluation of a product carry its significance in judging that product's quality. The aim of the study is to develop a simple, effective and inexpensive method for determination of IgA content in these preparations by ELISA technique, using WHO International Standard (IRS) (67/086) (IgA unitage of 0.8147 mg/ml) and reagents from Human IgA ELISA kit, Immunology Consultants Laboratory, Inc. (ICL) and Cygnus Technologies, USA. Both the kit reagents are verified by observing Linearity, Accuracy and Precision with reference to IRS at a concentration of 50ng/ml as a control sample. The %RSD observed for both procedures are 2.80% (ICL) & 3.57% (Cygnus Technologies). More than 25 samples of Human Immunoglobulins for Intravenous use preparations from different manufacturers tested using these reagents during this study. The results found within pharmacopoeia described specifications for all samples by both reagents. The results obtained for control sample using reagents from both sources analysed using GraphPad Prism software and found statistically not significant. The method described will help testing laboratories and manufactures for establishing this test in their laboratories. The results obtained assures that the methods are simple, effective and inexpensive. Based on this study, the method can also be recommended for pharmacopeia monograph, Human Immunoglobulin for Intravenous use preparations.

Key Words: *Human Immunoglobulin for intravenous use, WHO/NIBSC Reference Standard, IgA, ELISA, Quality Control.*

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I. INTRODUCTION

The market for plasma derived Human Immunoglobulin for intravenous use preparations (IVIG) is increasing significantly and the number of clinical conditions like autoimmune, neurological, immunodeficiency and various infections treated with Intravenous Immunoglobulin (IVIG) are expanding time to time. Consequently the need for Quality Controlled IVIG is remarkably in demand. There are 5 classes (M, D, G, A, E) of Immunoglobulins and are further classified as subclasses (IgG1–4, IgA1, 2). These Immunoglobulins play major contribution for the immune defence, and failure to produce certain antibodies results in susceptibility to infections [1–7]. Immunoglobulin deficiencies are classified as primary (congenital) and secondary (acquired), and they in due course of time resulting as immunodeficiency syndromes, most commonly a deficiency in IgG or IgA subclasses [1, 7–12]. In addition to Immunoglobulin deficiencies, many individuals face difficulties in producing antibodies against specific infectious agents [1, 13]. Purified preparations of plasma-derived antibodies/Immunoglobulins are used to treat conditions like Immunoglobulin deficiencies and it is also reported that administration of which have also been found to modulate some autoimmune diseases [1, 14–20].

The first process for Immunoglobulin isolation from human plasma was devised by Cohn et al. [21]. This method used graduated ethanol fractionation, and was later modified by Oncley [22] and Kistler and Nitschmann [23]. Various procedures are in practice for manufacturing of Human Immunoglobulins from human plasma (like enzymatic and/or chemical treatment as well as chromatographic techniques) [24–28]. Selection of donors, separation procedures and processes for inactivation or elimination of enveloped and non-enveloped viruses are of utmost important parameters in manufacturing of quality and safety plasma derived products. Manufacturing

process and product composition (like protein content, etc.) slightly differ for different Immunoglobulin preparations depending upon the route of administration i.e. for subcutaneous or intramuscular (SC/IMIG) and intravenous (IVIG) [24, 25]. A pool of plasma from minimum 1,000 healthy donors are required for manufacturing of Immunoglobulins and the plasma used for manufacturing should comply with the requirements of the monograph on *Plasma for Fractionation* [35]. Thus prepared product must be safe for patients in terms of transmission of infections and should be of protein concentration of 50–120 g/l (IVIG) or 160 g/l and 165 g/l (SCIG), along with a defined antiviral and antibacterial antibodies at a concentration at least three fold (IVIG) or ten-fold (SCIG) above that of the starting material [1, 24, 26, 35]. In addition, IVIG preparations should have a defined distribution of Immunoglobulin G (IgG) subclasses as well as display Fc functions of native Immunoglobulins [35]. The sum of the monomeric and dimeric IgG molecules should not be less than 90% and the total of polymers and aggregates should not be more than 3% [1, 24, 35].

Intravenous immunoglobulin (IVIG) preparations contain ≥ 95% IgG with a plasma like IgG subclass distribution and only traces of IgA and IgM [24, 29–31]. IVIGs in low doses are used for immunoglobulin deficiency (to support host immune response) or used in high doses (as immune modulator) for treatment of various clinical conditions of autoimmune and inflammatory disorders [24, 29–33]. The manufacturing guidelines and quality control criteria are set by the European/British/Indian Pharmacopeia for various Immunoglobulin preparations (SCIG, IMIG and IVIG). Immunoglobulins currently available in the market contain more than 90% monomeric IgG1–4 and only insignificant amounts of IgM and IgA molecules. These IVIG preparations with very low IgA concentration are also used in patients with manifest clinically relevant antibodies against IgA molecules [24, 34].

Quality Control of these preparations is very important in the interest of public health. Product specific monographs are available in Pharmacopoeia with details of tests to be performed on finished product along with acceptance criteria. Determination of IgA content in final product of IVIG is one of tests for quality control or batch release testing with pharmacopoeia specifications being "*should not be more than the maximum content stated on the label*" [35]. The aim of the present study was to develop a simple, effective and inexpensive method to estimate IgA content in IVIG based on ELISA technique. In this study we described a method to estimate IgA content in IGIV samples using international reference preparation (WHO/NIBSC-67/086) [36] and reagents from commercially available kits [37,38]. Reagents are used from Human IgA ELISA kit, Immunology Consultants Laboratory, Inc. (ICL), Portland, USA [37] and Immunoenzymetric assay for the measurement of Human Immunoglobulin A kit, Cygnus Technologies, USA [38]. The procedure is followed as per the instructions given by kit manufacturers for preparation of reagents, test performance and analysis of results. About 25 different IVIG samples using reagents from ICL and 25 samples using Cygnus Technologies, were tested. Results for all the samples found within acceptance criteria/ pharmacopoeia specifications [35]. The left over samples of batches received for testing in the laboratory are used in this study. The methods described in this report are verified by studying Linearity, Accuracy and precision. The % Accuracy and % Error are calculated using a control sample at 50ng/ml concentration in six performances. The correlation coefficient obtained in all performances is >0.99 and shows that the method is simple and effective. The results obtained are calculated applying dilution factor. The results obtained for control sample using reagents from both sources are further analysed using GraphPad Prism software.

II. MATERIAL AND METHOD:

The study is conducted in two sets using reagents from two different sources as described in this section.

MATERIAL:

i.) WHO International Standard (IRS) (67/086): (Unitage of IgA is 0.8147 mg/ml or 814,700 ng/ml)

ii.) Reagents from Human IgA ELISA kit (Catalogue No. E-80A), Immunology Consultants Laboratory, Inc., Portland, USA, like Running Buffer, Wash solution Concentrate, Enzyme-Antibody Conjugate (affinity purified anti-Human IgA antibody conjugated with horseradish peroxidase in a stabilizing buffer), Chromogenic Substrate (TMB) Solution, Stop Solution, affinity purified Anti-Human IgA coated ELISA micro plate provided in the kit are used for the testing.

iii.) Reagents from Human IgA ELISA kit (Catalogue No. F165), Cygnus Technologies, USA, like Wash Concentrate, Anti-human IgA-HRP Conjugate (affinity purified goat antibody to Human IgA conjugated with horseradish peroxidase (HRP) in a protein matrix with preservative), TMB Substrate, Stop Solution, Anti-Human IgA coated microtitre strips provided in the kit are used for the testing. In addition, Sample Diluent Buffer (Product No.1028), Make: Cygnus Technologies, USA is procured separately for preparation of standard and samples.

The instructions given by the kit manufacturers are strictly followed for storage, reagent preparation and use of these reagents.

METHOD:

Preparation of Standard: WHO International Standard (IRS) (67/086) with IgA unitage 0.8147 mg/ml was reconstituted as per instructions for reconstitution of standard given in its leaflet. Further the standard was diluted to a range of standards to construct standard curve. The dilutions details are shown in table 1.

Table 1: Preparation of dilutions for Standard Curve:

S.No.	ng/ml	Volume added to 1x Diluent	Volume of 1x Diluent
1.	81,470	100 µl of 814,700 ng/ml	900 µl
2.	8,147	100 µl of 81,470 ng/ml	900 µl
3.	814.7	100 µl of 8,147 ng/ml	900 µl
Further dilutions for the preparation of standards to construct standard curve:			
4.	100	123 µl of 814.7 ng/ml	877 µl
5.	50	500 µl of 100 ng/ml	500 µl
6.	25	500 µl of 50 ng/ml	500 µl
7.	12.5	500 µl of 25 ng/ml	500 µl
8.	6.25	500 µl of 12.5 ng/ml	500µl

Preparation of Samples: A set of 25 IVIG samples of different manufacturers are tested using reagents from ICL Kit and a set of 25 samples are tested using reagents from Cygnus Technologies. The samples are further diluted using 1x diluent based on the label claim for IgA content in it. In case of Cygnus Technologies samples and standard are prepared using Sample Diluent Buffer (Product No.1028). The dilution factor varies widely as the label claim is different from sample to sample. The details of dilution factor applied in each sample is shown in results section (Table 3 & 4). As in most of the samples the value shown on label is not estimated value by the manufacturer, hence the dilution factor applied accordingly to get IgA value in the range of standard curve.

Equipment: BIOTEK EPOCH 2 Micro plate Reader, Model – EPOCH 2 NS (Make: BioTek Instruments Inc., US) is used to take absorbance at 450 nm & 450nm/650nm dual wavelength and Micro plate Washer (Make: BIORAD; Model – PW40) is used for plate washing.

Procedure**:** The principle of double antibody sandwich ELISA is followed in this assay. The quantity of bound enzyme varies directly with the concentration of IgA in the sample tested, thus the absorbance at 450nm, is a measure of the concentration of IgA in the test sample. Accordingly, the quantity of IgA in standard and test samples was interpolated from the standard curve constructed using WHO International Reference Standard (IRS) (67/086) and corrected as per sample dilution. 50 ng/ml of standard solution is used as a control in every test to verify the test validity. The percentage recovery of same is shown in results to calculate %RSD, % Error and %Accuracy of the test and method.

A. Method for ICL Kit: 100 µl of standard and samples loaded in duplicate into wells including blank. Further the Microtiter plate was incubated at room temperature for 30 ± 2 minutes. After incubation, the contents of each well are aspirated, then filled with appropriately diluted Wash Solution & aspirated. Similarly the microtitre plate was washed four times. 100 µl of appropriately diluted Enzyme-Antibody Conjugate added to each well, followed by incubation at room temperature for 30 ± 2 minutes. Washing of microtitre plate again as described above. Then 100 µl of TMB Substrate Solution was added into each well, then incubated the plate in the dark at room temperature for about 10 minutes. After incubation for 10 minutes at room temperature, 100 µl of Stop Solution was added to each well. Then absorbance of the content of each well was read at 450 nm using ELISA reader.

B. Method for Cygnus Technologies Kit: 100 µl of standard and samples loaded in duplicate into wells. Then added 100 µL of anti-hIgA: HRP Conjugate. Further the Microtiter plate was incubated at room temperature for 3 hours. Following incubation, aspirated the contents of the wells. Then added approximately 350µL of 1x wash solution and aspirated. Repeated the same cycle for four times. Then added 100 µl of TMB Substrate Solution into each well, incubated at room temperature for 30 minutes. Then 100 µl of Stop Solution was added to each well. The absorbance of microtitre plate was taken at 450 nm/650 nm (with dual wavelength capability) blanking on the Zero standard.

The test method, protocol, plate map, standard curve, curve fit, detection method, read type, blank reduction, temperature, etc. parameters were all set in the ELISA Reader using Gen-5 (Version 2.07) software before taking the absorbance. End point read type and Polynomial regression degree 2 standard curve fit options are selected for calculation of results. The results obtained were further calculated applying dilution factor and conversion factor to represent results in units other than ng/ml as per requirement. The amount of hydrolyzed substrate is read on microtitre plate reader and is directly proportional to the concentration of human IgA content present in samples.

III. RESULTS:

Linearity, Accuracy and Precision (Repeatability) characteristics have been taken into consideration for verification of method performance. The study has been conducted using the left over samples of batches received for testing in the laboratory to develop and establish a common method to estimate IgA content in IVIG

manufactured by different indigenous and imported manufacturers for their Quality Control testing purpose. WHO/NIBSC international reference standard (67/086) is used for construction of standard curve and same is also used as a control sample at a concentration of 50ng/ml in both methods to verify the test performance. Percentage recovery observed for control sample in every assay is within 90-110% (Table 5) by both methods. Based on visual evaluation concept, this data will also support the verification of detection limit (DL) and quantitation limit (QL) of the method [39]. The test validity criteria are selected keeping in view of biological nature of test and control samples.

#***Test Validity Criteria:***

- **Linearity:** The linearity is the ability of the analytical procedure to produce test results which are proportional to the concentration of analyte in samples within a given concentration range. Linearity should be determined by using a minimum of five standards [39]. Correlation co-efficient should be greater than or equal to 0.95.
- **Accuracy:** Accuracy is to demonstrate that test results are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. The acceptable % recovery should be in between 90% to 110% for standard used as a control.

% Error = {(Obtained Mean Value – Actual Value)/ Actual Value} X 100 [40].

% Accuracy = 100% - % Error [40].

- **Precision** (Repeatability)**:** The precision of an analytical method is usually expressed as the standard deviation or RSD. The % RSD for observations should not be more than 5% for control sample.
- The content of the Immunoglobulin A is not more than the maximum content stated on the label for samples under test (IP current edition) [35].

Results using ICL Kit:

Six performances are taken to calculate %RSD, % Accuracy, % Error and Precision (Repeatability). Precision over reportable results/range, observed using a set of six readings for control sample with regard to the intended use of the procedure. The reportable range is confirmed by demonstrating that the analytical procedure provides results with acceptable accuracy, precision and specificity [39]. The range of reportable results should include the upper and lower specification limits or limits of reporting results, as applicable [39]. The results observed for Linearity, %RSD, %Error and %Accuracy are >0.99, 2.80, 1.66 & 101.66 respectively (Table 2), which are acceptable for the assay with reference to a set of validity criteria taken for consideration. To study Linearity, the correlation of coefficient obtained for a range of five standards [39] used i.e. starting from **6.25 ng /ml to 100 ng /ml** is >0.99 every time (Table 2). To study Accuracy of the method, reference material comparison concept (i.e. the analytical procedure is applied to an analyte of known purity (*e.g.*, a reference material, a well characterized impurity or a related substance) and the measured *versus* theoretically expected result is evaluated [39]) is followed. The combined measurement of uncertainty estimated based on results obtained for control sample is 49.17±0.2013 ng/ml @ k=2.

Table 2: Results of Control Sample using ICL and Cygnus Technologies Kits

S.No.	No. of Test	Correlation Of Coefficient	Concentration of IRS(WHO) NIBSC Code: 67/086 (50 ng/ml)	Acceptable (%) Range (90% - 110%)
Results obtained Using Reagents from ICL, USA.				
1.	Test 1	0.999	48.249	96.498 %
2.	Test 2	0.996	50.82	101.64 %
3.	Test 3	1	50.92	101.84 %
4.	Test 4	1	48.287	96.574 %
5.	Test 5	0.999	47.729	95.458 %
6.	Test 6	1	49.065	98.13 %
Mean			49.17	
SD			1.37	

%RSD			2.80	
% Error = {(Obtained Mean Value-Actual Value)/Actual Value} X 100			= {(49.17-50)/50} X 100 = (-0.0166) X 100 = -1.66 %	
% Accuracy = 100% - % Error			=100% - (-1.66) % =101.66 %	
Results obtained Using Reagents from Cygnus Technologies, USA.				
1.	Test 1	0.999	53.19	106.38 %
2.	Test 2	1	52.96	105.92 %
3.	Test 3	0.998	48.05	96.1 %
4.	Test 4	0.999	49.42	98.84 %
5.	Test 5	0.998	51.11	102.22 %
6.	Test 6	0.999	50.82	101.64 %
Mean			50.925	
SD			1.82	
%RSD			3.57	
% Error = {(Obtained Mean Value-Actual Value)/Actual Value} X 100			= {(50.925-50)/50} X 100 = (0.0185) X 100 = 1.85 %	
% Accuracy = 100% - % Error			=100% - (1.85) % = 98.15 %	

The test method is performed using about 25 different IVIG preparations and results obtained for all samples are within the acceptance criteria [35]. Details of dilution factor applied, manufacturer's label claim and results obtained are shown in Table 3. Based on the IgA content value given on the label, all the samples were diluted and accordingly dilution factor was applied to calculate the estimated IgA content. In some samples where the results were found below detectable limit, the dilution factor applied was further reduced and retested. Control sample is used in every test and percentage recovery observed is within set criteria i.e. 90-110%. The correlation co-efficient achieved in every performance is > 0.99.

TABLE 3: Details of results obtained including dilution factor applied and manufacturer's label claim of 25 different Human Normal Immunoglobulin for intravenous use preparations (IVIG) tested using reagents from ICL, USA:

Sample Number	Value obtained from Graph (ng/ml)	Dilution factor	Value after applying dilution factor and conversion factor	IgA Content on Label (as per Manufacturer)
1.	4.864	40,000	0.19456 mg/ml	≤ 2mg/ml
2.	18.456	1,20,000	2.21472 mg/ml	6 mg/ml
3.	13.409	36,000	482.724 µg/ml	≤ 1800 µg/ml
4.	24.307	80	1.94456 mg/L	< 4 mg/L
5.	7.356	40,000	0.29424 mg/ml	≤ 2mg/ml
6.	16.668	1,600	26.6688 mg/L	NMT 80 mg/L
7.	10.794	1,600	17.2704 mg/L	NMT 80 mg/L
8.	16.884	1,600	27.0144 mg/L	NMT 80 mg/L
9.	14.894	1,600	23.8304 mg/L	NMT 80 mg/L
10.	6.538	40,000	0.26152 mg/L	≤ 2mg/ml
11.	11.039	1,600	17.6624 mg/L	NMT 80 mg/L
12.	2.938	40,000	0.11752 mg/ml	≤ 2mg/ml
13.	8.703	3,200	27.8496 mg/L	≤ 160 mg/L
14.	16.173	1,600	25.8768 mg/L	NMT 80 mg/L
15.	10.758	1,600	17.2128 mg/L	NMT 80 mg/L
16.	16.918	1,600	27.0688 mg/L	NMT 80 mg/L
17.	17.398	1,20,000	2.08776 mg/ml	6 mg/ml
18.	24.607	1,20,000	2.95284 g/L	6 g/L
19.	23.892	1,20,000	2.86704 g/L	6 g/L
20.	23.996	1,20,000	2.87952 g/L	6 g/L
21.	8.593	1,400	12.0302 µg/ml	NMT 70 µg/ml
22.	6.074	1,400	8.5036 µg/ml	NMT 70 µg/ml
23.	5.777	1,400	8.0878 µg/ml	NMT 70 µg/ml

24.	9.116	1,600	14.5856 mg/L	NMT 80 mg/L
25.	17.998	1,20,000	2.15976 mg/ml	6 mg/ml
Complies / Not Complies (as per IP Specifications* & Test Validity Criteria#)			All results reported are complies as per IP Specifications* & tests met Test Validity Criteria#	

Results using Cygnus Technologies Kit:

Six performances are taken to calculate %RSD, % Accuracy, % Error and Precision (Repeatability) using reagents from Cygnus also. Similar procedure followed for analysis of results in both methods. The results observed for Linearity, %RSD, %Error and %Accuracy are >0.98, 3.57, 1.85 & 98.15 respectively (Table 2), which are acceptable for the assay with reference to a set of validity criteria taken for consideration. To study Linearity, the correlation of coefficient obtained for a range of five standards [39] used i.e. starting from **6.25 ng /ml to 100 ng /ml** is >0.98 every time (Table 2). The combined measurement of uncertainty estimated based on results obtained for control sample is 50.925 ± 0.608 ng/ml @ k=2.

Using Cygnus kit, 25 different IVIG are tested and results obtained for all samples were within the acceptance criteria [35]. Details of dilution factor applied, manufacturer's label claim and results obtained are shown in Table 4. As in case of ICL Kit, based on the IgA content value given on the label, all the samples were diluted and accordingly dilution factor was applied to calculate the estimated IgA content. Control sample is used in every test and percentage recovery observed is within set criteria i.e. 90-110%. The correlation co-efficient achieved in every performance is > 0.98.

TABLE 4: Details of results obtained including dilution factor applied and manufacturer's label claim of 34 different Human Normal Immunoglobulin for intravenous use preparations (IVIG) tested by reagents from Cygnus Technologies, USA:

Sample Number	**Value obtained from Graph (ng/ml)**	**Dilution factor**	**Value after applying dilution factor and conversion factor**	**IgA Content on Label (as per Manufacturer)**
1.	21.586	1,20,000	2.59032 mg/ml	6 mg/ml
2.	20.601	40,000	0.82404 mg/ml	≤ 2 mg/ml
3.	18.45	40,000	0.738 mg/ml	≤ 2 mg/ml
4.	20.02	40,000	0.8008 mg/ml	≤ 2 mg/ml
5.	24.051	1,400	33.6714 µg/ml	≤70 µg/ml
6.	23.473	1,20,000	2.81676 mg/ml	6 mg/ml
7.	23.352	1,20,000	2.80224 mg/ml	6 mg/ml
8.	20.854	40,000	0.83416 mg/ml	≤ 2 mg/ml
9.	19.288	40,000	0.77152 mg/ml	≤ 2 mg/ml
10.	18.37	1,400	25.718 µg/ml	≤70 µg/ml
11.	22.45	40,000	0.898 mg/ml	≤ 2 mg/ml
12.	20.82	40,000	0.8328 mg/ml	≤ 2 mg/ml
13.	21.95	400	8.78 mg/L	NMT 20 mg/L
14.	25.13	400	10.052 mg/L	NMT 20 mg/L
15.	31.14	400	12.456 mg/L	NMT 20 mg/L
16.	20.06	40,000	0.8024 mg/ml	≤ 2 mg/ml
17.	10.376	1,400	14.5264 µg/ml	≤70 µg/ml
18.	16.844	40,000	0.67376 mg/ml	≤ 2 mg/ml
19.	17.798	1,20,000	2.13576 mg/ml	6 mg/ml
20.	12.651	1,400	17.7114 µg/ml	≤70 µg/ml
21.	13.706	1,600	21.9296 mg/L	≤ 80 mg/L
22.	27.253	1,600	43.6048 mg/L	≤ 80 mg/L
23.	20.526	1,20,000	2.46312 mg/ml	6 mg/ml
24.	20.546	1,20,000	2.46552 mg/ml	6 mg/ml
25.	13.543	1,600	21.6688 mg/L	≤ 80 mg/L
Complies / Not Complies (as per IP Specifications* & Met Test Validity Criteria#)			All results reported are complies as per IP Specifications* & tests met Test Validity Criteria#)	

Results achieved using reagents from ICL & Cygnus Technologies are summarised in Table 5 and Fig.1. Results are within set validity criteria for all parameters considered, using both sources of reagents. Results for control samples obtained using reagents from both sources are further analysed using GraphPad Prism software. The results for P value and statistical significance are shown 0.1657 and not statistically significant. Same is also shown in Table.5.

Fig.1: Comparison of results obtained for control sample using ICL & Cygnus Kit Reagents:

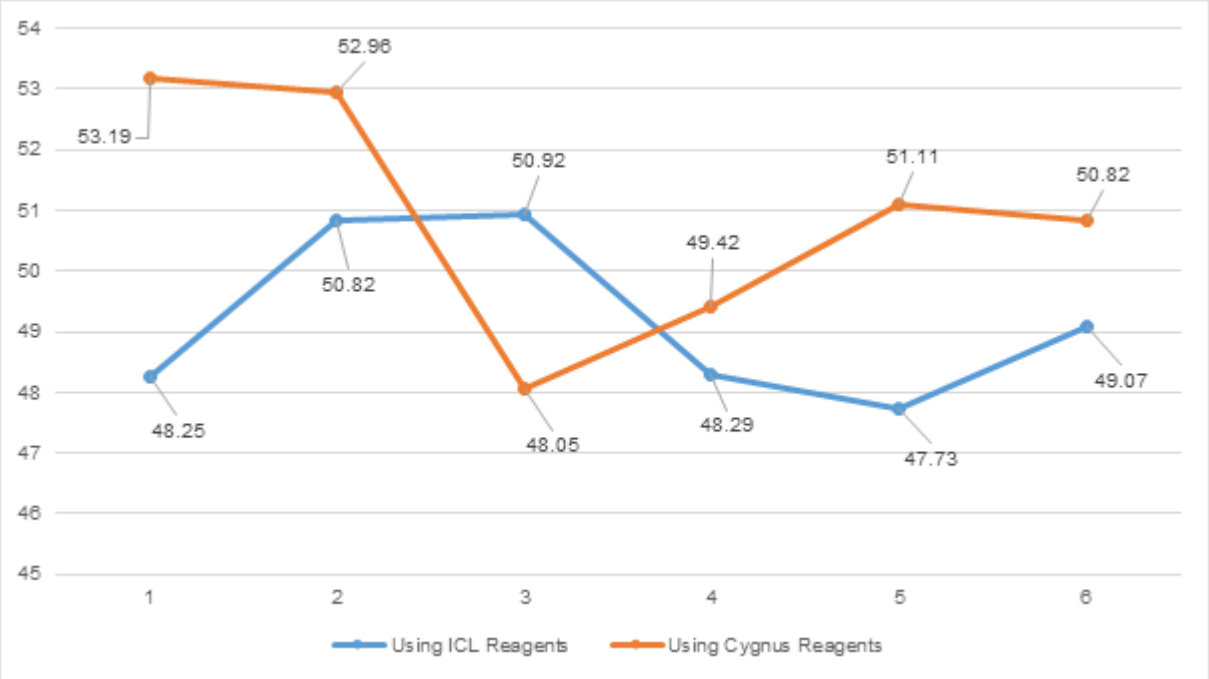

TABLE 5: Summary of Results observed for Method Verification Parameters in the Study (No. of tests taken into consideration = 6; using control sample at 50ng/ml concentration) along with Statistical Analysis of Results (Paired t test) using GraphPad prism Software:

Parameter	*Test Validity Criteria*	Value Obtained		Remarks
		Method-I	Method-II	
Mean	% recovery should be in between 90% to 110% for standard used as a control	49.17 (98.34%)	50.925 (101.85%)	Criteria met
Linearity	correlation co-efficient should not be less than 0.98	> 0.99	> 0.99	Criteria met
Accuracy (% recovery of control sample)	% recovery should be in between 90% to 110% for standard used as a control	95.5% - 101.8%	96.1% - 106.38%	Criteria met
% Accuracy	95% - 105%	101.66 %	98.15%	Criteria met
Precision (Repeatability)	% RSD for observations should not be more than 5% for standard used as a control	2.80	3.57	Criteria met

Outcome of Statistical Analysis of Results (Paired t test) using GraphPad prism Software:

Group	ICL Reagents	CYGNUS Reagents
Mean	49.1800	50.9250
SD	1.3776	1.9924
SEM	0.5624	0.8134
N	6	6

P value and statistical significance:
The two-tailed P value equals 0.1657
By conventional criteria, this difference is considered to be not statistically significant.

Confidence interval:
The mean of ICL Reagents minus CYGNUS Reagents equals -1.7450
95% confidence interval of this difference: from -4.5102 to 1.0202

IV. DISCUSSION & CONCLUSION:

Much data is not available regarding determination of IgA content in final product of IVIG for the purpose of their Quality Control testing. The two kits used in the present study mention that they are for "*research purpose only"*. Hence the methods described here will help Quality Control testing laboratories and manufactures of IVIG, for establishing this test in their laboratories.

The results obtained for linearity and accuracy assure that the method is simple and the very less concentration (i.e. in ng/ml) of reference standard and test samples, required to perform the test which establishes its cost effectiveness or inexpensive. The repeatability (Precision) observed for a set of six samples (Table 2) and

the results obtained for 25 samples using ICL Reagents (Table 3) and 25 samples using reagents from Cygnus Technologies (Table 4) promises that the method is effective and can be used for Quality Control or Batch Release testing of IVIG. Results for control samples obtained using reagents from both sources are further analysed using GraphPad Prism software. The results shown for P value as 0.1657 and not statistically significant (Table.5). Based on above conclusion, this method can also be recommended in Pharmacopoeia for determination of IgA content in IVIG. An orthogonal procedure comparison (i.e. Specificity/selectivity can be verified by demonstrating that the measured result of an analyte is comparable to the measured result of a second, well characterized analytical procedure [39]) could not be done due to lack of a well recommended procedure for Quality Control testing purpose. However, the same is tried to show through Accuracy.

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CONFLICT OF INTERESTS: The authors have no conflict of interest in this work.

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