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Comparison of two chromogranin A assays and investigation of nonlinear specimens

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ABSTRACT

Background: As a marker for functional and non-functional neuroendocrine tumors, serum chromogranin A (CgA) concentrations have shown value for detecting and monitoring disease. Here we describe a comparison between an established micro-titer plate assay (Cisbio CgA ELISA) and an analyzer-based assay (B·R·A·H·M·S CgA II KRYPTOR). Reference limits were established along with a performance evaluation of the KRYPTOR assay. Nonlinearity observed in approximately 0.03% of patients was also investigated.

Methods: Samples were tested according to kit manufacturer's protocols. Reference limits were established for both assays testing the same cohort of healthy volunteers. Potential causes of nonlinearity investigated were HAMA, macromolecule effects and elevated serum creatinine.

Results: KRYPTOR vs. Cisbio: slope=0.692, y-intercept=−40.0 (r^2=0.967, n=186). Upper reference limits were 160 and 103 ng/mL for the Cisbio and KRYPTOR assays, respectively. Linearity: slope=1.012 (r^2=0.998) with 95.0–105.5% recoveries. Precision: repeatability ≤2.4%, within-laboratory ≤3.1% (79 and 738 ng/mL). Limit of detection: 8 ng/mL. Strong nonlinear specimens (n=6) retested for HAMA interference generated differences (block-no block) ranging −3.2–4.2%. Polyethylene glycol precipitation recoveries ranged from 157 to >5714% for affected specimens versus 71–79% for normal specimens. Eight of 14 nonlinear specimens (57%) had elevated serum creatinine results (>1.20 mg/dL).

Conclusions: The CgA II KRYPTOR assay performs acceptably for quantifying CgA in human serum. While adequate correlation is observed against the Cisbio ELISA, there is significant disagreement overall. Efforts to identify a cause of the nonlinearity observed in a small percentage of patients were inconclusive, but neither HAMA interference, macromolecule effects nor renal failure appear as major factors.

1. Introduction

Chromogranin A(CgA) is a 49 kilodalton prohormone found in the secretory granules of endocrine and neuroendocrine cells [1].

Abbreviations: CgA, chromogranin A; ELISA, enzyme-linked immunosorbent assay; TRACE, time resolved amplified cryptate emission; PEG, polyethylene glycol.

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Containing several recognition sites for endopeptidases, CgA is cleaved into several functional peptides. Among these are: vasostatin I and II, pancreastatin, catestatin, and prochromacin [2,3].

As a marker for functional and non-functional neuroendocrine tumors, CgA has shown clinical value in patients suffering from neuroblastomas, pheochromocytomas, carcinoid tumors and endocrine pancreatic tumors in addition to tumors of the lung, prostate, colon and breast. Sensitivities for the diagnosis vary from 10 to 100% depending on tumor type, burden, and the presence or absence of metastatic disease, whereas specificities can range from 65 to 100% [1,4–8].

CgA has also shown value in monitoring tumor response during treatment. For example, a relationship has been reported between elevated serum CgA and prostatic carcinoma due to neuroendocrine differentiation [9,10]. Therefore, serum CgA levels can have implications in the type of therapy used for treatment [11,12].

The Cisbio Chromoa® Chromogranin A ELISA test kit (Cisbio Bioassays, Codolet, France) is a well-established enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of human CgA in serum or plasma. The assay is a solid-phase, 96-well microtiter plate formatted sandwich immunoassay incorporating two monoclonal antibodies targeting different epitopes of the unprocessed core domain of the human CgA molecule [13,14].

The B·R·A·H·M·S CgA II KRYPTOR® assay is a sandwich immunoassay that quantitatively measures CgA using the B·R·A·H·M·S KRYPTOR family of automated analyzers (B·R·A·H·M·S GmbH, Hennigsdorf, Germany). The assay is a newer version of the KRYPTOR CgA assay, incorporating new antibodies targeting different epitopes [15]. The test utilizes Time-Resolved Amplified Cryptate Emission technology, also known as TRACE™. TRACE involves the non-radiative energy transfer from a donor (europium cryptate) to an acceptor (Alexa Fluor® 647) when they are part of an immunocomplex. When the sample is excited with a nitrogen laser, TRACE measures the signal emitted from the immunocomplex with a time delay to eliminate nonspecific background signals [15,16].

During incubation, CgA molecules present in the sample are sandwiched between the donor and acceptor labeled antibodies, thus, forming the immunocomplex. Upon laser excitation, the fluorescent signal emitted is proportional to the CgA concentration in the sample. Comparison of the signal data with a standard curve allows for determining the CgA concentration of the sample [15,16]. According to the instrument's manufacturer, potential advantages of TRACE technology over ELISA are minimal processing steps, faster results, less waste and timely automated dilution.

Previous studies at our facility revealed an apparent high-dose-hook effect (nonlinearity upon dilution) observed in elevated specimens at a rate of approximately 15% in three other CgA immunoassays, but was absent using the Cisbio CgA ELISA [17]. However, after testing tens of thousands of specimens at neat and 5-fold dilution over the past several years using this assay, it was revealed that approximately 0.03% of specimens continue to exhibit this phenomenon. Comparable results were observed while evaluating the newer CgA II KRYPTOR assay, with the test kit manufacturer making a similar claim of 0.02% of specimens showing the effect [16].

Studies comparing the older KRYPTOR CgA assay with the established Cisbio CgA ELISA have been published [18]. However, literature addressing the second generation CgA II KRYPTOR versus the Cisbio assay is lacking. Here we report a split-sample method comparison between the Cisbio CgA ELISA and the automated CgA II KRYPTOR assay. Reference intervals were also established for both assays and compared. Additionally, performance characteristics, including linearity, precision, analytical sensitivity and carryover were evaluated for the newer KRYPTOR assay along with analyte stability. Finally, we report an attempt to discover why a very small number of specimens continue to exhibit nonlinearity upon dilution, although tested using two CgA assays that have demonstrated superior resilience to this apparent high-dose hook effect [16,17]. Because CgA is elevated in patients with renal failure due to decreased elimination [19], we sought to understand if patients with very elevated serum creatinine, likely due to kidney failure, were frequently observed in specimens with nonlinear CgA results. Other potential causes evaluated were heterophilic antibody/HAMA interference and macromolecule effects.

2. Materials and methods

2.1. Assay kits

Cisbio Chromoa Chromogranin A ELISA test kits were purchased from ALPCO® (Salem, NH). The B·R·A·H·M·S KRYPTOR compact PLUS and CgA II KRYPTOR kits were provided by Thermo Fisher Scientific Inc. (Waltham, MA).

2.2. Study specimens and testing

Serum specimens were collected using University of Utah Internal Review Board approved protocols and stored refrigerated short term (≤48 h) or frozen (−70 °C). CgA was measured according to each test kit's protocol. Automation of the ELISA testing was conducted using a TECAN Freedom EVOlyzer® liquid handler and TECAN Sunrise™ microplate reader controlled with Magellan™ data analysis software (Tecan US, Inc., Morrisville NC). Unless noted otherwise, ELISA results are the mean of duplicate measurements whereas singlet measurements were used for the KRYPTOR assay. Samples testing above the analytical measurement range (770 and 3000 ng/mL, Cisbio and KRYPTOR, respectively) were appropriately diluted using the test kit manufacturer's diluent and retested.

2.3. CgA II KRYPTOR evaluation

KRYPTOR linearity was evaluated by combining low and high CgA concentrated human serum pools at various ratios, creating 10 samples of varying concentrations. Samples were then tested in duplicate and measured results compared to the calculated expected

results.

Precision was assessed utilizing two serum pools of differing CgA concentration levels. Each pool was tested over five days, four determinations per day. Both repeatability and within-laboratory precision were calculated from the same dataset.

Analytical sensitivity was calculated from 10 measurements each of CgA free human serum (EMD Millipore, Burlington, MA) and a low CgA concentrated serum pool.

Carryover was evaluated using an extremely elevated and a low concentrated CgA serum specimen. The elevated specimen was tested followed immediately by two determinations of the low sample, with this testing pattern performed sequentially four times.

2.4. Analyte stability

Analyte stabilities at room temperature, refrigerated (4–8 °C) and frozen (−20 °C) were evaluated at two levels using two serum CgA pools of differing concentrations. Time intervals studied were zero to 48 h at room temperature, to 14 days refrigerated and to 4 weeks frozen. Freeze/thaw stability was also assessed up to four cycles.

2.5. Method comparison

A split-sample study was conducted utilizing 186 deidentified serum specimens. Results, CgA II KRYPTOR vs. the Cisbio ELISA, were analyzed by Deming regression.

2.6. Reference limits

For reference limit studies, the same cohort of serum specimens from healthy adult volunteers (n=125, ages 19–65 years), were tested using each assay. Non-parametric reference limits were calculated at the 95th percentile.

2.7. Nonlinear specimens

Specimens demonstrating the apparent high-dose hook using the Cisbio assay, were collected for approximately one year (n=20). These same specimens were then tested using the KRYPTOR assay at various dilution ratios as described previously.

2.8. HAMA studies

Nonlinear specimens were treated for HAMA using Heterophilic Blocking Tubes (Scantibodies Laboratory, Inc., Santee, CA). A 300 μL aliquot was placed into a blocking tube and capped. The tubes were then inverted five times followed by gentle vortex for approximately 2 s. After incubation at room temperature for 1 h, treated samples and their untreated counterparts were tested using the KRYPTOR CgA assay.

2.9. Macromolecule effect studies

Macromolecule effects were investigated using polyethylene glycol (PEG) precipitation techniques. Affected specimens and two normal samples were diluted with an equal volume of PEG 8000 solution (250 g/L in PBS, Sigma-Aldrich, St. Louis, MO). Samples were then vortexed for 30 s followed by incubation at room temperature for 10 min. Samples were then centrifuged at 10,000×*g* for 5 min followed by removal of the supernatant. Sample supernatants and their untreated (neat) counterparts were then tested using both the KRYPTOR and Cisbio CgA assays.

2.10. Serum creatinine studies

Serum creatinine measurements were conducted using the Creatinine plus ver.2 assay and a Roche cobas c702 (Roche Diagnostics, Indianapolis, IN).

2.11. Data analysis

Data analysis was performed using Microsoft® Excel® (Microsoft Corporation, Redmond WA), Analyse-it® Method Validation Edition (version 5.66, Analyse-it Software, Ltd., Leeds, United Kingdom) and Prism (Prism 9, GraphPad Software, San Diego, CA) software.

3. Results

3.1. CgA II KRYPTOR evaluation

Linear regression analysis generated a slope of 1.01 with an r^2 of 0.998 (Fig. 1). Recoveries of the expected concentrations ranged from 95.0 to 105.5%.

Precision at two levels of CgA concentration was evaluated over five days, four replicates per day per level. Analysis produced the following: Level I, CVs of 2.4 and 3.0% for repeatability and within laboratory, respectively (mean=79 ng/mL); Level II, CVs of 1.6 and 3.1% for repeatability and within laboratory, respectively (mean=738 ng/mL).

Method validation software generated a limit of blank of 5 ng/mL, and a limit of detection of 8 ng/mL.

For carryover, the mean for the low concentration pool results that immediately followed testing of the high concentration (~36,000 ng/mL) pool was 43.0 ng/mL. The mean for the low pool results immediately following a previous low pool test was 43.5 ng/mL. Thus, demonstrating carryover to be a nonissue for the assay.

3.2. Analyte stability

Serum CgA stability study results are summarized in Table 1. A percent difference of a result from time zero of ±10% was used for acceptable stability. Based on this criterion, CgA was found stable for a minimum of 48 h at room temperature, for three days refrigerated, a minimum of 10 weeks frozen (−20 °C), and over a minimum of four freeze/thaw cycles.

3.3. Method comparison

Deming regression analysis of the CgA II KRYPTOR versus the Cisbio CgA ELISA is presented in Fig. 2. Although the correlation between assays appears adequate (r^2=0.967), a significant bias is evident, with the KRYPTOR assay measuring significantly lower (−27.2%, Bland-Altman analysis).

3.4. Reference limits

Upper reference limits of 160 and 103 ng/mL were established for the Cisbio CgA ELISA and CgA II KRYPTOR assays, respectively (Fig. 3). No significant differences were evident between genders, generating p-values of 0.834 and 0.427 for the Cisbio and KRYPTOR assays, respectively (unpaired *t*-test, two-tailed).

3.5. Nonlinear specimens

After testing tens of thousands of specimens at neat and 5-fold dilution over several years using the Cisbio ELISA, approximately 0.03% of specimens were found to exhibit nonlinearity (high-dose hook) upon dilution. Further testing of these specimens at varying dilutions confirmed the observation. During the evaluation period of the Kryptor assay, 20 specimens were found exhibiting the nonlinearity phenomenon. These 20 specimens were then retested neat and at several dilution ratios using the KRYPTOR assay. KRYPTOR results were similar to those of the Cisbio ELISA that initially discovered these unique specimens. Results for six representative specimens are shown in Fig. 4.

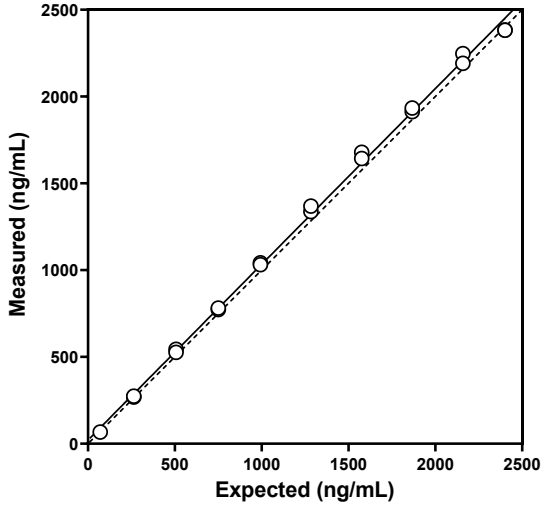

Fig. 1. CgA II KRYPTOR linearity. Linear regression of Measured vs. expected CgA concentrations, n=10 pools. Slope: 1.01 (r^2=0.998). Pool concentrations ranged from 70 to 2401 ng/mL.

Table 1
CgA analyte stabilities in serum.

Room Temperature (ng/mL)				
Hours	Level I	Level II	% Difference	
0	86	532	Level I	Level II
12	83	520	−3.5	−2.3
24	81	504	−5.8	−5.3
48	79	485	−8.1	−8.8
Refrigerated (ng/mL)				
Days				
0	94	521		
3	86	513	−8.5	−1.5
7	75	478	−20.2	−8.3
14	64	418	−31.9	−19.8
Frozen, −20 °C (ng/mL)				
Weeks				
0	62	494		
2	65	518	4.8	4.9
4	65	533	4.8	7.9
6	67	535	8.1	8.3
8	61	538	−1.6	8.9
10	63	502	1.6	1.6
Freeze/Thaw (ng/mL)				
Cycles				
0	62	485		
1	64	491	3.2	1.2
2	59	501	−4.8	3.3
3	57	495	−8.1	2.1
4	59	482	−4.8	−0.6

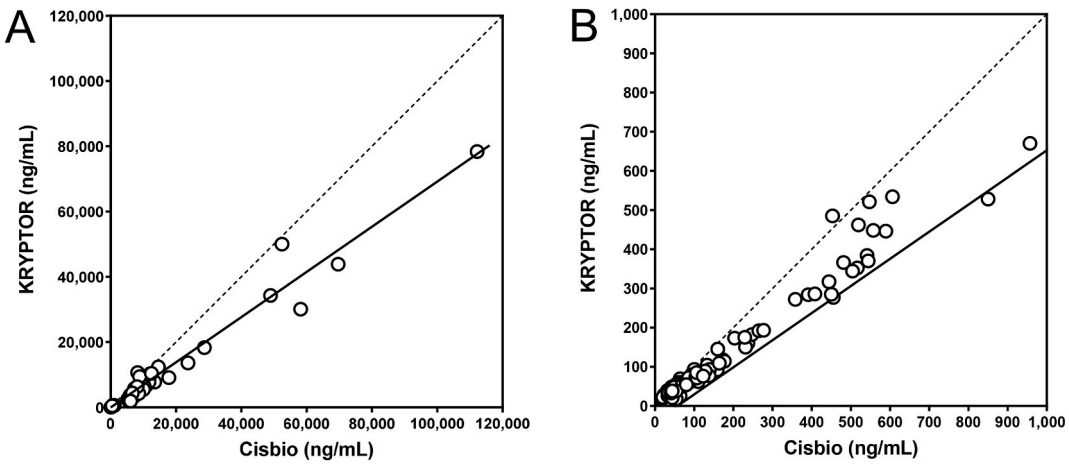

Fig. 2. CgA II KRYPTOR vs. Cisbio CgA ELISA. (A) Deming regression of results from a split-sample method comparison (n=186). Slope, 0.692; intercept, −40; r^2, 0.967; p<0.0001. (B) Expansion of plot A for results <1000 ng/mL, demonstrating data distribution and at lower CgA concentrations and near the reference limits.

3.6. HAMA studies

Specimens exhibiting strong nonlinearity/high-dose hook (n = 6) were tested for HAMA interference using the KRYTOR assay. CgA concentration differences (HAMA block treated-none treated) were small, ranging −3.2–4.2 ng/mL. Results suggest HAMA is not the cause of the observed nonlinearity.

3.7. Macromolecule effect studies

PEG precipitation results are shown in Table 2. CgA recoveries ranged from 157 to >5714% for the nonlinear specimens versus mean recoveries of 75% and 106% (Cisbio and KRYPTOR, respectively) for normal specimens. Because nonlinearity remained a

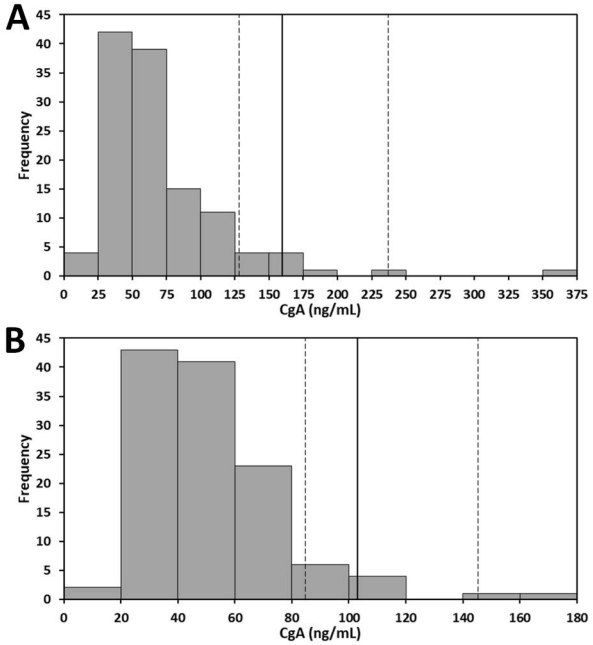

Fig. 3. Frequency histograms, CgA reference intervals. Non-parametric upper reference limits calculated at the 95th percentile (125 healthy adult volunteers, ages 19–65 yrs old, 61 males, 64 females). (A) Analysis for the Cisbio CgA ELISA, producing a limit of 160 ng/mL (90% CI, 128.0–237.0 ng/mL). (B) For the CgA II KRYPTOR assay, generating a limit of 103 ng/mL (90% CI, 84.7–145.4 ng/mL).

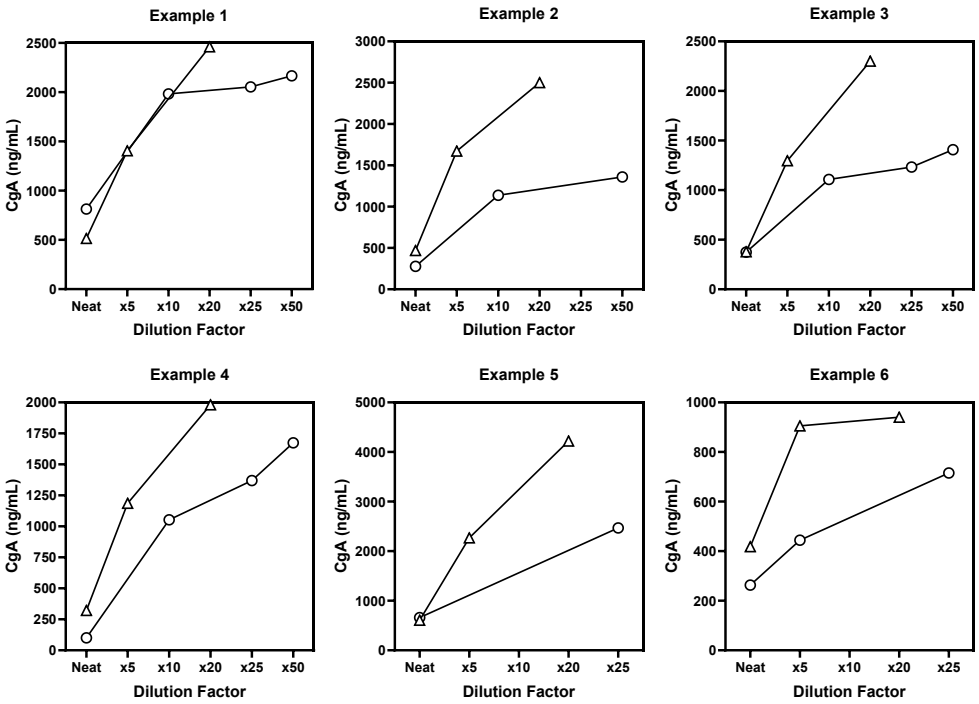

Fig. 4. Representative nonlinear CgA specimens. Specimens tested neat and at various dilution ratios using both the Cisbio CgA ELISA (△) and the CgA II KRYPTOR assay (◯).

significant issue in the PEG treated specimens, macromolecule effects appear to not be the cause.

3.8. Serum creatinine studies

Of 14 nonlinear specimens, eight (57%) produced elevated serum creatinine results (>1.20 mg/dL). However, five of the eight were only slightly elevated producing concentrations of 1.25–1.67 mg/dL. Only three of the 14 specimens were considered as extremely elevated, with creatinine ranging 5.13–9.19 mg/dL. Results suggest that the CgA nonlinearity is not caused by potential kidney failure.

4. Discussion

The Cisbio CgA ELISA is a well-established assay for determining patient serum CgA concentrations. Although studies comparing this ELISA with the first generation KRYPTOR CgA assay have been published [18], studies against the newer CgA II KRYPTOR assay appear deficient in the literature. Here, we address a comparison of the Cisbio ELISA with the newer CgA II KRYPTOR assay, including a performance evaluation of the latter. Finally, we attempted to find a cause for the very small percentage of specimens (0.03 and 0.02%, Cisbio and KRYTOR II assays, respectively) that continue to produce nonlinear results upon dilution, despite both the Cisbio and KRYOTPR II assays eliminating the vast majority of nonlinear results versus other CgA assays [16,17].

Evaluation of the CgA II KRYPTOR assay demonstrates acceptable performance as indicated by a slope of 1.012 (r^2=0.998) and excellent recoveries for linearity, precision CVs of ≤3.1%, and a limit of detection of 8 ng/mL. In comparison, our previous evaluation of the Cisbio Chromoa Chromogranin A ELISA generated a slope of 1.04 (r^2=0.997), precision CVs of ≤9.3%, with an equal limit of detection of 8 ng/mL [17]. Overall, the assays appear to perform equally except for precision, where the KRYPTOR produces better CVs. The automated pipetting of the KRYPTOR assay may account for the better precision versus the manual pipetting performed at the time of the Cisbio kit evaluation. The automated format also implied that carryover could potentially be an issue because the analyzer does not use disposable tips. However, carryover was found to be negligible.

In reference to CgA stability in serum, a difference was observed for refrigerated temperature. Our earlier validation of the Cisbio ELISA verified refrigerated stability for a minimum of 14 days [17]. However, stability was demonstrated for up to only three days in our KRYPTOR study here. Differences in refrigerated CgA stability between test kits is not new. The test kit we used previously to the Cisbio, distributed by Alpco® (Salem, NH) at the time, showed significant CgA degradation within 24–48 h at refrigerated temperatures even though the ambient stability was for days for the same kit. Although a combination of CgA protein conformational changes at refrigerated temperatures and epitope locations were theorized as a possible cause, no studies were taken to prove or disprove the theory. Overall, the three-day refrigerated analyte stability for the KRYPTOR assay is still reasonable for clinical applications, as long as transport and storage are conducted while frozen. Also of note is the minimum of four freeze/thaw cycles for which CgA stability was verified for the KRYTOR in this study.

As shown, the method comparison of the CgA II KRYPTOR versus the Cisbio CgA ELISA showed a decent correlation (r^2=0.967) but poor agreement (slope=0.692, −27.2% bias). Of interest is that a prior study comparing the first generation KRYPTOR CgA assay with the Cisbio ELISA, indicated a much closer agreement (slope=1.05, intercept=−0.20, Passing-Bablok regression), with KRYPTOR results reading slightly higher [18]. Our study described here using the newer second generation KRYPTOR II CgA assay, demonstrates observable performance differences versus the first-generation assay. One possible reason for these discrepancies could be the different antibodies utilized between the first and second generation KRYPTOR assays [15]. CgA fragmentation due to extracellular proteolysis and/or degradation is well known and can vary between tissue and tumor type [20–24]. Hence, numerous epitopes are possible, generating varying antibody binding characteristics that may produce dissimilar results among different assays using different antibodies [25–29]. Additional potential reasons for the discrepancies are different calibration strategies, unexpected variances with the Cisbio ELISA and/or KRYPTOR assays, or other variables inherent to diverse study sites.

The upper reference limits established for both the Cisbio and KRYPTOR assay using the same cohort of specimens also showed a significant difference. As shown previously, the Cisbio ELISA reference limit of 160 ng/mL is much higher than the value of 103 ng/mL for the KRYPTOR II assay. This was not totally unexpected however, based to the method comparison results as described above. Obviously, results between the two assays should not be used interchangeably, especially in the clinical setting.

Our previous evaluation and validation of the Cisbio Chromoa CgA ELISA led us to believe that the assay completely eliminated the apparent high-dose-hook effect we had observed in approximately 15% of specimens using other CgA assays we had investigated [17]. However, that Cisbio evaluation was over a short period of time relative to the years that followed utilizing the assay in clinical practice. Over time, it was revealed that approximately 0.03% of specimens still exhibit the effect using the Cisbio ELISA. Moreover, evaluation of the CgA II KRYPTOR demonstrated an equal ability to minimize nonlinearity upon dilution, producing the phenomenon in essentially the same small cohort of specimens as the Cisbio ELISA. As a result, we decided to see if it was possible to find a similar attribute specific among this very small number of unique specimens that might be the cause. As our results show, neither HAMA, macromolecule effects nor elevated creatinine (renal impairment) was found to cause the phenomenon. Therefore, the cause remains unresolved, but these potential sources of the effect appear to be eliminated. Fortunately, using either the Cisbio or KRYPTOR assays, the effect is observed in such a small number of patients (0.02–0.03%) that it considered a rare occurrence. That cannot be said for other CgA assays we have evaluated in the past [17].

5. Conclusions

The B·R·A·H·M·S CgA II KRYPTOR automated assay performs acceptably for quantifying CgA in human serum. A good correlation

Table 2
PEG precipitation. Cisbio CgA ELISA..

Specimen	Dilution Factor	CgA (ng/mL)		Percent Recovery	Nonlinearity Observed?
		Raw Result	× Dilution		
1	1	617	617		
1 w/PEG	2	>750	>1500	>243%	Yes
2	1	467	467		
2 w/PEG	2	568	1136	243%	Yes
3	1	147	147		
3 w/PEG	2	295	590	401%	Yes
Normal 1	1	119	119		
Normal 1 w/PEG	2	52	104	87%	No
Normal 2	1	48	48		
Normal 2 w/PEG	2	30	60	125%	No
			Mean Normal Recovery:	106%	No
KRYPTOR CgA II Specimen	Dilution Factor	CgA (ng/mL) Raw Result	× Dilution	Percent Recovery	
1	1	785	785		
1 w/PEG	2	618	1235	157%	Yes
2	1	277	277		
2 w/PEG	2	285	569	205%	Yes
3	1	104	104		
3 w/PEG	2	145	291	281%	Yes
4	1	105	105		
4	2	>3000	>6000		
4 w/PEG	2	>3000	>6000	>5714%	Yes
Normal 1	1	100	100		
Normal 1 w/PEG	2	36	71	71%	No
Normal 2	1	54	54		
Normal 2 w/PEG	2	21	43	79%	No
			Mean Normal Recovery:	75%	No

exists between the well-established Cisbio Chromoa CgA ELISA and the newer CgA II KRYPTOR assay. However, the agreement is suboptimal, as indicated by the −27% bias between these assays. The bias is further evident by the significant difference in upper reference limits established in this study, 160 and 103 ng/mL for the Cisbio and KRYPTOR II, respectively. Therefore, as with other CgA assays, test results between these two assays should not be used interchangeably.

Additionally, evaluation of the CgA II KRYPTOR assay demonstrates equal performance as the Cisbio ELISA regarding the high-dose-hook effect (nonlinearity upon dilution) observed in a very small cohort (0.02–0.03%) of specimens. Although the cause of this effect remains unknown for these unique specimens, our studies suggest that HAMA interference, macromolecule effects or renal impairment are not key factors.

Author statement

J. Alan Erickson: Conceptualization, Validation, Formal Analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Fang-I Chiang:** Validation, Investigation, Resources, Writing – Review & Editing. Chelsie M. Walker: Validation, Investigation, Resources, Writing – Review & Editing. **Jonathan R. Genzen:** Conceptualization, Validation, Formal Analysis, Writing – Review & Editing, Visualization, Supervision. **Kelly Doyle:** Conceptualization, Validation, Formal Analysis, Writing – Review & Editing, Visualization, Supervision.

Declaration of competing interest

I have no conflict of interest to report.

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