Method Comparison and Reference Limits for Two Chromogranin A (CgA) Assays

J. Alan Erickson[1], Fang-I Chiang[2], Chelsie M. Walker[2], Jonathan R. Genzen[1,3]

[1]ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories, Salt Lake City, UT; [2]ARUP Laboratories, Salt Lake City, UT, [3]University of Utah School of Medicine, Department of Pathology, Salt Lake City, UT.

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ABSTRACT

Background: Chromogranin A (CgA) is a 49 kD prohormone found in the secretory granules of neuroendocrine cells. The protein contains several recognition sites for endopeptidases that cleave the protein into several functional peptides. Among these are vasostatin I and II, pancreastatin, catestatin, and prochromacin. As a marker for functional and non-functional neuroendocrine tumors, studies have shown serum CgA concentrations to be useful clinically for the detection and monitoring of disease. Here we describe a method comparison between a CgA micro-titer plate ELISA (Chromogranin A ELISA kit, Cisbio Bioassays) and an analyzer based assay (B·R·A·H·M·S CgA II KRYPTOR, Thermo Fisher Scientific, Inc). Reference limits for each assay were also established.

Methods: Specimens were collected according to Institutional Review Board approved protocols. Serum CgA was measured according to each assay manufacturer's testing protocol. A method comparison was conducted utilizing 149 specimens. Reference limits were established for each assay by testing the same cohort of 125 healthy adult volunteers, ages 19 – 65 yrs old. Data analysis was performed using Analyse-it Method Validation Edition (version 4.65, Analyse-it Software, Ltd.) and Prism (Prism 5, GraphPad Software).

Results: Deming regression of the KRYPTIOR vs. the Cisbio CgA assays generated a slope of 0.695 and y-intercept of 7.215, with an r^2 = 0.947. Non-parametric reference limits at the 95th percentile were determined to be 160 and 103 ng/mL CgA for the Cisbio and KRYPTOR assays, respectively. No significant differences were identified between genders, with p-values of 0.834 and 0.427 for the Cisbio and KRYPTOR assays, respectively. CgA concentrations increased slightly but significantly with age (Cisbio: r^2 = 0.095, p = 0.0006. KRYPTOR: r^2 = 0.111, p = 0.0002) but the increases were deemed clinically insignificant.

Conclusions: An adequate correlation is observed between the Cisbio Bioassays Chromogranin A ELISA and the B·R·A·H·M·S CgA II KRYPTOR assay. However, there is significant disagreement overall. As a result, reference limits cannot be used interchangeably.

INTRODUCTION

Chromogranin A (CgA) is a 49 kilodalton prohormone found in the secretory granules of endocrine and neuroendocrine cells (1, 2). Containing several recognition sites for endopeptidases, CgA is cleaved into several functional peptides. Among these are: vasostatin I and II, which inhibit vasoconstriction and suppress the release of parathyroid hormone; pancreastatin, which inhibits insulin release, promotes hepatic glycogenolysis, and diminishes glucose uptake by skeletal muscle; catestatin, which inhibits catecholamine release from the adrenal medulla; prochromacin, which exerts bacteriolytic and antifungal effects (3, 4).

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 of these neoplasms vary from 10–100% depending on tumor type, burden, and the presence or absence of metastatic disease, whereas specificities can range from 65–100% (1, 5-9).

CgA has also shown value in monitoring tumor response during treatment. Example, a relationship has been reported between elevated serum CgA and prostatic carcinoma due to neuroendocrine differentiation (10, 11). Therefore, serum CgA levels can have implications in the type of therapy used for treatment (12, 13).

The Cisbio CgA ELISA kit is an enzyme-linked immunosorbent assay for the quantitative measurement of human CgA in serum or plasma. The assay is a 96-well microtiter plate formatted sandwich immunoassay incorporating two monoclonal antibodies targeting different epitopes of the unprocessed core domain of the human CgA molecule. Solely targeting this core domain allows measurement of both intact and fragmented CgA. The first antibody, bound to the microtiter well, captures the CgA proteins contained in the calibrators and samples. The second, conjugated to horse-radish-peroxidase (HRP), binds to the captured CgA protein which then provides a means of detection and quantification of CgA in the sample (14).

Calibrators, controls and patient serum samples are diluted in a buffer and then added to the assigned microtiter wells. After an incubation and wash step to remove unbound materials, HRP labeled antibody is added to each well. After a second incubation period and washing, a chromogenic substrate is added. During this final incubation step, color is produced by HRP turnover of the substrate generating a direct relationship between the color intensity and the CgA concentration of each calibrator and sample. An acidic stopping solution is added to terminate the reaction. The color intensity (optical density) of each sample is subsequently measured spectrophotometrically at 450 nm. Lastly, a calibration curve is constructed (optical density vs. CgA concentration) and the unknown sample CgA concentrations calculated from the curve. Final results are expressed as ng/mL CgA (14).

The B·R·A·H·M·S CgA II KRYPTOR® assay quantitatively measures CgA using the B·R·A·H·M·S KRYPTOR family of automated analyzers. The test incorporates time resolved amplified cryptate emission technology also known as, "TRACE™" (15).

TRACE involves the transfer of non-radiative energy from europium cryptate (donor) labeled to an antibody, to a fluorophore (acceptor) also labeled to an antibody, when both are bound to the analyte (in this case, CgA). The energy transfer is possible due to the close proximity of the donor and acceptor in the immunocomplex. This energy transfer results in a considerably lengthened emission signal of the fluorophore that is measured after a time delay when other unspecific signals (e.g. unbound fluorophores and other light emitting molecules) have terminated. The measured long-lived signal of the fluorophore (647 nm) is proportional to the concentration of CgA in the sample. The signal produced by the cryptate (620 nm) is also measured simultaneously, thus, acting as an internal reference. This allows for sample characteristics that may interfere with light transmission to be compensated for by means of the ratios of the intensities at these two wavelengths, which are calculated automatically by the analyzer. Because the ratio remains constant, an accurate measurement of CgA is provided irrespective of sample turbidity, color or other such characteristics. Final results are reported in ng/mL. The advantages of using TRACE over ELISA are minimal processing steps, faster results and less waste (15, 16).

In this report, we describe a method comparison study between the Cisbio Chromogranin A ELISA kit and the automated analyzer based B·R·A·H·M·S CgA II KRYPTOR assay. CgA reference limits were also established for the assays and compared.

MATERIALS AND METHODS

- Cisbio Chromoa® Chromogranin A ELISA kits were purchased from ALPCO® (Salem, NH).
- B·R·A·H·M·S KRYPTOR compact PLUS automated immunoanalyzer and B·R·A·H·M·S CgA II KRYPTOR kits were acquired from Thermo Fisher Scientific Inc. (Waltham, MA).
- Serum specimens were collected using University of Utah Internal Review Board approved protocols.
- Specimens were stored refrigerated short term (≤48 hours) or frozen until use.
- 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).
- Serum CgA was measured according to each assay manufacturer's testing protocol, with ELISA results the mean of duplicate measurements and singlet measurements for the KRYPTOR assay.
- Data was analyzed using Analyse-it Method Validation Edition (version 4.65, Analyse-it Software, Ltd., Leeds, United Kingdom) and Prism (Prism 5, GraphPad Software, San Diego, CA).

RESULTS

- **Figure 1.** A method comparison (split-sample) study utilizing 149 deidentified serum specimens. Deming regression analysis of the KRYPTOR CgA II vs. Cisbio CgA ELISA generated a slope of 0.695 and y-intercept of 7.215, with r^2 = 0.947.
- **Figure 2.** Bland-Altman analysis of the percent difference (KRYPTOR – Cisbio) vs. average of the method comparison results produced a bias of -23%.
- **Figure 3.** Non-parametric reference limits calculated at the 95th percentile (125 healthy adult volunteers, ages 19 – 65 yrs old). Reference intervals: 160 and 103 ng/mL for the Cisbio CgA ELISA and KRYPTOR CgA II assays, respectively.
- No significant differences in reference limits were found between genders, generating p-values of 0.834 and 0.427 for the Cisbio and KRYPTOR assays, respectively.
- **Figure 4.** Analysis of reference limit results vs. age showed a slight but significant increase in CgA concentration with age for each assay, with p-values of 0.0006 and 0.0002 for the Cisbio and KRYPTOR assays, respectively. These minor increases however, were regarded as insignificant clinically.

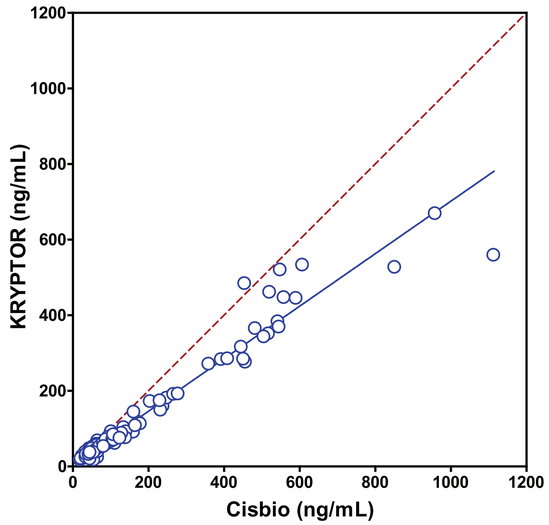

Figure 1. KRYPTOR CgA II vs. Cisbio CgA ELISA. Deming regression of 149 split samples. Slope = 0.695 ± 0.014 (95% CI, 0.668 – 0.722); y-intercept = 7.215 ± 3.186 (95% CI, 0.919 – 13.51); r^2 = 0.947.

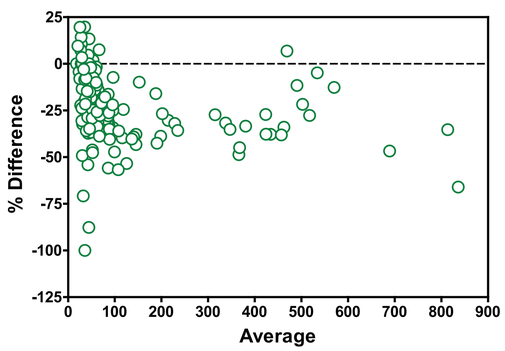

Figure 2. Bland-Altman Plot, KRYPTOR CgA II vs. Cisbio CgA ELISA. Percent difference of the KRYPTOR - Cisbio CgA method comparison results vs. average. Bias = -23% (95% limits of agreement: -61 to 15%).

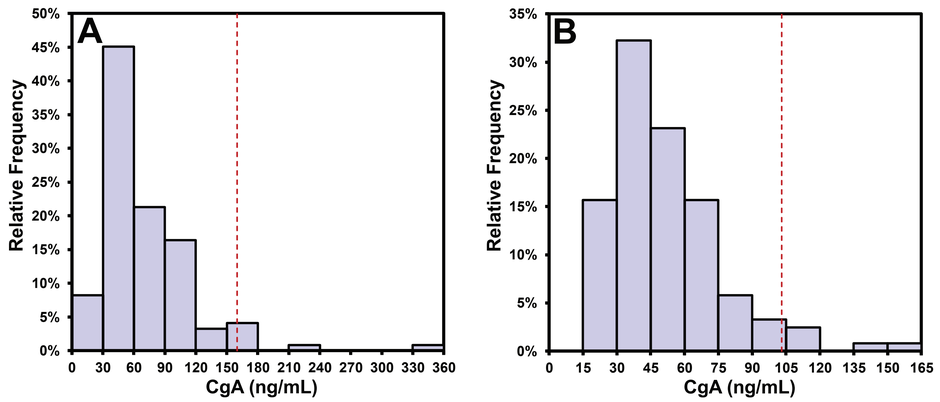

Figure 3. CgA Reference Limits. Non-parametric reference limits calculated at the 95th percentile. (A), Cisbio CgA ELISA, 160 ng/mL (90% CI, 128 – 237 ng/mL). (B), KRYPTOR CgA II, 103 ng/mL (90% CI, 85 – 145 ng/mL). Reference limits indicated by red dashed lines.

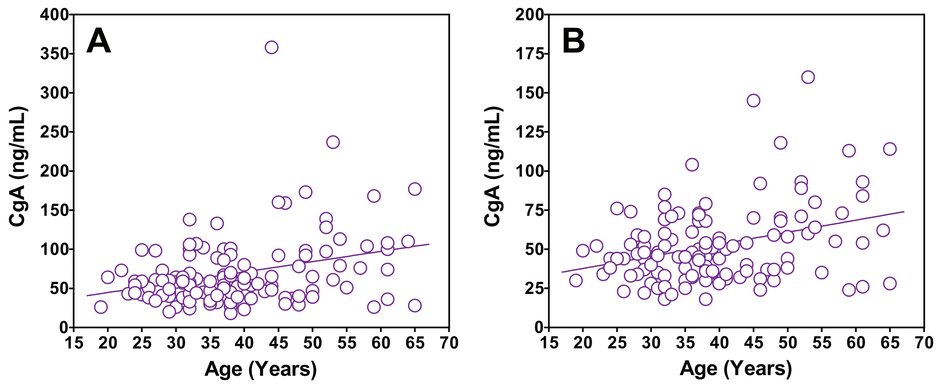

Figure 4. CgA vs. Age. Reference limit CgA results vs. volunteer age. Slight but significant increases in CgA concentration were observed in the assays. Cisbio, p = 0.0006 (r^2 = 0.095); KRYPTOR, p = 0.0002 (r^2 = 0.095). However, these increases were considered clinically insignificant.

CONCLUSIONS

- There is good correlation between the Cisbio Bioassays Chromogranin A ELISA and the B·R·A·H·M·S CgA II KRYPTOR assay.
- Although the correlation between assays is adequate, the agreement is poor.
- Reference limits for the Cisbio and KRYPTOR CgA assays cannot be interchanged because of the substantial disagreement between assays.
- Although gender does not appear to influence CgA concentrations, a slight but clinically irrelevant increase is observed with age for both assays.

ACKNOWLEDGMENTS

We would like to thank Kisha Fisher for volunteer recruitment and specimen collection. We also thank the ARUP Special Chemistry technical section and Chris Ciotti of Thermo Fisher Scientific for technical assistance.

Financial support provided by the ARUP Institute for Clinical and Experimental Pathology.

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