Biological variation of neuroendocrine tumor markers chromogranin A and neuron-specific enolase

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ARTICLE INFO

Article history:
Received 19 April 2012
received in revised form 6 September 2012
accepted 10 September 2012
Available online 18 September 2012

Keywords:
Biological variation
Chromogranin A
Neuron-specific enolase

ABSTRACT

Objectives: Chromogranin A (CgA) and neuron-specific enolase (NSE) are biomarkers for neuroendocrine tumors. Although the knowledge of their biological variation (BV) is critical, only one study for CgA and no data for NSE are available. We report a definitive assessment of BV components of these biomarkers in the same cohort of subjects by an accurately experimental protocol.

Design and methods: We collected five blood specimens from each of 22 healthy volunteers (10 men and 12 women, 23–54 years) on the same day every two weeks for two months. Serum specimens were stored at −80 °C until analysis and analyzed in a single run in duplicate. Data were analyzed by ANOVA.

Results: Serum CgA concentrations were significantly higher for women than for men (P = 0.01), whereas no difference was found for NSE. Intra-individual variance was not different between genders for both biomarkers. Within- and between-subject CVs were 16.3% and 33.5% for CgA and 13.6% and 11.5% for NSE, respectively. CgA showed marked individuality, suggesting that the use of population-based reference limits is inadequate for its interpretation. Conversely, the low individuality of NSE allows the use of a single reference interval. Reference change values were 46% for CgA and 39% for NSE. Desirable analytical goals for imprecision, bias, and total error were <8.2%, ±9.3%, and ±22.8% for CgA, and <6.8%, ±4.5%, and ±15.7% for NSE, respectively.

Conclusion: In this study, we defined BV components of serum CgA and NSE and derived indices that may improve the clinical use of these biomarkers.

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Introduction

Chromogranin A (CgA) is a hydrophilic glycoprotein released by post-ganglionic sympathetic neurones and adrenal chromaffin cells. CgA acts as a pro-hormone that undergoes maturation by proteolysis, leading to the production of bioactive peptides with different paracrine, autocrine, and endocrine functions. Neuron-specific enolase (NSE) is a glycolytic enzyme located in central and peripheral neurons and neuroendocrine cells, as well as in erythrocytes and platelets. Both markers have been recommended for evaluation of neuroendocrine tumors [1,2].

In spite of the clinical role of CgA and NSE, aspects related to their biological variation (BV) have not received enough attention. Only one study determining BV of CgA is available in literature [3], while no data are available for NSE. BV information is essential for the definition of analytical goals for biomarker measurements [4]. Furthermore, the knowledge of BV is the necessary prerequisite for the calculation of reference change value (RCV), i.e. the minimal difference that must be exceeded for a change in two consecutive marker results in the same individual to become clinically relevant, which depends on both analytical and within-subject biological (CV) CVs [4]. Finally, the utility (or lack of it) of conventional population-based reference intervals (RI) for biomarker interpretation can be assessed from its BV [5]. Here we report an assessment of BV of CgA and NSE in the same cohort of subjects by paying extra attention to pre-analytical sources of variability and statistical analysis of data.

Abbreviations: CgA, chromogranin A; NSE, neuron-specific enolase; BV, biological variation; RCV, reference change value; CV, within-subject CV; RI, reference interval; TRACE, Time-Resolved Amplified Cryptate Emission; HI, hemolysis index; CI, Cochran’s test; II, index of individuality; n, number of specimens required to ensure that the mean biomarker result is within ±15% of the individual’s homeostatic set point; IH, index of heterogeneity.

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http://dx.doi.org/10.1016/j.clinbiochem.2012.09.005
Materials and methods

Study design

We collected five blood specimens from each of 22 apparently healthy volunteers (10 men and 12 women; ages 23–54 years) on the same day every two weeks for two months. In accordance with the Helsinki II Declaration, the design and execution of the experiment were explained thoroughly to the subjects, and informed consent was obtained. The inclusion criteria were that the subjects should be within 80–120% of ideal body weight, have no previous or present gastro-pancreatic, cerebral, or pulmonary disorders, and for women, that they have regular menstrual cycles and not use hormonal contraceptives. None took any medication, consumed substantial (>10 g of ethanol/day) quantities of alcohol or was smoker. Venous blood was obtained between 0900 and 0930 from subjects who had overnight fasted and had not exercised that morning. Samples were collected by the same phlebotomist with minimal stasis in 4-mL vacuum collection tubes with no anticoagulant and polymer gel for serum separation (Becton Dickinson Vacutainer® ref. 369032). Serum specimens, separated by centrifugation, were aliquoted and stored at −80 °C until analysis. When all specimens were available, they were thawed, mixed, centrifuged, and analyzed in a single run in duplicate in random order. To further minimize analytical variation, a single analyst performed all the assays using single lots of reagents. CgA was determined on ThermoFisher Kryptor® analyzer using Brahms immunoassay. The assay uses Time-Resolved Amplified Cryptate Emission (TRACE™) technology. NSE was measured on Modular Analytics EVO platform (Roche Diagnostics) by an electrochemiluminescent immunoassay. Before assaying specimens, each serum aliquot was checked by estimating hemolysis index (HI) on Roche Integra 800 platform to exclude significant interference by erythrocyte NSE. As previously reported [6], visible hemolysis is defined for free hemoglobin concentrations in serum above 0.30 g/L, which approximately correspond to an HI of 30 on the Integra system. Analytical systems were employed in strict accordance with the manufacturer’s instructions.

Statistical analysis

The analytical, within-subject, and between-subject components of variation were calculated by nested ANOVA from replicate analyses [4]. Formulae used to estimate these and other variables have been reported [7]. Briefly, analytical variance was estimated from the duplicate results for each specimen, within-subject biological variance from the total within-subject variance (referred to as the variance of the mean of duplicate assays) minus one-half the analytical variance, and between-subject biological variance from the total variance of the data minus the analytical and intra-individual components. All the components of variance were then transformed to the relevant CV using the overall means. Cochran’s test (Ct) was performed for outlier identification among observations and within-subject variances, whereas Reed’s criterion was used for identification of outliers among mean values of subjects [4]. After outlier exclusion, a normality test (Shapiro–Wilk test) was applied separately to the set of results from each individual to check data distribution and validate the normality hypothesis. Student’s unpaired t-test was used to compare the mean values and the F-test was applied to assess the difference in intra-individual variances. The index of individuality (II), yielding information about the utility of conventional population-based RI, the RCV, and the number of specimens (n) that should be collected to estimate the homeostatic set point of an individual within ±15% were estimated [7]. To study the heterogeneity of within-subject variation, we estimated the index of heterogeneity (IH), i.e. the ratio of the observed CV of the set of individual variances (including analytical variance) to the theoretical CV, which is \[\frac{2}{(k-1)} \] where k is the number of specimens collected per subject. The SD of the difference between this ratio and its expected value of unity (under the hypothesis of no heterogeneity of true variance).
within-subject variances) is $1/(2k)^{1/2}$. A significant heterogeneity is present if the ratio differs from unity by at least twice the SD [4]. In our study, with five data for each subject, an $\text{HI} \leq 0.632$ indicates that the within-subject data are homogeneous. Finally, optimal, desirable, and minimum analytical goals for imprecision, bias and total error for CgA and NSE determination were obtained from BV components [8].

Results

The study involved collection of 110 specimens for both CgA and NSE, each assayed in duplicate yielding 220 analytical results. For NSE determinations, no sample was found affected by visible hemoly-

sis [mean HI (±SD) = 9.8 (±4.9)]. Regression analysis between HI values and mean NSE concentrations gave a coefficient of determination of 0.22, indicating a negligible relationship between the two vari-

ables in our samples. No observation was removed as statistical outlier (Ct value, 0.08 for CgA and 0.04 for NSE; $P>0.05$). One within-subject variance was detected as statistical outlier for CgA. After removing the outlier subject, Ct was repeated and no further within-subject variance was found as outlier (Ct value, 0.19; $P>0.01$). Using Reed’s criterion, one NSE mean value was detected as statistical outlier. After removing the outlier subject, the Reed’s cri-

terion did not find any further outlier. In summary, the suitable sub-

jects for BV estimate were 21 (10 men, 11 women) for CgA and 21 (9 men, 12 women) for NSE, respectively. As the Shapiro–Wilk test accepted the hypothesis of normality in a substantial proportion of subjects (86% for CgA and 91% for NSE), the parametric statistical model was considered appropriate for the estimation of variance components.

Table 1 gives overall mean and absolute range of values for CgA and NSE in studied subjects, whereas Table 1 gives overall mean values and estimated average analytical and biological variations (as CV) for all subjects, and separately for men and women. II, RCV, $n$, and analytical goals are also reported. Serum CgA concentrations were significantly ($P=0.01$) higher for women than for men, whereas no difference was found for NSE. CV$_i$ was not different between genders for both biomarkers ($P=0.30$ for CgA and $P=0.45$ for NSE, respectively). The calculated HI (0.768 for CgA and 0.805 for NSE) did not fulfill the homogeneity condition and, although RCVs docu-

mented in Table 1 may be used as a simple single figure to guide clinical decision making, they are not ubiquitously valid.

Discussion

In the sole study evaluating BV of CgA, plasma-EDTA samples were used [3]. This is not a marginal issue as blood collection into tubes containing EDTA could introduce a source of pre-analytical variability in CgA determination. Previous studies have shown markedly higher CgA values in plasma when compared with serum [9]. Thus, to avoid possible interferences we used tubes containing no anticoagu-

lants or gel separator. Except for the sample type, the protocol by Dittadi et al. [3] was similar to ours and the obtained results were quite comparable (CV$_i$ and between-subject CV were 16.3% and 33.5% in our study vs. 12.8% and 26.3% in the previous study, respec-

tively). In our study, CgA concentrations were higher in women than in men. Two previous publications [3,10] reported that CgA concen-

trations were not gender dependent, whereas another paper de-

scribed higher CgA concentrations in men [11].

The II provides information about the utility of using population-

based RI for test interpretation [5]. If the II is low ($<0.6$), then the use of RI is of very limited value in the detection of unusual results for a particular individual and may be misleading. If II is $>1.4$, then the RI is of significant utility. For CgA, II was $<0.4$ indicating that classical RI has little use in the interpretation of CgA results. In addition, five samples are required to estimate an individual’s CgA value within 15% of the true mean value. This substantially high individuality of CgA exposes the flaw in the argument that this marker should be utilized to diagnose neuroendocrine tumors. Furthermore, condi-
tions such as treatment with proton pump inhibitors, heart failure, renal dysfunction or chronic atrophic gastritis can significantly interfere with CgA diagnostic value [12]. CgA may be a useful adjunct only in monitoring neuroendocrine tumors where RCV and results of recent analyses for an individual are used as a pathology guide in that individual. On the other hand, for NSE (II = 1.44), population-based RI may be of value in assessing patients’ results.

In our study, analytical goals were derived from BV data according to the conventional model of Fraser et al. [8]. However, some authors have questioned this approach, by proposing an alternative model in which the maximum allowable bias and imprecision are interrelated and described in a curve and the allowable total error calculated from each point of the graph [13]. Using this approach, an overestimation of the al-

lowable total error according to the classical Fraser’s model was demonstrated [14]. Differences in magnitude of calculated allowable total error may influence, e.g., the evaluation of performance of participating labora-

tories in External Quality Assessment Schemes, so that the possible impact of an allowable total error overestimation has to be considered in assessing quality of laboratory measurements. Even with some limi-
tations, the Fraser’s model remains, however, the most widely used ap-

proach for definition of analytical goals in Laboratory Medicine [15].

Acknowledgments

We thank Doriana Basco for skilful technical assistance and all those who provided blood specimens for their willing and enthusiastic participation. We also thank ThermoFisher Brahms for the generous loan of the Kryptor® instrument and CgA reagents to carry out the study.

References