Biological Variation and Reference Change Value Data for Serum Neuron-Specific Enolase in a Turkish Population

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Background: Neuron-specific enolase (NSE) is a recognized biomarker for the assessment of cerebral injury in neurological disorders. This study aims to report a definitive assessment of the biological variation (BV) components of this biomarker, including within-subject BV (CVI), between-subject BV (CVG), index of individuality (II), and reference change value (RCV), in a cohort of Turkish participants using an experimental protocol. **Methods:** Six blood specimens were collected from each of the 13 apparently healthy volunteers (seven women, six men; ranging in age from 23 to 36) on the same day, every 2 weeks for 2 months. Serum specimens were stored at -20°C until analysis. Neuron-specific enolase levels were evaluated in serum samples using an electrochemiluminescence (ECLIA) immunoassay kit with a Roche

Cobas e 411 auto-analyser. ANOVA test was used to calculate the variations. **Results:** The CVI and CVG for NSE were 21.5% and 28.8%, respectively. Analytical variation (CVA) was calculated as 10.2%. Additionally, II and RCV were calculated as 0.74 and 66% (95% confident interval, CI), respectively. **Conclusion:** As the performance index (PI) was found to be less than 2 ($\text{PI} = 0.95$), it is concluded that the NSE measurements have a desirable performance for analytical imprecision. Since the II was found to be less than 1 ($\text{II} = 0.74$), the reference values will be of little use. Thus, RCV would provide better information for deciding whether a significant change has occurred. *J. Clin. Lab. Anal.* **30**:1081–1085, 2016. © 2016 Wiley Periodicals, Inc.

Key words: analytical variation; between-subject BV; index of individuality; neuron-specific enolase; reference change value; within-subject BV

INTRODUCTION

Neuron-specific enolase (NSE) is one of the biomarkers that are most frequently used for the assessment of cerebral injury in neurological disorders (1, 2). NSE is released into the systemic circulation after neurologic damage, intracerebral hemorrhage, including ischemic stroke, and traumatic or hypoxic parenchymal brain injury (1, 3). Several studies have reported an association between increased serum NSE levels or cerebrospinal fluid (CSF) NSE levels and central nervous system (CNS) damage and neurological diseases [acute ischemic stroke (4), traumatic brain injury (5), Alzheimer's disease (6), delirium (7) and epileptic seizures (8)]. In addition to cerebral injury, increased serum NSE levels have also been found to be associated with essential hypertension (9), lung

cancer (10), acute carbon monoxide poisoning (11), pediatric cancer (12), renal cell carcinoma (13), diabetic peripheral neuropathy (14), and multiple myeloma (15).

Enolases are dimers that result from the association of three distinct subunits (α , β , and γ). NSE is the γ isoform of the glycolytic enzyme enolase with a molecular weight of 78 kDa, expressed primarily in neurons in the central and peripheral neuroendocrine cells (1, 16). Moreover, a small amount NSE is found in

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erythrocytes and platelets (17, 18), which explains its physiologically low concentrations in blood (18). NSE is not actively secreted into the bloodstream; rather, it is passively released by cell destruction (1). In addition to indicating the progression of primary damage, NSE levels also indicate the progression of secondary damage. Therefore, NSE has perfect theoretical potential as a long-term prognostic biomarker and therapeutic indicator in neurological intensive care (19).

Biological variation (BV) data are part of the comprehensive evaluation required for analytes that are measured in a clinical laboratory, and they are an essential prerequisite for the introduction of new analytes (20, 21). Information about BV is also a prerequisite for the calculation of reference change value (RCV), that is, 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 variations (CV_A) and within-subject biological variations (CV_I) (22–24). Unfortunately the use of non-standardized methodology and terminology in BV studies is still a significant and problematic issue. Recently, two different research groups have reported on methodology criteria that can be used in BV studies (25, 26).

Interestingly, BV issues have not received enough research attention in spite of the clinical importance of NSE. Very few studies examining the BV components of NSE are available in the literature (22, 27). To our knowledge, this present study is the first research study conducted using the new methodology criteria for BV studies. Without information on BV, it is difficult to objectively assess serial patient results as well as data on internal control and quality assessments. In order to correctly evaluate the potential use of this new biomarker, NSE, sufficient knowledge about the BV components of the analyte and the analytical quality of the method is needed. For this purpose, we aimed to calculate detailed BVs and determine the parameters of NSE.

MATERIALS AND METHODS

Study subjects

The study group consisted of 13 healthy volunteers (seven women, six men; ranging in age from 23 to 36) from Southern Turkey. The study protocol was approved by the local ethics committee, and all of the participants provided their written informed consent to participate in the study. Interviews were conducted, health questionnaires were distributed, and routine blood and biochemical tests were administered to ensure that all of the participants led healthy lifestyles,

had a relatively healthy medical status and did not take any medications. The participants' weight and height were measured using a standard protocol, and their body mass indexes (BMIs) were calculated; the BMIs of all of the participants were in the normal range: 18.5–24.9. Additionally, none of the enrolled women were pregnant or currently menstruating during the blood collection. During the study period, the participants maintained their normal lifestyles, which involved no excessive consumption of alcohol, tea, or tobacco products, and no strenuous exercise.

Specimen collection

Blood was collected under standardized conditions in order to minimize sources of pre-analytical variation. We collected six blood specimens according to Wu AHB's (28) sampling criteria from each of 13 healthy volunteers, on the same day every 2 weeks for 2 months, between April 2015 and June 2015. All of the samples were collected by the same phlebotomist between 8:00 and 9:00 in the morning; the participants had not engaged in any morning exercise, and the samples were taken after the participants had been resting for 5 min in a seated position. The samples were collected with minimal stasis in 5-ml vacuum collection tubes with no anticoagulant and polymer gel for serum separation (Becton Dickinson Vacutainer® ref. 369032). All of the blood samples with visible hemolysis were discarded. Serum specimens, separated by centrifugation within 1 h, at 3000 g for 10 min, were aliquoted and stored at -20°C until analysis (within 10 weeks). When all of the specimens were available, they were thawed, mixed, again centrifuged (at 3000 g for 10 min) and analyzed at room temperature in a single run, in duplicate, and in random order.

Measurement of the analyte

To further minimize analytical variation, the same lots of reagents, standards, and quality control materials were used throughout the analysis, and all of the analyses of a particular analyte were performed by the same analyst. Serum NSE levels were analyzed with electrochemiluminescence immunoassay (ECLIA) using a standard assay kit for in vitro diagnostics (NSE kit catalog number: 12133113122, Roche Diagnostics GmbH, Mannheim, Germany). Reactions and quantitation were performed in duplicate using a fully automatized Cobas e 411 analyser (Roche Diagnostics GmbH, Mannheim, Germany). The quality of the assay was monitored using internal software and controls provided by the manufacturer. The NSE γ subunit was analyzed using the manufacturer's protocol.

The measurement values of the NSE method ranged from 0.05 ng/ml to 370 ng/ml. Functional sensitivity was set at 0.25 ng/ml, and the expected normal values were <17.0 ng/ml.

Statistical analyses

Microsoft Office Excel 2007 and ANOVA tests were used for the calculations. Analytical variation (CV_A) was calculated from the duplicate results of each sample. Mean and standard deviation (SD) of the duplicate results were calculated, and the following formula, $CV_A = SD/mean \times 100$, was used to calculate the within-run $CV_A\%$. Before performing calculations on the results, the Cochran test was used to exclude the outlying values from the individual participants, and the Reed test was used to eliminate the mean outlying values. The mean, median, SD, and variation in the NSE results were calculated for each study participant. The ANOVA test was used to estimate CV_A plus CV_I (CV_{A+I}). CV_I was calculated using a subtraction step with the two previous variables ($CV_{A+I} - CV_A$). Between-subject BV (CV_G) was obtained by subtracting the CV_{A+I} from the total variation (CV_T), which was found using all of the data from all of the study participants (Fig. 1).

The index of individuality (II) was calculated using the CV_I/CV_G ratio (Fig. 1). The RCVs for the analytes at various probabilities, which were applied to either a single quantity or to two combined quantities, were calculated using the following formula: $RCV = 2^{1/2} \times Z \times (CV_A^2 + CV_I^2)^{1/2}$ (Fig. 1).

RESULTS

The CV_I and CV_G for NSE were 21.5% and 28.8%, respectively. CV_A was calculated as 10.2%. The desirable performance index (PI) was defined as $CVA/0.5CVI < 2.0$ (20, 29). These results showed that our

data was reliable for calculating BV. The II of the NSE was 0.74. When the index was less than 1, two consecutive results from a subject could be outside the RCV, but well within the population-based reference interval. Thus, the reference values would be of little use, in particular, for deciding whether a significant change had occurred. For this reason, RCV was calculated and found to be 66% for NSE (at 95% CI). The results of the NSE calculations for CV_A , CV_I , CV_G , RCV, and II (CV_I/CV_G) are presented in Table 1. Additionally, Fig. 2 shows the median and distribution range of the NSE concentrations for each study participant.

DISCUSSION

In laboratory medicine, it is essential to consider BV in order to provide reliable results. Thus, clinical laboratories should minimize all the sources of variations related to laboratory processes, estimate the components of BV and appropriately manage them during the entire process leading to the laboratory report (30). Information about the BV components of newly discovered biomarkers can be found in the primary literature. Most studies on BV are conducted on an appropriate population of healthy participants. It is essential to understand the variations in healthy situation before the test results can be applied to diseased patients (28).

In this present study, we analyzed the CV_A , CV_I , CV_G , RCV, and II of NSE. In addition, we compared

TABLE 1. Analytical variation (CV_A), within-subject (CV_I) and between-subject (CV_G) biological variations, index of individuality (II), and reference change values (RCV) of NSE

	CV_A (%)	CV_I (%)	CV_G (%)	II	RCV (%)
NSE	10.2	21.5	28.8	0.74	66

$$\begin{aligned}
 CV_I &= (CV_{A+I}^2 - CV_A^2)^{1/2} \\
 CV_G &= (CV_T^2 - CV_I^2 - CV_A^2)^{1/2} \\
 II &= CV_I / CV_G \\
 RCV &= 2^{1/2} \times Z \times (CV_A^2 + CV_I^2)^{1/2}
 \end{aligned}$$

Fig. 1. Formulas used to calculate CV_I , CV_G , II, and RCV. CV_I : Within-subject BV, CV_{A+I} ; Total within-subject BV, CV_A ; Analytical variation, CV_G ; Between-subject BV, CV_T ; Total variation, II; Index of individuality, RCV; Reference change value. Z; probability selected for statistical significance ($Z = 1.96$ at 95% confident interval, CI).

Fig. 2. The median (dark points) and the distribution range of the NSE concentrations for 13 healthy individuals.

the BV of NSE with the data obtained from previous studies (22, 27). In our study, CV_A and PI was calculated as 10.2% and 0.95, respectively. This result showed that our data could be used to calculate BV. The CV_A , CV_I , and CV_G results for NSE in this present study were higher than the results in the study conducted by Qi et al. (27). The difference between those results and our results could be due to the different analytical systems that were used to measure NSE. Additionally, in our study the II of the NSE was found to be 0.74. Similarly, in Qi et al. (27) the II of NSE was 0.78. When the II is <1 , the reference interval is inappropriate (27). Because the II for NSE is less than 1 (II: 0.74) in this present study, the conventional reference values for the NSE biomarker might have limited usefulness in a clinical setting, in particular when deciding whether the changes observed in an individual are clinically significant. Instead, the use of RCV would provide better information for deciding whether a significant change has occurred (31, 32). For this reason, we calculated RCV and found it to be 66% for NSE (at 95% CI). On the other hand, in the study conducted by Qi et al. the RCV of NSE was found to be 37.05% (at 95% CI). The difference between our result and that study's result might be due to differences in the CV_A value obtained in each of the studies. In our study, the CV_A of NSE was found to be 10.2, and the CV_A of NSE in Qi et al.'s study was 2.12. The use of different methodologies could also be an important reason for the differences in the CV_A results between our study and the Qi et al. study. In contrast, Braga et al. (22) calculated the II of NSE as 1.44, although they stated that population-based reference intervals could be better for evaluating NSE results. Currently, several commercially available NSE immunoassays are available from different manufacturers, but there is no common standard. Several analytical and pre-analytical factors might affect the measured result (33). It is known that these measuring methods result in significant differences in NSE findings (17). Therefore, in our study, to minimize the effects of pre-analytical variations the samples were obtained and processed by the same phlebotomist. Moreover, the collection schedule for each of the study participants was designed at the same time. To reduce the analytical variation, one analyst performed all the analyses, using the same reagent batch lots, quality control material, and calibrators. The control material was included in each of the analytical series to guarantee that the analytical imprecision was within the acceptable limits according to our internal quality control protocol. In addition, an external quality assurance analysis (Bio-Rad Program, CA) was performed on all of the analytes during the study period.

CONCLUSION

In conclusion, knowledge of the BV parameters of NSE might be important for the optimum use of this test in clinical laboratories. All of the possible BVs of NSE should be considered in order to increase the reliability of the results. Additionally, future studies that focus on the RCV values of NSE in different ongoing clinical conditions are needed, which could be more helpful for clinicians.

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