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Biological Variation in Protein C, Protein S and Antithrombin Concentrations in Plasma of Healthy Subjects

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Summary: The analytical, intra- and inter-individual components of biological variation were estimated for protein C, protein S and antithrombin over a period of 6 weeks in a cohort of 17 apparently healthy subjects. Expressed as percentage activity (protein C and antithrombin) and as percentage concentration in normal human plasma (protein S), the mean values for men and women show no significant differences ($p > 0.05$) for any of the analytes studied. Calculated analytical goals (CV, %) for precision required for optimal patient care are: protein C, 2.9; protein S, 2.9 and antithrombin 2.7. A single numerical index, called "index of fiduciality", was also calculated to demonstrate that the analytical performance of our method was satisfactory. The generally applicable differences (% activity or % concentration) required for two results to be significantly different ($p \leq 0.05$) were calculated as: protein C, 17; protein S, 16 and antithrombin, 16. The usefulness of critical differences as guidelines for the interpretation of changes in serial results was investigated using an "index of heterogeneity" of intra-individual variation.

The marked degree of individuality demonstrated for all the quantities indicates that, if conventional population-based ranges are used uncritically, major changes in analyte concentration may not be correctly identified for some patients, because observed values continue to lie within the reference range. The utility of conventional population-based reference intervals was determined by calculating a single numerical index, called "index of individuality". For protein C, protein S and antithrombin we found that five of a patient's specimens are required to achieve a 95% chance that the mean is within ± 5% of the true value.

Introduction

Data on the biological variation of analyte concentrations have many valuable uses in clinical chemistry (1), including setting desirable performance standards for imprecision (2), determining the true significance of changes in serial results from a single patient (3) and assessing the usefulness of conventional population-based reference ranges (4).

Although many data are available on the biological variation of analytes in serum or plasma (5–7), the true biological variation of the three naturally occurring anticoagulant proteins, protein C, protein S and antithrombin (8–9), has not been investigated in depth. A previous study on the biological variation of antithrombin has been reported (3), but in that article the discussion was limited to the intra-individual variation and critical difference.

Since the values of these quantities are widely used in clinical decision making, we believe that their biological variation should be examined.

We therefore assessed their biological variation in a cohort of 17 apparently healthy subjects, collecting six specimens from each subject over a period of six weeks.

Materials and Methods

Subjects

Seventeen apparently healthy hospital staff members (nine females and eight men, ages 21 to 52 years), informed about the objective of the study, were recruited. They were non-smokers, and had taken no medication in the preceding two months. They maintained their usual lifestyles throughout. None of the women was pregnant or using oral contraceptives.

Specimen collection and handling

Preparation of the subjects before blood collection was carefully controlled, and blood collection was performed in standardized conditions to minimize sources of preanalytical variation. After an overnight fast, a blood specimen was taken with minimal venous occlusion, between 8 and 10 a. m. to avoid the influence of circadian variation on the plasma concentrations of the studied quantities. Six consecutive blood samples were taken weekly from each subject. All samples were drawn by the same phlebotomist from subjects in a supine position. The first 4 ml were discarded, and venous blood was then sampled into evacuated tubes containing 129 mmol/l trisodium citrate (Venoject VT-050SCBS, Terumo-Europe, Leuven, Belgium). Plasma was obtained by centrifugation (2000 *g*, and 4 °C for 10 min) and stored at −80 °C until analysis. At the end of 6 weeks, all frozen samples were transferred into a

water bath at 37 °C for 15 minutes and then handled at room temperature.

Analytical techniques

The analytical protocol was designed to minimize analytical variance both within and between batches of analyses. Each set of specimens from an individual was assayed in duplicate, in no specific sequence, within the same analytical batch. Single lots of reagents, standards and quality-control materials were used throughout, and analyses were performed by a single operator. Comparability between runs was ensured by rigorous quality control. The activities of protein C and antithrombin were measured on a Hitachi 911 analyzer using a synthetic chromogenic substrate (antithrombin and Stachrom protein C, Boehringer Mannheim, Mannheim, Germany), while quantitative determination of protein S concentration was performed using a microlatex particle-mediated immunoassay (Liatest protein S, Boehringer Mannheim, Mannheim, Germany).

Statistical analysis of data

The detailed approach advocated by *Fraser & Harris* (1) was followed. After exclusion of outliers, nested analysis of variance (ANOVA) was applied. The total variance was divided into the components attributable to analytical, intra- and inter-individual variation. Since, in assay procedures, quality control materials may not behave in the same way as specimens from patients (10), we calculated analytical variance from the results of duplicate analyses of each specimen. We used only the first of each duplicate result to calculate the average intra-individual variance and the inter-individual variance. *Student*'s unpaired t-test was used to assess whether the means for men and women were different.

Results and Discussion

Analytical results

The mean values for men and women showed no significant difference ($p < 0.05$) for each of the analytes studied (fig. 1). The data generated were therefore treated as a single set of data.

Table 1 shows the mean values and estimated analytical (CVA), intra- (CVG) and inter-individual (CVI) variation as coefficients of variation of protein C, and antithrombin percentage activity and protein S percentage concentration in normal human plasma.

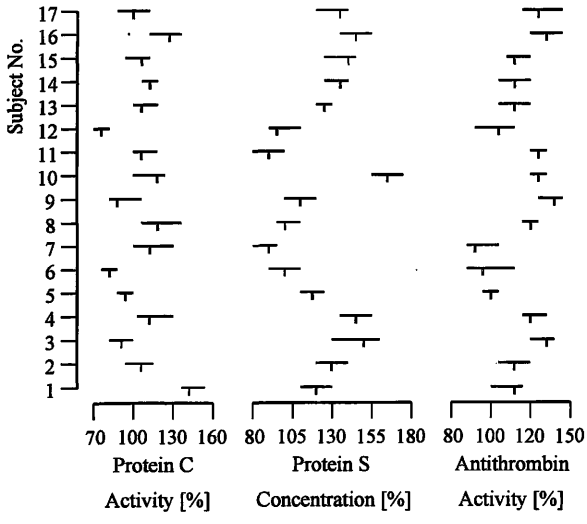

Fig. 1 Means and ranges for protein C, protein S and antithrombin in sera from 17 healthy subjects. 1–8: male; 9–17: female.

The data in the table 1 were used to:

- derive analytical goals for each quantity analysed,
- determine the changes required in serial results, termed critical difference (3), before significance can be claimed,
- assess the usefulness of conventional population-based reference values,
- estimate the number of specimens required to produce a more precise estimate of the homeostatic set point of an individual.

Analytical goals

Stipulated standards of performance are necessary for the objective analysis of the data generated during the analytical investigation. It is widely accepted that analytical goals, the standards of analytical performance required to provide optimal patient care, are best derived from data on biological variation (11). Goals for imprecision were derived as 1/2CVI (2). The goals for imprecision for all quantities were met in this study, as shown in table 2.

A single numerical index, called "index of fiduciality", was also suggested to demonstrate whether a method met the analytical goals or not (12). It is calculated as the total analytical error achieved (CVA) divided by the analytical goal. If this index is ≤ 1, then the analytical goal is met. The calculated indices of fiduciality, de-

Tab. 1 Mean values, and estimated average analytical (CVA), intra-individual (CVI) and inter-individual (CVG) variation in healthy subjects for protein C, protein S and antithrombin.

Analyte	Mean[a] (%)	CVA (%)	CVI (%)	CVG (%)
Protein C	106.8	2.1	5.8	55.2
Protein S	117.6	2.0	5.8	63.4
Antithrombin	119.1	1.8	5.5	25.2

[a] Mean values are expressed as percentage activity for protein C and antithrombin and percentage concentration in normal human plasma for protein S.

Tab. 2 Estimated indices of individuality, heterogeneity, fiduciality, analytical goals for precision, critical differences between serial results and required numbers of specimen collections to produce a more precise estimate of the homeostatic set point in healthy subjects for protein C, protein S and antithrombin.

	Protein C	Protein S	Antithrombin
Index of individuality	0.1	0.1	0.2
Index of heterogenity	0.65	0.64	0.64
Index of fiduciality	0.72	0.68	0.66
Analytical goal, CV (%)	2.9	2.9	2.7
Critial difference (%)	17	16	16
No. of specimens required	5.8	5.7	5.1

tailed in table 2, provide evidence that the analytical performance of our method was satisfactory.

Critical difference

The results of biochemical tests are often used in monitoring patients. It is therefore important to know the magnitude of the changes in serial results from an individual that amount to statistically significant differences. Such changes may be due not only to improvement or deterioration of the patient but also to analytical imprecision and intra-individual biological variation. Therefore, for a change to be significant, it must exceed the critical difference, which can be defined as the total variation required in serial results from an individual before significance can be claimed. For $p \leq 0.05$ the critical difference can be calculated as $2.77(CVA^2 + CVI^2)^{1/2}$ (3). The calculated critical differences for protein C, protein S and antithrombin are listed in table 2.

Use of the average CVI to calculate critical differences is truly valid only if all subjects have the same CVI. This was investigated using an "index of heterogeneity" of intra-individual variation. This index is defined as the ratio of the observed CV of a set of individual variances $(CVA^2 + CVI^2)^{1/2}$ to the theoretical CV if no heterogeneity existed, namely $[2/(n-1)]^{1/2}$, where n is the average number of observations per subject (13). If there were no heterogeneity of intra-individual variances, this ratio would be 1.00. When the index differs from unity by more than 2 SD, defined by $2/(2n)^{1/2}$, then significant heterogeneity exists. The calculated indices of heterogeneity for the analytes investigated are described in table 2. Since no index exceeds 1.00 by more than 2 SD, we believe that the critical differences detailed here are useful in clinical practice as guidelines in the interpretation of changes in serial results.

Our data on the critical difference and the CVI of antithrombin monitored over a six weeks period appear to be of the same order as those observed over six months by *Costongs* et al. (i.e., critical difference, 19.6%; CVI, 6.6%) (3). In *Costongs'* work the intra-individual variations and the critical differences of 9 coagulation quantities were investigated during short-term (within-one-day) and long-term (six months) periods. The antithrombin long-term CVI was found to be much smaller than the short-term one (16.3%), highlighting the fact that variations over the day should therefore be interpreted carefully.

Usefulness of conventional population-based reference values

Reference intervals are of particular importance when a test is used for screening or in making an initial diagnosis, when no previous data are available on an individual. The dispersion of conventional reference intervals is due to a composite of analytical, intra- and inter-individual variation. Conventional population-based reference values are of use only when the intra-individual variability exceeds the inter-individual variability. The ratio of the intra- to inter-individual biological variation, known as the "index of individuality" provides information on the utility of conventional population-based reference intervals (14). When this index is less than 0.6, reference values are of limited utility, but when it is more than 1.4, such values are of considerable use. Table 2 shows the indices of individuality for protein C, protein S and antithrombin. For all the quantities, the index is < 0.6. This high degree of individuality means that subjects could have values that are very unusual for them but still lie within the conventional reference interval. Thus, the analytes examined in this study, although commonly assayed in clinical chemistry laboratories, will be of little use in the diagnosis of early or latent disease, or as general population-screening tests.

Number of specimens required

Since many analytes show considerable intra-individual variation, analyses of multiple specimens will produce a more precise estimate of the homeostatic set point. The required number of specimen collections needed to estimate the mean to within a desired precision, may be calculated from a rearrangement of the usual formula for the standard error of the mean:

$$n = [Z(CVA^2 + CVI^2)^{1/2}/D]^2,$$

where n is the number of specimens required, Z the number of standard deviates required for a stated probability under the normal curve, and D is the desired percentage closeness to the homeostatic set point (1).

For protein C, protein S and antithrombin we found that five of a patient's specimens are required to achieve a 95% chance that the mean is within ± 5% of the true value.

Conclusions

Data on analytical, intra- and inter-individual variation support the following conclusion:

– analytical goals derived from data on biological variation for protein C, protein S and antithrombin indicate that the standard of analytical performance achieved at present may be adequate for ideal patient care;

– the intra-individual variation exhibited by protein C, protein S and antithrombin means that, even when analytical goals for precision are met, relatively large differences (i.e., 16–17%) between sequential results are re-

quired before two values can be said to be significantly different;

– population-based reference ranges are of limited value for the interpretation of results of measurements of protein C, protein S and antithrombin, because of the high degree of individuality for these quantities in plasma. Thus assays for protein C, protein S and antithrombin in plasma are likely to be useful in monitoring individuals but not in detecting disease with the desired nosological sensitivity.

– five specimens should be collected for protein C, protein S and antithrombin to estimate the homeostatic set point of an individual.

References

1. Fraser CG, Harris EK. Generation of data on biological variation in clinical chemistry. Crit Rev Clin Lab Sci 1989; 27:409–37.
2. Harris EK. Statistical principles underlying analytic goal-setting in clinical chemistry. Am J Clin Pathol 1979; 72:374–82.
3. Costongs GMPJ, Bas BM, Janson PCW. Short-term and long-term intra-individual variations and critical differences of coagulation parameters. J Clin Chem Clin Biochem 1985; 23:405–10.
4. Harris EK. Effects of intra- and inter-individual variation on the appropriate use of normal ranges. Clin Chem 1974; 30:1535–42.
5. Ross JW. Evaluation of precision. In: Werner M, editor. Handbook of clinical chemistry, Vol. 1. Boca Raton, FL: CRC Press, 1982:391–422.
6. Fraser CG. The application of theoretical goals based on biological variation in proficiency testing. Arch Pathol Lab Med 1988; 112:404–15.
7. Fraser CG. Biological variation in clinical chemistry. An update: collated data, 1988–1991. Acta Pathol Lab Med 1992; 116:916–23.
8. Bertina RM. Prevalence of hereditary thrombophilia and the identification of genetic risk factors. Fibrinolysis 1988; 2:7.
9. High KA. Antithrombin III, protein C, and protein S. Arch Pathol Lab Med 1988; 112:28–36.
10. Fraser CG, Peake MJ. Problems associated with clinical chemistry quality control materials. Crit Rev Clin Lab Sci 1980; 12:59–86.
11. Fraser CG. Desirable performance standards for clinical chemistry. Adv Clin Chem 1983; 23:299–339.
12. Fraser CG, Browning MCK. The "index of fiduciality" proposed for use in evaluation and comparison of methods. Clin Chem 1988; 34:1356–7.
13. Harris EK. Distinguishing physiologic variation from analytic variation. J Chron Dis 1970; 23:469–80.
14. Harris EK. Statistical aspects of reference values in clinical pathology. Prog Clin Pathol 1981; 8:45–66.

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