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Importance of Preanalytical Blood Collection Methods in Determining Measured Levels of Brain-derived Neurotropic Factor (BDNF) in Human Plasma and Serum

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Abstract

Importance of Preanalytical Blood Collection Methods in Determining Measured Levels of Brain-derived Neurotropic Factor (BDNF) in Human Plasma and Serum

Purpose: Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors and has been shown to be a potentially valuable biomarker for monitoring prognosis, and pharmacodynamics of drug treatment, with a broad range of diseases such as stroke, Alzheimer's, type 2 diabetes, and inflammation. This study was undertaken to define the blood collection techniques that yield consistent and maximal levels of soluble BDNF from human blood samples.

Methods: BDNF in serum and plasma was quantitated using an ELISA kit obtained from R&D Systems. Plasma and serum were obtained from a commercial source and from normal blood donors at MicroConstants. The blood samples from in-house personnel were collected using various Becton Dickinson (BD) vacutainer tubes, including K_3EDTA for plasma, as well as those with and without clotting activator and gel separator for serum collection. The samples were processed immediately and assayed for BDNF without freeze-thaw.

Results: The BDNF ELISA method displayed acceptable accuracy, precision, sensitivity (LLOQ 7 ng/mL before 1:20 minimal required dilution, MRD), linearity of dilution, and selectivity (100% of spikes passed at the LLOQ). It was confirmed using differential cellular centrifugation, and varied methods for initiation of clotting, that most BDNF in blood was contained in platelets. Normal serum (after clotting) yielded ~10-fold higher BDNF levels (median ~19.0 ng/mL) vs. parallel samples collected as cell-free plasma (~0.5-2.0 ng/mL). Whole blood collected with K_3EDTA vacutainer tubes and centrifuged at 150g yielded platelet-rich plasma (PRP). Treatment of PRP with detergent (Triton x-100) to lyse platelets, or with CaCl2 to initiate clotting, yielded very high levels of released BDNF. Centrifugation of whole blood plasma at 2000g yielded platelet-poor plasma (PPP). Treatment of PPP with detergent or CaCl2 failed to liberate substantial BDNF, although clotting was induced by the latter treatment. Variable levels of BDNF were observed in sera depending on the serum collection tube used. Tubes with clot activator, with or without gel separator, produced the highest levels of BDNF. Collection of sera in tubes lacking additive yielded significantly lower levels (~50% less) after clotting vs. that seen in parallel sera samples collected with clot activator. Very low levels of BDNF were observed in sera from a commercial vendor, in line with the common practice of producing serum from platelet-poor plasma by addition of cations. A screen of 20 normal human sera samples collected under optimal conditions (i.e., with clot activator) yielded 20.0 ± 6.26 ng/mL BDNF, range 13.8-31.1 ng/mL.

Conclusions: These results confirm that preanalytical variables can have dramatic effects on measured biomarker levels, especially those stored in platelets. Sera levels of BDNF can vary in samples obtained from the same individual, obtained at the same time, depending on the method of collection.

Materials and Methods

Principal of the BDNF ELISA. The assay is based on the sandwich enzyme-linked immunoassay technique (R&D Systems, BDNF ELISA Kit, Cat# SBD00). A monoclonal antibody specific for BDNF has been pre-coated onto the wells of the plate provided with the kit and pre-blocked. Following adding Assay Diluent RD6P, standards, QC, and samples are pipetted into the wells. Standards and QC are prepared in BDNF-poor sera pool. After incubation and no washing, the wells are probed for bound BDNF by addition of HRP-labeled anti-BDNF monoclonal antibody. After another wash step to remove any unbound antibody-enzyme conjugate, a substrate solution of tetramethylbenzidine (TMB) is added to the wells to detect the bound enzymatic activity. The color development is stopped by addition of an acidic solution and the absorbance is read at 450 nm using a microplate reader. The intensity of the color is inversely proportional to the concentration of BDNF in the sample and the concentrations in the QC and study samples were determined by 4-PL regression interpolation using SoftMax Pro GxP.

Plasma and Sera Preparation Procedures. Off-the clot normal human serum samples were purchased from BioReclamation, as was K_3EDTA plasma. Donors were equally divided between male and female. Normal human plasma and sera were also obtained from in-house donors using 1) Covidien Monoject K_3EDTA and serum blood collection tubes, Cat. No. 8881311743 and 8881301413, respectively, 2) BD Vacutainer RST™, rapid serum tube with thrombin and gel separator, Cat. No. 368774, 3) BD Vacutainer SST™, with silica clot activator and gel separator, Cat. No. 367981, and 4) BD Vacutainer, serum, with silica clot activator, Cat. No. 367820.

Serum samples were allowed to clot by incubation at room temp for 30 minutes, then centrifugation at 375g for 20 minutes. Clotting of plasma samples was induced by addition of $CaCl_2$ to a final concentration of 25 mM, followed by incubation for 1 hour at room temperature. The samples were centrifuged at 10000g for 5 minutes, unless indicated otherwise relative to Figure 1, with the supernatant fluid being retained to a fresh tube. The samples were either used immediately or stored at -20°C for future use.

Preparation of Standards and QC. Standards and QC were prepared in a pool constructed with serum samples derived from plasma treated with $CaCl_2$ as described above. All QC and calibrators were prepared in >95% matrix. The standard curve range, considering a 1:20 minimal required dilution (MRD), was 7.0 to 96.0 ng/mL, with a 3.5 ng/mL anchor point.

Calculations. A standard curve was generated for each run using recombinant human BDNF. SoftMax Pro, v. 5.4.4 software (Molecular Devices) was used to fit the data using a four-parameter logistic (4-PL) curve-fit of mean OD values versus nominal BDNF calibrator values. Concentrations of BDNF in study and QC samples were calculated by interpolation along this calibrator curve. Calculations for percent recovery in spiked, off-the-clot serum samples, used the subtraction method to account for endogenous BDNF in the samples. Briefly, the concentration of the tested biomarker observed in the matrix blank was subtracted from the amount observed in the spiked serum sample, and the net difference vs. nominal spiked value was used to calculate percent analytical recovery (%AR).

Background and Purpose

Brain-derived neurotropic factor (BDNF) is a biomarker of increasing importance in drug development for neurological conditions such as cerebral ischemia, hypoglycemia, and other conditions of neurotoxicity. Levels of BDNF typically decrease with the onset of such conditions and, as such, could serve as a useful tool to monitor pharmacodynamics (PD) associated with drug candidate administration. Promising areas where monitoring of BDNF PD could help improve drug development include diseases like stroke, Alzheimer's, Huntington's and Parkinson's diseases, as well as type 2 diabetes mellitus and inflammatory conditions.

The Immunology Department at the San Diego facility of MicroConstants, Inc. has developed a method for the determination of BDNF concentrations in human serum samples based on a R&D Systems BDNF ELISA kit. The method is applicable for measuring BDNF concentrations in normal human serum (NHS) ranging from 7.00 to 96.0 ng/mL.

BDNF in typical NHS is normally present in the range of 15-40 ng/mL and decreases by about 50% in the aforementioned disease conditions. Thus, in contrast to most biomarker assays which are designed to measure increased levels with disease or treatment, the present immunoassay was designed to capture both increased and decreased serum BDNF fluctuations.

To fully exploit the lower limit of quantitation (LLOQ) of the immunoassay in serum exhibiting relatively high endogenous BDNF levels, it was necessary to develop a sera pool with relatively little of the biomarker. It has previously been shown that most of the BDNF in serum samples is derived from platelets. We used this information to prepare BDNF-poor serum as an alternative to typical BDNF-replete serum expected as the matrix in planned human clinical trials. Thus, $CaCl_2$ was added to K_3EDTA human plasma to induce clotting, which yielded a sera pool with < 1 ng/mL BDNF. This alternative matrix was used in the preparation of standards (calibrators) and QC, allowing for accurate quantitation of the biomarker in the lower range of concentrations required.

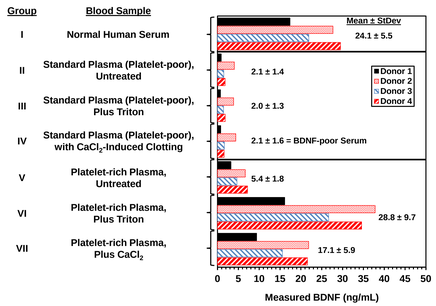
The suitability of this approach was established, as described herein, by demonstration of 1) congruency of calibrator performance in assay buffer with calibrators in BDNF-poor matrix pool at the intended LLOQ of 7.0 ng/mL through the ULOQ of 96.0 ng/mL, 2) parallelism in quantitation of authentic, endogenous BDNF in multiple off-the-clot NHS samples with high BDNF levels, and 3) 100% passing of selectivity at the LLOQ in such sera samples. It can be concluded that the BDNF ELISA, as configured with BDNF-poor serum matrix for standards and QC, should be suitable for quantitation of the biomarker in human clinical sera samples subsequent to full bioanalytical method validation.

Results

Figure 1 demonstrates differences in BDNF levels depending on the method used for blood collection, as well as conditions for release of the biomarker from platelet-rich plasma. Serum and plasma were collected from 4 normal human donors using SST and K_3EDTA BD vacutainer tubes, respectively. As shown by Group I, untreated sera samples exhibited a range of 15-30 ng/mL BDNF, while untreated K_3EDTA (Group II) exhibited about 10% as much of the biomarker. Treatment of standard plasma with detergent (Group III), or induction of clotting by addition of $CaCL_2$ (Group IV), failed to increase BDNF levels. Serum prepared by treatment of platelet-poor K_3EDTA plasma with $CaCl_2$ is the source of the BDNF-poor serum used as matrix for standards and QC described herein.

Platelet-rich plasma (PRP), while demonstrating little BDNF without treatment (Group V), but with substantial release of the biomarker into the fluid phase following treatment with Triton to lyse platelets (Group VI), or with $CaCl_2$ to induce clotting (Group VII).

Figure 1
Blood BDNF is Contained Primarily in Platelets and is Released Upon Platelet Lysis or Clotting Activation



Panel A of Figure 2 demonstrates the performance characteristics of the BDNF calibrators in assay buffer and in BDNF-poor sera pool; with results shown after a 1:20 minimum required dilution (MRD). The two curves were largely indistinguishable in the range of 7.0 to 96.0 ng/mL. **Panel B** shows that the recombinant human BDNF calibrator curve in BDNF-poor sera pool could accurately quantitate authentic endogenous BDNF across multiple dilutions, a successful demonstration of parallelism.

Figure 2
BDNF Calibrators in BDNF-poor Sera Pool; Demonstration of 1) Congruency with the Buffer Curve, and 2) Parallelism with Authentic Endogenous BDNF

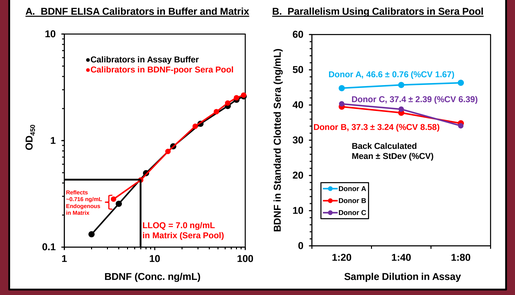


Table 1 shows typical accuracy and precision (A&P) results using validation samples prepared at 5 levels (LLOQ, LQC, IQC, HQC, ULOQ) in BDNF-poor sera pool. The VS spanned the range of the calibrator curve (7.0 to 96.0 ng/mL), with all results meeting standard acceptance criteria.

Table 1
Validation Sample Performance Characteristics in BDNF-poor Human Sera Pool

VS/QC	Target BDNF Conc. (ng/mL)	Mean Observed (ng/mL)	Observed Standard Deviation	%CV for Observed	%DEV Observed vs. Target
ULOQ	96.0	93.4	4.34	4.6%	-2.7%
HQC	76.8	77.7	1.59	2.0%	1.1%
IQC	40.0	47.3	1.29	2.7%	18.3%
LQC	21.0	22.0	0.32	1.5%	-5.0%
LLOQ	7.00	6.64	0.22	3.3%	-5.2%

Figure 3 presents the validation parameter of selectivity which further confirmed two important points about the suitability of this BDNF immunoassay for the analysis of human clinical sera samples for endogenous BDNF, including 1) the ability to selectively, and accurately, quantitate BDNF as spiked into standard off-the-clot sera samples at the proposed LLOQ of 7.0 ng/mL, and 2) confirmation of the target LLOQ of 7.0 ng/mL.

Figure 3
Spiked Recovery Confirms Selectivity of the BDNF ELISA as well as an LLOQ at 7.0 ng/mL

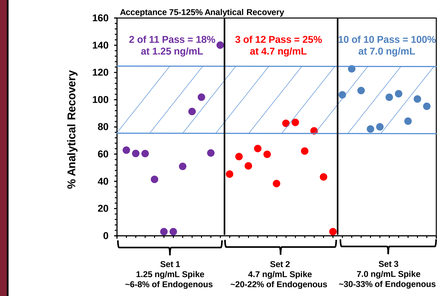


Figure 4 examines the optimal method to obtain the highest levels of BDNF in serum. Four serum vacutainer collection tube types were evaluated. K_3EDTA plasma was included for comparison purposes. Four normal blood donors were included in this study, the same donors shown in Figure 1. Plasma (Group I) demonstrated little BDNF as expected. Collection of serum without additives (Group II) yielded substantially higher levels of BDNF than observed with plasma, although inclusion of clot activator generated substantially higher levels of the biomarker with and without gel separator (Groups III-V). These results indicate that comparable levels of BDNF can be obtained with the 3 different blood collection tube types.

Figure 4
Optimal Platelet Release of BDNF into Serum Requires Clot Activator: Results from Multiple Blood Donors

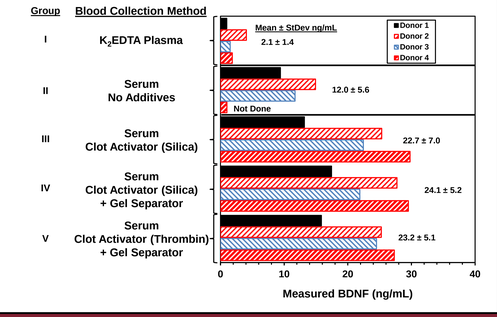
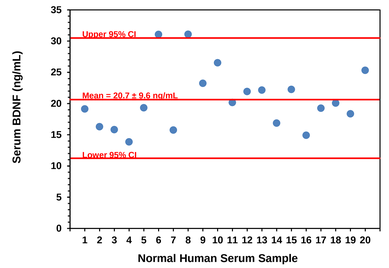


Figure 5 illustrates yield of BDNF from 20 normal human serum donors using blood collection tubes with clot activator and gel separator, as measured using the BDNF ELISA with BDNF-poor sera pool as matrix for standards and QC. A mean of ~20.7 ng/mL was observed, with a range of 13.8 to 31.1 ng/mL, for this cohort of 20 individuals. These results are consistent with BDNF levels reported by others using similar methods for blood collection and immunoanalysis.

Figure 5
Serum BDNF Levels Observed in 20 Normal Sera Samples following Platelet-involved Clotting



Conclusions

A. The R&D Systems' BDNF ELISA is sufficiently sensitive, accurate, and precise for measuring BDNF in human clinical trial serum samples.

B. BDNF in serum is largely derived from platelets.

C. BDNF-poor serum can be readily prepared by induction of clotting in K_3EDTA plasma that has been appropriately centrifuged to eliminate platelets.

D. BDNF-poor serum is a suitable alternative to serum with normally high levels of the biomarker because calibrators in BDNF-poor matrix demonstrated:

1. Similar curve characteristics to calibrators in assay buffer over the concentration range of interest, 7.0 to 96.0 ng/mL BDNF;
2. Parallelism of authentic endogenous BDNF; and
3. Selectivity with normal human serum samples.

E. Maximal yield of BDNF in serum can be achieved using blood collection methods involving clot activator.