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# Stability of Abnormal D-dimer Levels in Platelet-Poor Plasma Stored at -20°C and -70°C

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## Background

The products of clot degradation can prove useful in clinical situations for evaluation of suspected thromboembolism. During one of the final stages of thrombus formation, fibrin monomers are cross-linked by factor XIII to form a stable clot[1, 2]. Degradation of these monomer chains results in characteristic by-products including D-dimers. Elevated D-dimer levels indicate that a cross-linked clot has been formed and that it has since been lysed by plasmin[3]. Therefore elevated D-dimer levels are seen in conditions of pathologic coagulation, including deep vein thrombosis (DVT)[4] and pulmonary thromboembolism (PE).

It is important that DVT be accurately diagnosed and treated to avoid its progression to PE, a potentially fatal condition. Diagnosis of DVT can be challenging because its incidence is low and physical symptoms or pain, swelling and tenderness are common and fairly non-specific. The use of objective tests is required in the evaluation of DVT. Recently, the measurement of plasma D-dimer levels has been established as a single laboratory test capable of excluding DVT and PE in patients who have a low “pretest” clinical probability of thromboembolism.[4]

The specificity of the quantative D-dimer assays is fairly low (39-53%) because a number of conditions, such as infection, inflammation, vasculitis, pregnancy, trauma, hemorrhage, malignancy and recent surgery can result in elevated D-dimer levels[5, 6]. Conversely, the sensitivity of the assay is 96-100% and the negative predictive value is

93-100%[7, 8] in patients with a low to moderate pretest probability of DVT. A negative test can therefore be used to exclude thromboembolism from the differential diagnosis, eliminating the need for further testing such as compression ultrasonography. D-dimer evaluation has also shown promise in the evaluation of suspected recurrent DVT[9] and PE[6],as well as in diagnosis of acute aortic dissection[10].

For normal clinical evaluation, D-dimer measurements are performed on fresh plasma. However for research and quality assurance purposes, platelet-poor plasma is often frozen and stored for later batch testing. The Clinical and Laboratory Standards Institute (formerly the NCCLS) guidelines state that platelet-poor plasma samples may be stored for up to two weeks at -20°C and up to six months at -70°C [11]. The manufacturer's instructions for the STA®-Liatest®, a particular D-dimer assay marketed by Diagnostica Stago (Asnieres-sur-Seine, France), indicate that plasma samples may be stored at 20 $\pm$ 5°C for up to 8 hours or for up to one month at -20°C [12].

Most research studies have employed storage of samples at -70$^{o}$C[4 7] or even -80 $^{o}$C[13] for platelet-poor plasma samples. However, adequate research is lacking to show the stability of platelet-poor plasma during long term storage at a range of temperatures. Previous studies have investigated stability of coagulation markers at -20$^{o}$C, but these studies have been either small or of short duration[4, 14]. Bates *et al* found stable D-dimer levels after storage for two months at -20 °C. Woodhams *et al* demonstrated D-dimer stability at -74 $\pm$ 2°C as well as -24 $\pm$ 2°C in a study of six normal patients.

Clear demonstration of D-dimer stability in platelet-poor plasma during storage at -20 °C would simplify the storage of samples used for laboratory research.

Previous work on this project investigated storage of samples of platelet-poor plasma with normal D-dimer levels at both -20 °C and -70 °C. D-dimer levels were assayed at intervals for up to one year and no significant change in D-dimer levels were observed over the storage period. However, this previous investigation, as well as others, has only investigated D-dimer stability in samples with normal D-dimer levels (i.e. among asymptomatic subjects). In the current study, we tested the hypothesis that elevated D-dimer levels, as measured in the platelet-poor plasma of symptomatic subjects, will not change significantly over a period of 6 to 12 months, whether stored at -20 °C or -70°C.

## Methods

The sample size was estimated using the Student t distribution, assuming a 2-tailed statistical model for determination of sample size. Under these assumptions, it was estimated that at least 37 subjects would be required. The samples were obtained consecutively as they are identified during clinical evaluation of symptomatic patients at UNMH. All samples were collected under the approved UNM HRRC protocol #04-230.

All blood samples were collected into sodium citrate Vacutainers™ with all personal identifying information removed (with the exception of sex and age) prior to sample processing. Eligible samples are defined as those with D-dimer levels >0.5 μg (FEU)/mL, the upper normal limit used to exclude venous thromboembolism. When

such a sample was identified, technicians set aside the blood sample that would ordinarily be discarded. The blood sample was refrigerated at 4°C until processing. Within 8 hours of draw, the specimen was centrifuged at room temperature for at least 15 minutes at 2500 g. After being centrifuged, the platelet-poor plasma was dissociated from any personal identifying information and separated into 250μL aliquots in six or eight labeled Eppendorf tubes. The Eppendorf tubes were labeled with the specimen number, sex of the patient (M/F 1, 2, 3, etc.), at what month the D-dimer level will be re-evaluated (1, 3, 6 and 12 months) and the temperature in which the specimen will be stored. The specimens were then separated and stored at either -20°C or -70°C. Samples were initially stored at the lab at UNM Hospital, and then transferred to TriCore Laboratories within one month where they were stored and analyzed for the duration of the study.

All baseline values were established on the day of the draw. After storage for one, three, six and possibly twelve months the D-dimer levels were again tested. The specimens were thawed for three minutes at 37°C prior to the D-dimer level measurement with the STA®-Liatest® D-dimer assay.

Data analysis:

Univariate analysis of numeric variables was carried out using standard methods. D-dimer change over time was calculated by subtracting the baseline D-dimer level from the value measured at each time. Multivariate analysis of variance was used to assess differences in D-dimer change over time for each storage temperature.

Calculations:

All calculations were performed on an Intel Pentium-based microcomputer with a clock speed of 1.8 GHz. Statistical calculations were made with Statgraphics Plus for Windows Version 4.1, Manugistics, Inc., Rockville, MD. Data management was carried out using Microsoft Excel 2002 (Microsoft Corporation, Redmond, WA.) Two-tailed tests and a Type I error rate of 0.05 were employed throughout.

## Results

Samples from 46 patients with elevated D-dimer levels were identified and included in this study. Subject ages varied from 17 to 83 with a mean age of 43. There were 30 females (65%) and 16 males (35%). There were 299 samples available for analysis. There was one outlying point, which was preceded and followed by consistent D-dimer levels. This point was considered spurious and its value was set to the mean of that subject's remaining D-dimer data points.

The mean D-dimer differences at each storage temperature and month of storage ranged from -0.18 to 0.26 μg (FEU)/mL (see table 1) with an overall positive trend during the storage time. The temperature at which the samples were stored was not associated with a significant change in d-dimer difference (p=0.67) (see figure 1). The d-dimer level did not change appreciably over time (p=0.68).

| | 1 | 3 | 6 | 12 |
|---|---|---|---|---|
| -20°C | 0.06 | 0.04 | 0.00 | 0.02 |
| -70°C | 0.07 | -0.18 | 0.26 | 0.23 |

Table 1. Mean D-dimer difference from baseline at each temperature and month. Units are μg (FEU)/mL.

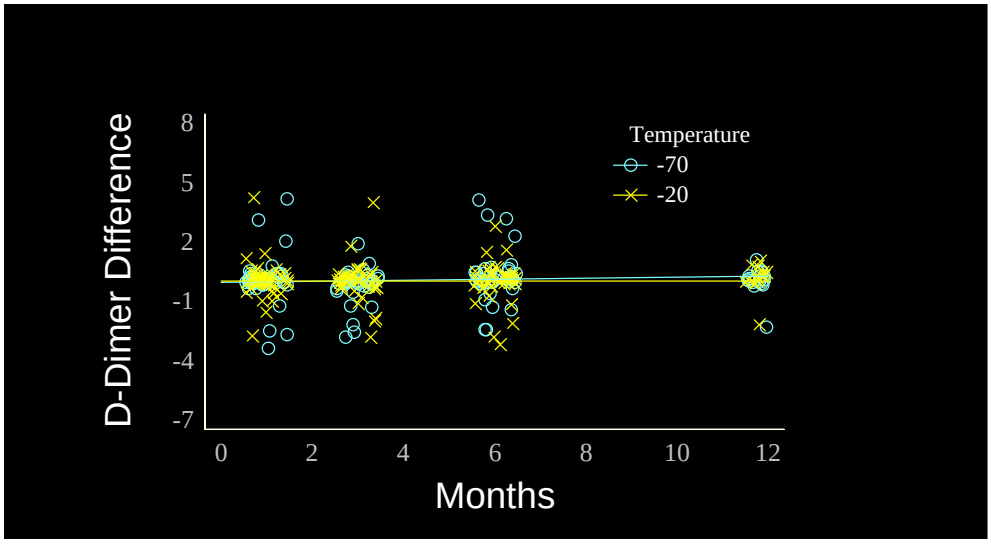


Figure 1. Difference in D-dimer levels from baseline at 1, 3, 6 and 12 months at storage temperatures of -20°C and -70°C. The units of difference are μg (FEU)/mL. Regression lines shows trend over time for each temperature.

## Discussion

This study demonstrates that D-dimer levels in platelet poor plasma from patients with an elevated D-dimer level do not change significantly when stored at -20°C compared to -70°C for up to 12 months. Previously published studies have analyzed plasma with normal D-dimer levels and have been either small[14] or short in duration[4] and have studied patients with normal D-dimer levels.

The trend over the 12 month storage period for the change from baseline D-dimer level was generally positive for both -20°C and -70°C. However, at the end of 12 months, the average change in D-dimer concentration was 0.02 for those samples stored at -20°C, a

**Comment [A.N.1]:** Your point is well taken. Is it better with what I added in the results section? I added this as an intro to the later discussion.

**Comment [a2]:** you are putting results in the discussion section – it is OK to do this only if you're RE-STATING something already presented in the results section.

change which is unlikely to be clinically significant (p= 0.99). The p value for the overall influence of temperature was 0.67. The mean change in samples stored at -70º was 0.23 after storage for 12 months. Although the magnitude of change was higher for the samples stored at -70ºC, the p value for the influence of storage time was 0.34 for the samples stored at -70ºC. Overall, these data indicate that platelet-poor plasma samples can be stored at -20°C rather than -70°C without clinically or statistically significant change.

**Comment [A.N.3]:** This is the information I was trying to get from Dr. Tandberg. The values I have in the files he sent to me have the overall p value rather than the breakdown. This is the value I got from my ANOVA.

The samples that were stored -70°C had to be moved from one freezer to another during the course of storage due to a freezer malfunction. This could have lead to slight thawing and refreezing of samples prior to testing; however, we believe any such variance was minimal because technicians were alerted to the problem prior to any significant thawing of the freezer contents. Moreover, because samples were collected in a staggered fashion, this move would not have affected all samples stored at this temperature.

One limitation of this study is that our protocol for processing the blood samples may be different than is done in different institutions. The protocol we designed allowed whole blood to be refrigerated at 4°C for up to 8 hours prior to sample processing. Protocols elsewhere may differ from this. Additionally, our centrifugation of the samples at 2500g for at least 15 minutes may be different from protocols elsewhere and may result in differing platelet concentration of the plasma. Because of these differences, our findings may not be generalizable to all platelet-poor plasma samples.

## Conclusion

Our findings demonstrate that platelet-poor plasma from patients with elevated D-dimer levels can be stored for up to 12 months at -20°C rather than -70°C without a statistically significant change in D-dimer concentration as measured by the STA®-Liatest® D-Di.


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## References


1. Goodnight S, WE H. *Disorders of Hemostasis and Thrombosis: A clinical guide*. Second ed. New York: McGraw-Hill; 2001.
2. McClatchey K, Hackel E. *Clinical laboratory medicine*. Second ed. Philadelphia: Lippincott Williams & Wilkins; 2002.
3. De Loughery T. *Hemostasis and Thrombosis*. Austin, TX: Landes Bioscience; 1999.
4. Bates S, Grand'Maison A, Johnston M, Naguit I, Kovacs M, Ginsberg J. A Latex D-Dimer Reliability Excludes Venous Thromboembolism. *Arch Intern Med.* 2001;161(3):447-453.
5. Bockenstedt P. D-Dimer in Venous Thromboembolism. *N Engl J Med.* 2003;349(13):1203-1204.
6. Brown M, Rowe B, Reeves M, Bermingham J, Goldhaber S. The accuracy of the enzyme-linked immunosorbent assay D-dimer test in the diagnosis of pulmonary embolism: A meta-analysis. *Ann Emerg Med.* 2002;40(2):133-144.

**7.** Houbouyan-Reveillard L, Mihoubi A, Houdijk W, et al. Preliminary Evaluation of two New Rapid Immunoturbidimetric D-dimer Assays in Patients with Clinically Suspected Venous Thromboembolism (VTE). *Thromb Haemost.* 2000;84(5):770-774.

**8.** Bucek R, Koca N, Reiter M, Haumer M, Zontsich T, Minar E. Algorithms for the diagnosis of deep-vein thrombosis in patients with low clinical pretest probability. *Thromb Res.* 2002;105(1):43-47.

**9.** Rathburn S, Whitsett T, Raskob G. Negative D-dimer to exclude recurrent deep venous thrombosis: a management trial. *Ann Intern Med.* 2001;141:839-845.

**10.** Weber T, Hogler S, Auer J, et al. D-dimer in Acute Aortic Dissection. *Chest.* 2003;123(5):1375-1378.

**11.** Standards, NCCLS. *Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays; Approved Guidelines.* Vol 23. Fourth ed. Pennsylvania: NCCLS; 2003.

**12.** STA®-Liatest® D-Di [package insert]. Asnieres-sur-Seine, France: Diagnostica Stago; 2002.

**13.** Iverson L, Thorlacius-Ussing O. Short-time stability of markers of coagulation and fibrinolysis in frozen plasma. *Thromb Res.* 1996;81(2):253-261.

**14.** Woodhams B, Giradot O, Blanco M, Golesse G, Gourmelin Y. Stability of coagulation proteins in frozen plasma. *Blood Coagul Fibrinolysis.* 2001;12(4):229-236.