Influence of Sample Collection and Storage on the Detection of Platelet Factor 4–Heparin Antibodies

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Abstract

Heparin-induced thrombocytopenia is a life-threatening thrombotic disorder caused by antibodies to platelet factor 4 (PF4) and heparin. Commercial immunoassays are frequently used for the detection of PF4-heparin antibodies, and several studies have reported that higher antibody titers are more frequently associated with adverse events. It is not known if conditions involving sample preparation and/or storage affect the operational characteristics of PF4-heparin immunoassays. We compared the detection of PF4-heparin antibodies from 48 patient samples collected concordantly in serum separator tubes or tubes containing EDTA or sodium citrate. We also examined the effects of extended sample storage on whole blood collected in serum separator, EDTA, or citrate tubes at 4°C for up to 96 hours on antibody detection. We noted that serum or plasma anticoagulated with sodium citrate or EDTA yielded comparable results. In addition, we could not demonstrate any significant sample deterioration after storage at 4°C in any medium for up to 4 days. These findings suggest that PF4-heparin antibodies are largely insensitive to the effects of sample preparation and storage.

Heparin-induced thrombocytopenia (HIT) is a life-threatening thrombotic disorder caused by pathogenic antibodies directed against complexes of platelet factor 4 (PF4) and heparin. HIT is diagnosed on the basis of clinical criteria and laboratory demonstration of PF4-heparin antibodies.[1] Antibodies to PF4-heparin can be identified by immunologic or functional assays. The functional assays measure the platelet-activating effects of PF4-heparin antibodies, whereas immunoassays detect the presence of antibodies.[2] In most clinical laboratories, immunoassays using enzyme-linked immunosorbent assays (ELISAs) for PF4-heparin antibodies are preferred over functional assays due to their high sensitivity, technical simplicity, and rapid turnaround time.

Although immunoassays have high sensitivity, they frequently seem to detect antibodies in the absence of clinical manifestations of the syndrome, particularly in certain clinical settings, such as in patients undergoing cardiac surgery.[3-5] Two studies have reported that higher PF4-heparin antibody titers are more specific for a diagnosis of HIT and may be predictive of thrombotic risk.[6,7] Assay performance and reproducibility are, therefore, important in the identification of PF4-heparin antibodies.

It is recognized that the performance characteristics of immunoassays can be significantly influenced by sample preparation and/or use of an anticoagulant such as sodium citrate or EDTA.[8,9] Additives used for blood collection can interfere with various aspects of immunoassays, including protein-protein interactions,[10] stability of proteins,[11] and/or binding properties.[12]

At present, it is not known if immunoassays for the detection of HIT antibodies are sensitive to the effects of sample preparation. The manufacturers of the 2 commonly used commercial immunoassays for HIT, PF4 Enhanced (GTI,

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Waukesha, WI) and Asserachrom HPIA (Diagnostica Stago, Asnières, France), recommend serum (GTI and Stago) or plasma (Stago) for the detection of PF4-heparin antibodies. However, there has been no published comparison of various collection media for the detection of PF4-heparin antibodies.

In the present study, we systematically examined the effects of various collection media (serum or blood anticoagulated with sodium citrate or EDTA) on the detection of PF4-heparin antibodies. In addition, we characterized the stability of PF4-heparin antibodies in collection media stored at 4°C.

Materials and Methods

Patient Samples

To characterize the effects of sample preparation on PF4-heparin antibody detection, we studied samples from patients who underwent recent cardiopulmonary bypass for cardiac surgery. This patient population was selected on the basis of high rates of seroconversion for PF4-heparin antibodies.[3,5,13] We included only subjects who had matched samples collected in serum separator, EDTA, and sodium citrate available for study on postoperative day 5 or later. We used deidentified blood samples that would otherwise have been discarded. After collection, blood was centrifuged at 800*g*, and serum or plasma was separated and stored immediately at –80°C.

To delineate the effects of specimen storage on PF4-heparin antibody detection, samples were collected from 4 subjects diagnosed with HIT[14] and 2 healthy volunteers after informed consent was obtained. Specimens were centrifuged at 800*g* and stored at 4°C in the original collection tube. A 100-μL aliquot was removed from each tube on the day of collection and subsequently on a daily basis for 4 additional days (up to day 4). Timed aliquots were immediately frozen at –80°C and thawed at the time of ELISA.

The Duke University Institutional Review Board (Durham, NC) reviewed and approved both protocols, and the research was carried out according to the principles of the Declaration of Helsinki.

PF4-Heparin ELISA Assays

The PF4 Enhanced ELISA was performed according to the manufacturer's instructions. An optical density (OD) of 0.4 or more is considered positive for the assay.

Statistical Analysis

To ensure comparable dilutions across the 3 collection media, results obtained in citrate samples were corrected by 11.1% to account for the ratio of 4.5 mL of whole blood to 0.5 mL of sodium citrate in each collection tube. Data were analyzed using (nonparametric) Spearman correlation, and linear regression was performed for presentation purposes. The paired *t* test and Bland-Altman plots were used to assess the agreement between measurements obtained in EDTA and serum, EDTA and citrate, and serum and citrate.[15] Contingency tables were used to analyze the frequency of discordant classification in which a sample is considered "negative" in one assay and "positive" in another. *P* values were calculated by using the Fisher exact method. Where appropriate, bootstrap analysis with 10,000 iterations was used to estimate confidence intervals for the frequency of discordant classification. A mixed-effects model, treating patients as random and days of storage and type of medium as fixed effects, was used to determine whether the variance of OD results for any given patient was significantly affected by the period of sample storage and/or the different collection media. Averages and SDs for replicates were calculated by using Microsoft Office Excel 2003 (Microsoft, Redmond, WA). All other analyses were performed using R 2.2.1 (R: A Language and Environment for Statistical Computing 2004, R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org). Raw data are available from us on request.

Results

Collection Medium Does Not Affect PF4-Heparin Antibody Detection

We performed PF4-heparin immunoassays on serum or plasma derived from specimens collected in sodium citrate (n = 48), potassium EDTA (n = 48), or serum separator tubes (n = 44). Four serum samples were unavailable for testing. The signals obtained in serum or plasma (EDTA or citrate) were highly concordant with one another. The Spearman correlation coefficients for PF4-heparin antibody levels obtained in EDTA and citrate (0.957), EDTA and serum (0.930), and citrate and serum (0.919) were high **Figure 1**.

To affirm that strong correlation was due to agreement in the various sample preparations, Bland-Altman plots were constructed for pairs of media **Figure 2**. **Table 1** shows the average differences among results obtained in the different media for individual patients. There was no significant difference between results obtained in EDTA and citrate (P = .843), EDTA and serum (P = .835), or citrate and serum (P = .857). However, if the results obtained in citrate samples were not corrected for the dilution, they were significantly less than the results obtained in the other media, by up to 0.13 OD (data not shown).

Although our results showed significant concordance among the various collection media, discordant results were obtained in samples from 3 patients. The following estimates for the frequency of discordant classification were noted: 2

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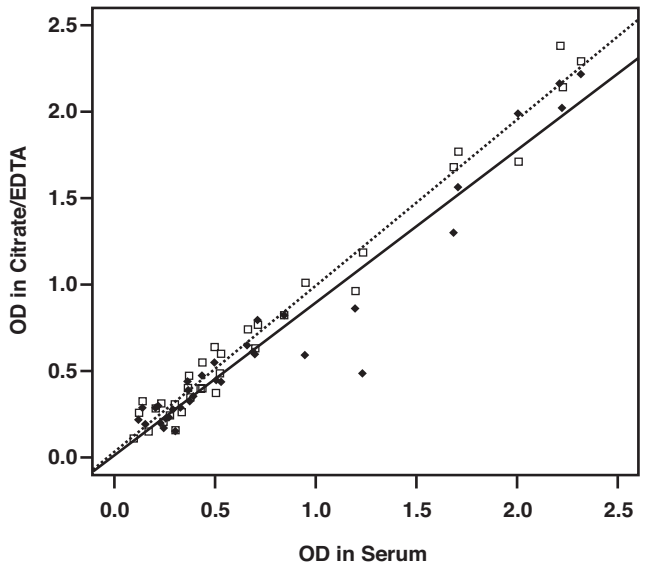

Figure 1 Detection of platelet factor 4–heparin antibodies in serum, EDTA, or citrate. For presentation purposes, the optical density (OD) in citrate (solid line) is regressed on the OD in serum (diamonds; $R^2 = 0.94$). The OD in EDTA (dotted line) is regressed on the OD in serum (squares; $R^2 = 0.98$). Lines of least squares are included. Results for paired EDTA and serum samples are closer to the line of equality than results for paired citrate and serum samples. (The line of equality is not shown. R^2 is the proportion of the variance explained by the line of least squares.)

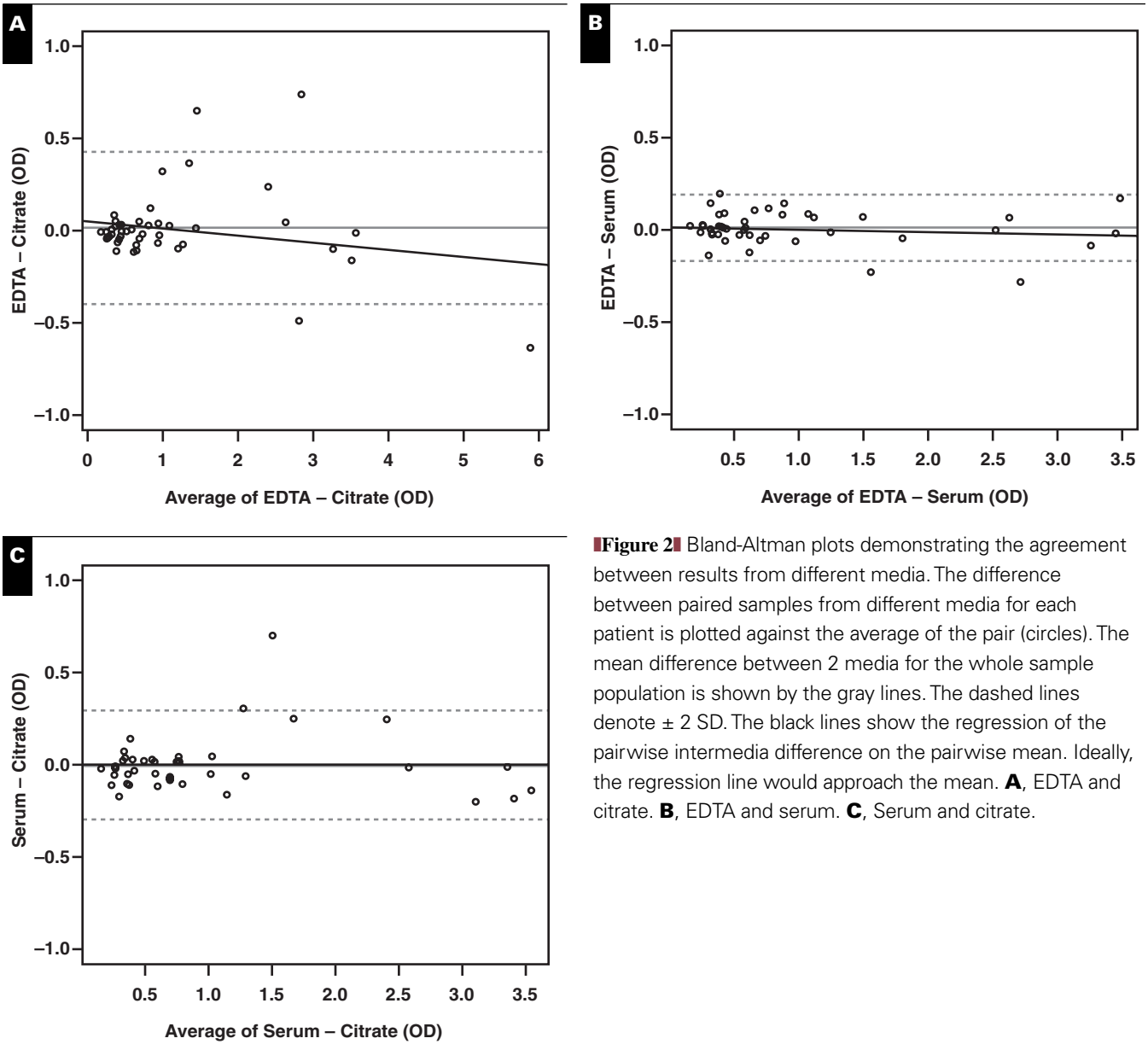

Figure 2 Bland-Altman plots demonstrating the agreement between results from different media. The difference between paired samples from different media for each patient is plotted against the average of the pair (circles). The mean difference between 2 media for the whole sample population is shown by the gray lines. The dashed lines denote ± 2 SD. The black lines show the regression of the pairwise intermedia difference on the pairwise mean. Ideally, the regression line would approach the mean. **A**, EDTA and citrate. **B**, EDTA and serum. **C**, Serum and citrate.

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Table 1
Comparison of Within-Patient Results in Different Media

	Within-Patient Optical Density Difference	
	Mean (95% Confidence Interval)	**Median (Interquartile Range)**
EDTA-citrate*	0.006 (–0.053 to 0.065)	–0.009 (–0.058 to 0.029)
Serum-citrate*	–0.004 (–0.049 to 0.041)	–0.018 (–0.084 to 0.020)
EDTA-serum	0.003 (–0.024 to 0.030)	0.003 (–0.033 to 0.064)

* Applying an 11.1% correction to account for the ratio of 4.5 mL of whole blood to 0.5 mL of sodium citrate in each collection tube.

(4.2%) of 48 patients for paired EDTA and citrate samples (95% confidence interval [CI], 0%-8.3%), 1 (2.3%) of 44 patients for paired serum and citrate samples (95% CI, 0%-6.8%), and 3 (6.8%) of 44 patients for paired EDTA and serum samples (95% CI, 0%-13.6%). Of note, discordant results for a given medium were close to the 0.4 cutoff.

We also investigated whether there was a higher rate of discordance at an OD cutoff of 1.00, a level reported to be associated with a higher risk for thrombotic events.[7] Discordant results were obtained in samples from 3 patients, with the following estimates for the frequency of discordant classification: 2 (4.2%) of 48 patients for paired EDTA and citrate samples (95% CI, 0%-8.3%), 2 (4.5%) of 44 patients for paired serum and citrate samples (95% CI, 0%-9.1%), and 2 (4.5%) of 44 patients for paired EDTA and serum samples (95% CI, 0%-9.1%). Heuristically varying the cutoff OD above which samples were considered positive in any medium could not improve on the rate of discordant classification.

Sample Storage in Collection Media Does Not Affect PF4-Heparin Antibody Detection

To determine the effects of specimen storage on PF4-heparin antibody detection, we examined whether the OD obtained for any given patient would change significantly depending on whether the sample was tested immediately after collection or stored in the original specimen tube as a centrifuged sample at 4°C for a period of up to 4 days. As shown in **Figure 3**, we noted no significant change in PF4-heparin antibody signals over time in patients with HIT or healthy subjects. By using a mixed-effects model, we could not demonstrate any significant diminution in signal with respect to storage time (P = .89) or medium (P = .56) in seropositive or seronegative patients. The antibodies were no more likely to degrade in one medium compared with any other (P = .66 for the interaction between medium and days of storage).

Discussion

In the present study, we demonstrated that detection of PF4-heparin antibodies is not affected by additives used for blood preparation. In particular, we could not show any significant differences in signals when samples from patients suspected of having HIT were collected in tubes that yield serum or plasma (sodium citrate or EDTA tubes, Figure 1). Moreover, we also showed that PF4-heparin antibodies remain stable at 4°C over time in blood in the presence or absence of additives.

Our study suggests that not any one medium is superior or inferior to another for PF4-heparin antibody detection. Although GTI recommends using serum in the PF4 Enhanced ELISA,

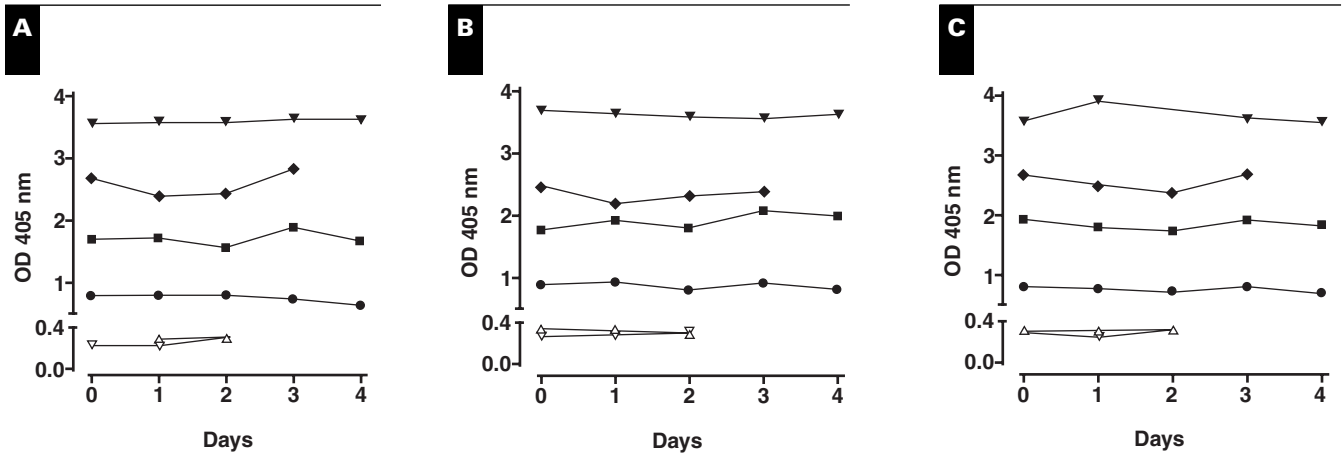

Figure 3 Optical density (OD) signals during 4 consecutive days of storage at 4°C in citrate (**A**), EDTA (**B**), and serum (**C**). Samples from 4 subjects with heparin-induced thrombocytopenia (solid symbols) and 2 negative control subjects (open triangles) are shown. Platelet factor 4–heparin antibody signals spanned a range from low-positive (circles), to moderate (squares, diamonds), to high-positive (triangles).

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EDTA or citrate can be substituted for serum. It is important to correct for the dilutional effect of the volume of sodium citrate present in the tube itself (0.5 mL), or antibody titers will be systematically underestimated if using the latter medium.

Although some large discrepancies in signals were noted on occasion from a given patient, between serum and citrate or EDTA and citrate, these discrepancies occurred only at high OD signals and did not alter the classification of seropositivity. In the most extreme example, 1 subject had an EDTA signal of 2.141 compared with a citrate OD signal of 1.411, both of which were markedly higher than the positive cutoff value of 0.4. In patients with OD results around the cutoff of 0.4 or around the higher cutoff of 1.0, minor fluctuations led to altered classification of patients. In our study, this occurred in 3 (2.1%) of 144 samples at the lower and higher cutoffs (95% CI, 0%-4.9%). In these cases, obtaining serial antibody levels and using a clinical prediction rule might be helpful in reaching decisions about therapy. These findings underscore the importance of combining clinical judgment with laboratory results.

Studies have shown that the PF4 concentration in serum is approximately 3-log-fold higher (5,334 ng/mL) than in plasma anticoagulated with sodium citrate or EDTA (1.8 ng/mL).[16] The increased concentration of PF4 in serum could theoretically disrupt formation of preformed antigenic complexes and diminish binding of PF4-heparin antibodies. However, we did not observe any significant differences in signal between samples collected in serum or plasma, indicating that this potential cause of discordant results had no impact on the ELISA. It is likely that the high concentrations of PF4 found in serum were reduced through sample dilution. In commercial assays, samples are diluted 50-fold, which lowers the final concentration of PF4 to levels that are likely to have negligible effects on PF4-heparin multimolecular assembly (0.05-0.1 μg/mL).[17,18] In addition, the GTI assay uses covalent linkage of PF4 to polyvinyl sulfate, which is unlikely to dissociate or bind additional PF4 that may be present in solution.

Our study also examined the stability of PF4-heparin antibodies in various collection media. Our results indicate that antibodies stored at 4°C in serum or anticoagulated blood remain stable with no deterioration in signal occurring for up to 96 hours. These findings suggest that PF4-heparin antibodies do not become adsorbed to cell surface glycosaminoglycans or to PF4 released from platelets contained in the specimen.

These findings provide important laboratory validation of conditions involved in PF4-heparin antibody testing and have implications for clinical and research studies. The comparability of blood preparations (serum, citrate, and EDTA) for PF4-heparin antibody detection suggests that clinical testing for HIT can be batched with other tests (eg, CBC counts and/or prothrombin time and activated partial thromboplastin time) to minimize the volume of blood collected from an individual patient and charges incurred for phlebotomy. Because most clinical laboratories store blood collected for CBC counts, prothrombin and activated partial thromboplastin times, and chemistry tests for a period after testing is performed (2-7 days), our findings on the stability of PF4-heparin antibody signals also suggest that testing for PF4-heparin antibodies can be "added on" to existing specimens. The ability to add on a test for PF4-heparin antibodies may be particularly useful when preexisting PF4-heparin antibodies are suspected in patients in whom rapid-onset HIT develops.[19,20]

In summary, we found that testing for PF4-heparin antibodies using the GTI assay yields comparable OD values whether assays are performed on serum samples or plasma anticoagulated with citrate or EDTA. There is no clinically significant difference if samples are run after 2 to 4 days of cold storage. Our data imply that comparisons are valid for the results of studies performed with different types of samples and that clinical decisions about diagnosis and management can be based on values obtained from testing samples in whichever medium is most convenient.

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