# The Influence of Free Hemoglobin and Bilirubin on Heparin Monitoring by Activated Partial Thromboplastin Time and Anti-Xa Assay

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• ***Context.***—**Elevated free hemoglobin (Hb) and bilirubinemia complicate extracorporeal membrane oxygenation and could affect unfractionated heparin (UH) therapy monitoring by anti-Xa assay and activated partial thromboplastin time (aPTT).**

***Objectives.***—**To compare in vitro response of anti-Xa and aPTT assays to UH in samples with artificial hyperbilirubinemia and hyperhemoglobinemia and to estimate if this interference is also observed in vivo in pediatric extracorporeal membrane oxygenation.**

***Design.***—**Measurement of aPTT and anti-Xa activity in plasma spiked with UH and increased concentration of free Hb and/or conjugated bilirubin. All samples with anti-Xa activity, antithrombin, free Hb, and bilirubin determination and infused dose of UH from inpatients on extracorporeal membrane oxygenation were extracted from the clinical patient database and analyzed.**

***Results.***—**Each increment of free Hb by 100 mg/dL significantly shortened aPTT, whereas an increment of bilirubin by 6 mg/dL caused significant prolongation of aPTT and stepwise increase of free Hb and/or bilirubin in plasma decreased anti-Xa activity by 0.03 to 0.05 IU/mL. Extracorporeal membrane oxygenation samples with free Hb 50 mg/dL or greater had significantly lower anti-Xa activity compared with normal ones: 0.33 (0.25–0.42) versus 0.4 (0.31–0.48) IU/mL ($P = .01$), despite the identical UH infusion and similar antithrombin activity. Moderate increase of free Hb by 59 mg/dL was associated with absolute decrease of anti-Xa activity by 0.07 IU/mL.**

***Conclusions.***—**Activated partial thromboplastin time and anti-Xa assay are affected by elevated level of free Hb and/or bilirubin in the presence of UH, and lower anti-Xa activity is noted in extracorporeal membrane oxygenation patients with elevated free Hb. Severe hemolysis and/or hyperbilirubinemia could compromise UH monitoring based on these assays.**

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Introduction of extracorporeal membrane oxygenation (ECMO) support into clinical practice has increased survival in children and adults with cardiopulmonary failure.[1] However, mortality is still high, ranging from 35% to 40%, and the main causes of increased morbidity remain bleeding and thrombosis.[2,3] In order to prevent activation of the hemostatic system into the circuit, unfractionated heparin (UH) is usually administered for patients on ECMO and therapeutic anticoagulation is monitored by various laboratory tests: activated clotting time, activated partial thromboplastin time (aPTT), anti-Xa assay, and thromboelastography.[4,5] Our previous experience in laboratory monitoring of heparin therapy in neonatal ECMO has shown poor correlation among anti-Xa, aPTT, and activated clotting time, suggesting no single laboratory test is useful to determine appropriate anticoagulation management in these settings.[6] Indeed, ECMO support is frequently complicated with mechanical hemolysis followed by elevated plasma free hemoglobin (Hb) and hyperbilirubinemia,[7,8] which could affect the abovementioned assays and affect UH monitoring. Currently the anti-Xa assay used in our hospital is claimed to be insensitive to free Hb (up to 200 mg/dL) and bilirubin (up to 6.6 mg/dL).[9] It is also unknown if heparin monitoring by aPTT is affected by elevated bilirubin or free Hb.

The aim of our study is to compare in vitro response of anti-Xa and aPTT assays to therapeutic levels of unfractionated heparin in citrated plasma samples with artificially created hyperbilirubinemia and hyperhemoglobinemia and to estimate if this interference is also observed in vivo in a pediatric ECMO setting.

## MATERIALS AND METHODS

Synthetic conjugated bilirubin (ditaurobilirubin, Lee Biosolutions, St Louis, Missouri) was dissolved in phosphate-buffered

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| Activated Partial Thromboplastin Time (aPTT) Results in Nonheparinized Samples With Bilirubinemia and Elevated Free Hemoglobin (Hb) | | |
|---|---|---|
| | aPTT, Median (Interquartile Range), s | *P* Value |
| Bilirubin | | |
| Baseline | 29.3 (27.5–29.5) | .01 |
| 6 mg/dL | 31.1 (28–32.5) | |
| 12 mg/dL | 32.8 (29.1–34.9) | |
| Free Hb | | |
| Baseline | 29.8 (28.1–31.7) | .003 |
| 100 mg/dL | 29.4 (27.9–30.7) | |
| 200 mg/dL | 29.1 (27.6–30.3) | |

saline, pH 7.4 (final concentration 300 mg/dL), aliquoted, and frozen at –80°C. Free Hb was prepared from red blood cells: they were washed twice by isotonic saline and frozen (–80°C) and thawed 3 times; then, the debris from membranes was sedimented at 12 000*g* for 30 minutes and supernatant was collected (final free Hb concentration 30 g/dL) and kept at –80°C before using in experiments. Blood samples from 6 normal donors were collected into blue-top BD Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, New Jersey) containing buffered 3.2% sodium citrate according to the standard protocol after informed consent was obtained. Citrated plasma samples were prepared from whole blood by centrifugation at 1500*g* for 15 minutes and used for spiking experiments: they were spiked with 6 and 12 mg/dL of conjugated bilirubin or 100 and 200 mg/dL of free Hb and with 0.3 and 0.6 U/mL of UH (100 U/mL; heparin sodium, APP Pharmaceuticals LLC, Schaumburg, Illinois). In addition, dual-spiked (Hb 50 or 100 mg/dL plus bilirubin 3 or 6 mg/dL) heparinized samples were prepared. In order to minimize the possible dilutional effect on results, all baseline samples were spiked with identical volume of vehicle solutions (phosphate-buffered saline or isotonic saline). Activated partial thromboplastin time was measured using PTT STA reagent and anti-Xa activity was measured using Rotachrom reagent (detection limit 0.1 IU/mL, intra-assay coefficient of variation across the measured range <5%) on the STA-R analyzer (reagents and analyzer from Diagnostica Stago, Parsippany, New Jersey).

A prospective observational study approved by institutional review board was conducted among children on ECMO hospitalized at Texas Children's Hospital, Houston. All samples for which anti-Xa assay and antithrombin activity were performed simultaneously with free Hb and bilirubin determination from inpatients on ECMO receiving intravenous UH were extracted from the clinical patient database together with the daily infused dose of UH.

Statistical analysis was performed using repeated-measures analysis of variance for in vitro experiments, and clinical data were analyzed using Mann-Whitney test, Pearson correlation (SPSS Statistics for Windows, Version 19.0, IBM Corp, Armonk, New York). All results are expressed as median (interquartile range), and statistical significance between groups was defined as a *P* value of less than .05.

## RESULTS

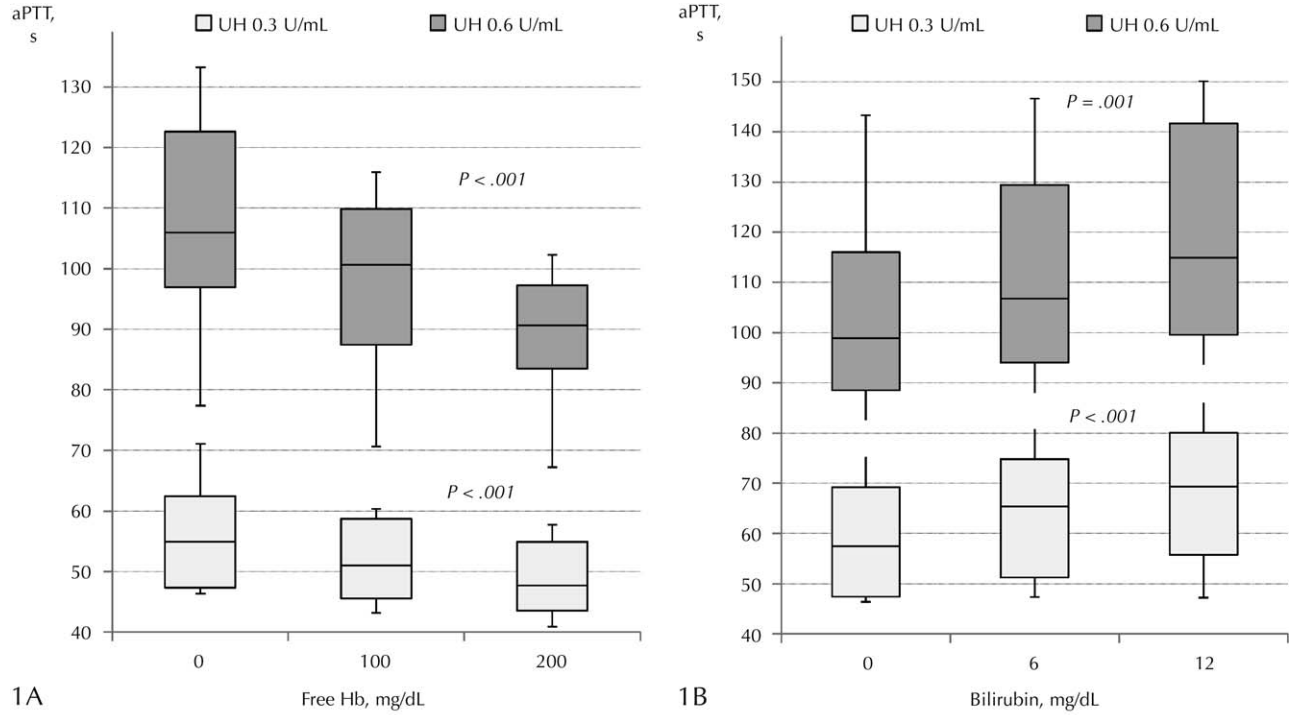
In the absence of UH, increased concentrations of bilirubin led to small but statistically significant prolongation of aPTT, whereas free Hb shortened significantly aPTT values (Table). Activated partial thromboplastin time also was significantly affected by bilirubin and free Hb in heparinized samples: it was shortened by 7% to 9% with each increment of free Hb by 100 mg/dL (Figure 1, A) and prolonged by 7% to 8% with increased bilirubin concentration by 6 mg/dL (Figure 1, B). Bilirubin and free Hb also affected the anti-Xa assay: anti-Xa activity decreased by 0.03 to 0.05 IU/mL with each increment of free Hb concentration by 100 mg/dL (Figure 2, A) or with bilirubin increment of 6 mg/dL (Figure 2, B). Accuracy of aPTT and anti-Xa activity measurement across the therapeutic range of UH exceeded 10% at free Hb level higher than 150 mg/dL or bilirubin level higher than 10 mg/dL. Dual spiking of heparinized plasma samples with both compounds was accompanied with a similar decrease of anti-Xa activity by 0.035 IU/mL with each increment of free Hb 50 mg/dL plus bilirubin 3 mg/dL, but did not significantly affect aPTT measurement (data not shown).

One hundred forty-nine specimens from 20 patients (65% male; median age, 1.8 months [interquartile range, 1 day–5.1 years]) on ECMO for 7.5 (5–9) days were analyzed: heparin infusion rate was 25 (18–30) units/kg/h, anti-Xa activity was 0.38 (0.29–0.46) IU/mL, antithrombin activity was 93% (82%–104%), and free Hb concentration was 22 (8–59) mg/dL. Samples with elevated free Hb (n = 50, defined as ≥50 mg/dL) had significantly lower anti-Xa activity (Figure 3) despite the identical infusion rate (25 [17–33] versus 25 [22–28] units/kg/h, $P = .96$) of UH or similar antithrombin level (95% [85%–107%] versus 90% [82%–102%], $P = .08$). Moderate increase of median value of free Hb by 59 mg/dL between two groups was associated with absolute decrease of median anti-Xa activity by 0.07 IU/mL. We found that patients with elevated free Hb were out of the target UH level range more frequently: anti-Xa activity was less than 0.3 IU/mL in 32% of cases, compared with 21% for the group with normal Hb values. There were no sufficient data to compare the influence of bilirubin on anti-Xa assay, because this analysis was not performed routinely in patients on ECMO, but from available data (n = 16) we noted that elevated free Hb 143 (99–198) mg/dL in plasma samples was accompanied with increased total bilirubin 10 (5–13) mg/dL; however, the correlation between these variables were poor ($r = .04$, $P = .88$).

## COMMENT

Hemolysis is frequently observed in the pediatric ECMO setting accompanied with pronounced elevation of free Hb (peak values 140–220 mg/dL) and severe hyperbilirubinemia (peak values 8–15 mg/dL) observed within the first 7 days of ECMO.[10] We found that hyperbilirubinemia and elevated level of free Hb in plasma samples provoked mild underestimation of heparin activity by anti-Xa assay. Colorimetric assays might be sensitive to high free Hb or high bilirubin in the specimen, and the anti-Xa assay Rotachrom is claimed to be insensitive to these substances unless bilirubin is higher than 6.6 mg/dL or Hb higher than 200 mg/dL.[9] Our data suggested that assay sensitivity could depend on heparin concentration; that is, anti-Xa activity is already underestimated by more than 10% in the presence of 6 mg/dL of bilirubin or 100 mg/dL of free Hb if anti-Xa activity is low at 0.2 to 0.3 IU/mL.

We found aPTT is slightly shorter in the samples with elevated free Hb, in agreement with previous findings,[10–12] and aPTT values are further shortened by 5 to 20 seconds, depending on initial heparin and free Hb concentration in plasma samples. Although the exact mechanism of this finding is not known, direct activation of coagulation factors (V, VII, X) is suggested.[11] In addition, free Hb has been shown to bind UH between tetrameric Hb and polyanionic UH stoichiometrically[13] that might lead to partial neutralization of UH anticoagulant activity in vivo and contribute to the shortened aPTT values and anti-Xa activity in heparin-



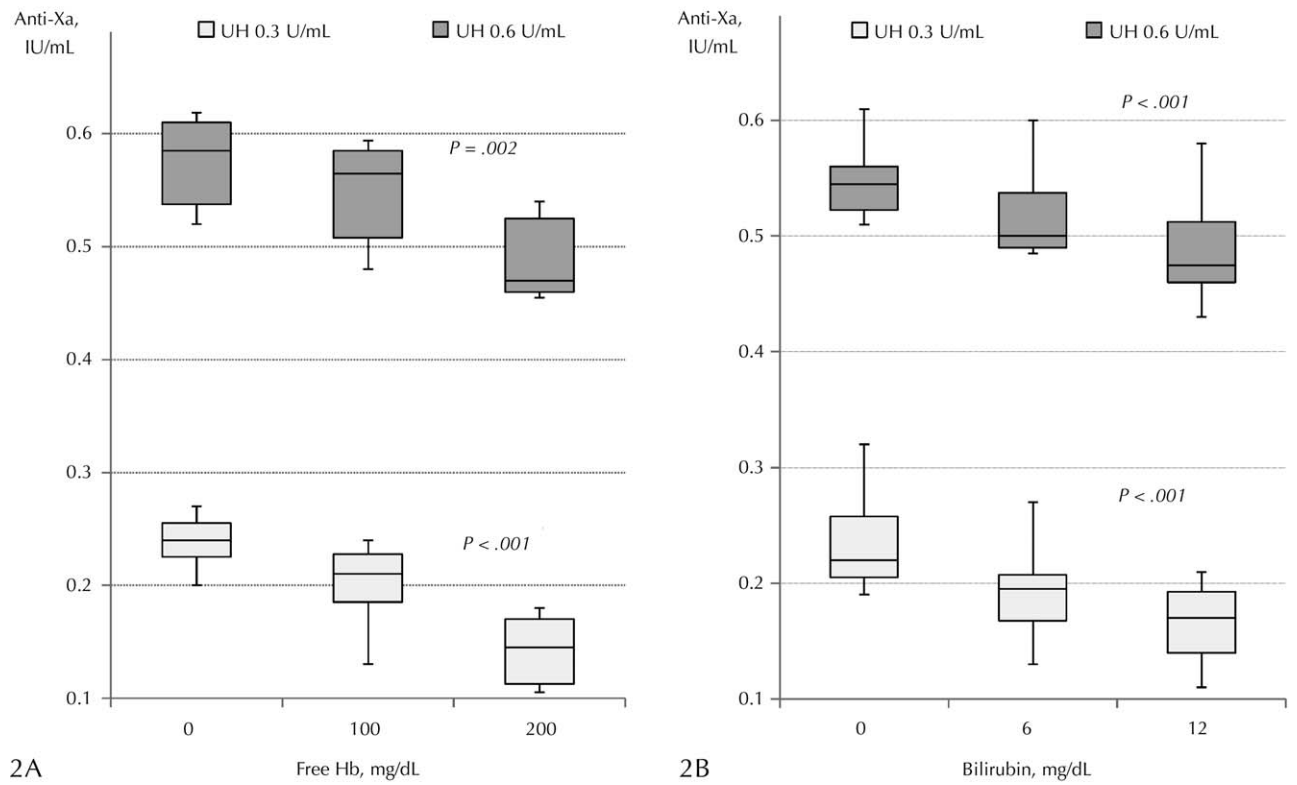




**Figure 1.** *Influence of free hemoglobin (Hb) (A) and bilirubin (B) on activated partial thromboplastin time (aPTT) in heparinized (final unfractionated heparin [UH] concentrations 0.3 and 0.6 U/mL) samples (median and interquartile range; n = 6).*

**Figure 2.** *Influence of free hemoglobin (Hb) (A) and bilirubin (B) on anti-Xa activity in heparinized (final unfractionated heparin [UH] concentrations 0.3 and 0.6 U/mL) samples (median and interquartile range; n = 6).*

ized samples. It is not clear if hemolysis in patients on ECMO is the cause or rather a consequence of increased activation of the clotting system[14] and if higher anticoagulation by heparin would be beneficial in this situation. Monitoring of heparin therapy by aPTT or anti-Xa assay in the ECMO setting complicated with severe hemolysis might lead to underestimation of anticoagulant heparin activity in plasma and increased risk of heparin overdose.

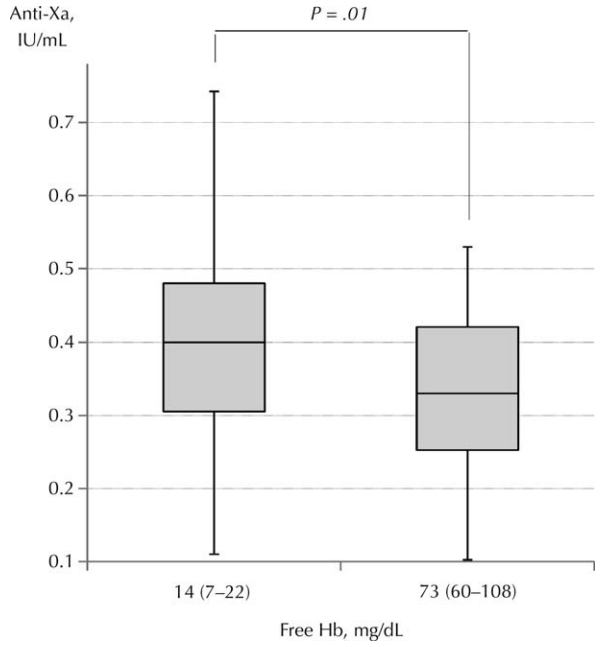



**Figure 3.** *Anti-Xa activity in plasma samples from patients on extracorporeal membrane oxygenation with free hemoglobin (Hb) below 50 mg/dL (n = 99) and above 50 mg/dL (n = 50), median and interquartile range.*

Surprisingly, we found that conjugated bilirubin slightly prolonged aPTT in citrated plasma, and more prominent prolongation was observed in heparinized samples. Hyperbilirubinemia is not considered an important interfering substance on coagulation tests according to a recent review.[15] However, early publications suggested that bilirubin infused intracardially to rabbits was associated with prolongation of clotting times in plasma mixed with heparin (heparin tolerance test) or after recalcification at plasma bilirubin level higher than 6 mg/dL and decreased activity of factors II and VII.[16] The addition of bilirubin to human plasma was associated with a mild decrease of factor V activity,[17] and slightly prolonged aPTT values in neonates with hyperbilirubinemia were reported.[18] Monitoring of heparin therapy by aPTT and anti-Xa assay in the ECMO setting complicated with severe hyperbilirubinemia could cause disproportionately prolonged aPTT relative to anti-Xa value and lead to underestimation of anticoagulant heparin activity in plasma based on anti-Xa assay.

Laboratory data analysis from pediatric ECMO charts is in agreement with in vitro experiments: higher free Hb is associated with lower anti-Xa activity in heparinized plasma samples despite the similar UH infusion rate. Absolute difference of UH anti-Xa level by 0.07 IU/mL in the clinical ECMO setting was similar to an observed in vitro decrease when plasma sample was simultaneously spiked with 100 mg/dL of free Hb and 6 mg/dL of bilirubin.

Our study has several limitations. Citrated plasma spiked in vitro with UH overestimates the degree of aPTT prolongation when compared with ex vivo patients' plasma samples treated with UH.[19] That is why aPTT prolongation in such patients with hyperbilirubinemia or aPTT shortening with elevated free Hb is expected to be less impressive, as we showed in our experiments. The free Hb concentrate used in our experiments could contain residual red blood cells membranes or microparticles and might contribute to the shortened aPTT found in our experiments. However, it has previously been shown that 94% removal of these microparticles by filtration did not significantly affect aPTT.[20]

In conclusion, both aPTT and anti-Xa assay are affected by an elevated level of free Hb or bilirubin in the presence of a therapeutic level of UH. Clinicians who are taking care of patients on ECMO should be aware of compromised heparin monitoring and anticoagulant activity based on these assays in case of severe hemolysis and/or hyperbilirubinemia.

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