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LETTER TO THE EDITOR

Effect of pH on thrombin activity measured by calibrated automated thrombinography

We would like to thank Kristensen et al,[1] who have recently reported the effect of increasing pH on reduced thrombin generation (TG) parameters by calibrated automated thrombogram (CAT). pH change in plasma has resulted in stabilization of commercially pooled and lyophilized plasmas with the addition of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Interestingly, the authors demonstrate that the addition of HEPES buffer to plasma can help normalize pH and modulate TG parameter results. Clearly, pH change can influence coagulation, ultimately posing a risk for the accurate assessment of TG parameters. We would like to expand upon the work of Kristensen et al by addressing additional points that should be considered in regard to pH and CAT.

- **Lactic acid for pH normalization:** An alternative to HEPES buffer for plasma pH normalization is the addition of lactic acid. Supplementing plasma with 10% lactic acid corrects for pH > 8, making pH stable at 7.1-7.4 for 24 hours.[2] Further, lactic acid is an endogenous component found in blood, providing a more physiological and stable means of normalizing pH.
- **Ensuring pH stability *during* CAT assay:** Kristensen et al found that TG parameters in plasma without HEPES were lower than in HEPES-treated plasma despite both having normal pH at the start of the experiment. This can be explained by the poor stability of untreated plasma in the open, moving microplate inside the heated microplate reader. From this perspective, pH stabilization prior to the TG experiment or use of a CO_2-controlled microplate reader can offer a more accurate TG assessment.
- **Matching plasma pH conditions for thrombin calibrator:** It is well established that pH affects the activity of enzymatic reactions, including coagulation factors. Indeed, thrombin activity

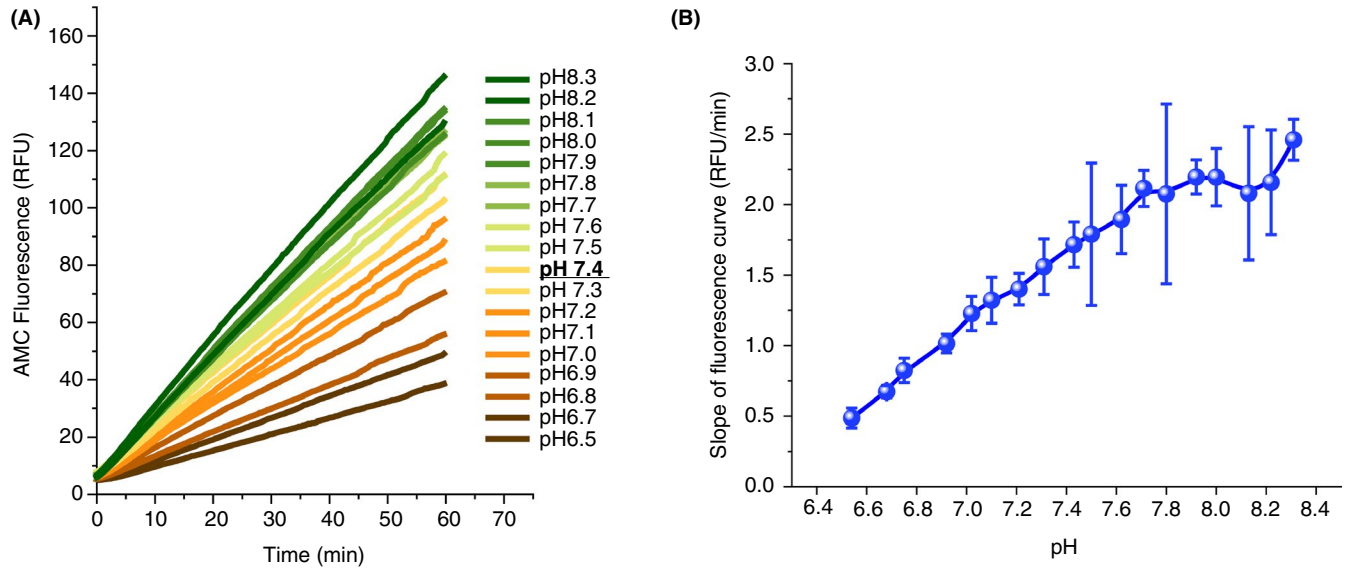

FIGURE 1 Thrombin-mediated substrate cleavage is dependent upon pH. Thrombin activity was measured by calibrated automated thrombogram (CAT; Stago, Parsippany, NJ, USA) in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer system of varying pH. (A) Differing fluorescence relative light unit (RLU) measurements over time are observed from thrombin-mediated substrate consumption. Thrombin generation is low in HEPES buffer with low pH (<pH 7.0) and is increased as pH increases. (B) The average rate of thrombin activity for the indicated pH values, was observed to be higher as pH increased

toward the fluorogenic substrate of the CAT assay is clearly pH dependent (Figure 1). Hence, the internal CAT thrombin calibrator (thrombin-α_2 macroglobulin complex) may be helpful in correcting the effects of pH. It will be interesting to compare TG in plasma samples, where pH is modulated via CO_2 release, when calibrated to either 1 calibrator well or to pH-matched calibrator wells. Unfortunately, CAT assay reagents tend to influence the pH of plasma; for example, FluCa often reduces the pH (data not shown). Therefore, differences in the impact of CAT assay reagents, such as thrombin calibrator and tissue factor reagent, can complicate this analysis.

- **Normalization may not be suitable for patients with abnormal blood pH:** There are situations where normalization may not be the most ideal method to reflect the patient hemostasis potential. For example, poor TG in acidosis was studied by Mitrophanov et al[3] as a potential factor contributing to the pathology of acidosis patients. We therefore raise the notion that perhaps the normalization of pH may not be suitable for plasmas with abnormal pH, such as in acidosis conditions, for example, due to the changes in TG results associated with normalization.

Thus, many conflicting considerations should be taken when performing CAT analysis on samples that have a pH change, highlighting the possibility that simple solutions are not possible.

RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

JWJ wrote the letter. SSS performed experiments and data analysis. YL and LAP contributed to study design and interpretation. MVO designed the study and helped write and revise the letter.

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The contributions of Yideng Liang, Leonid A. Parunov, and Mikhail V. Ovanesov are informal communications and represent our own best judgment. These comments do not bind or obligate the FDA.

REFERENCES

1. Kristensen SR, Nybo J, Pedersen S. The effect of pH on thrombin generation-an unrecognized potential source of variation. Res Pract Thromb Haemost. 2020;4(2):224–9.
2. Sinauridze EI, Volkova RI, Krasotkina YV, Sarbash VI, Ataullakhanov FI. Dynamics of clot growth induced by thrombin diffusing into nonstirred citrate human plasma. Biochim Biophys Acta. 1998;1425(3):607–16.
3. Mitrophanov AY, Rosendaal FR, Reifman J. Mechanistic modeling of the effects of acidosis on thrombin generation. Anesth Analg. 2015;121(2):278–88.