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BRIEF REPORT

The effect of pH on thrombin generation–An unrecognized potential source of variation

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Abstract

Background: When CO_2 escapes from plasma, the pH of the plasma increases. In samples left open or kept in long-term storage, the pH may increase considerably. Assays in which the ratio of plasma sample relative to the total volume including reagents is high may be sensitive to the pH of the plasma sample.

Objective: The aim was to investigate the effect of the pH of plasma samples used in the calibrated automated thrombin generation (CAT) assay in which the ratio (plasma sample) / (total volume) is high.

Methods: Plasma pH was increased by allowing CO_2 to escape in open beakers before the CAT analysis. The effect of pH was also investigated by mixing plasma with buffers with different pH levels.

Results: At a pH close to 8.0, endogenous thrombin potential (ETP) and peak decreased considerably, whereas lagtime and time-to-peak were modestly increased. Mixtures of plasma and buffer with pH levels between 7 and 8 showed that ETP and peak decreased at alkaline pH; lagtime and time-to-peak were higher at acidic pH levels but were shortened, partly in contrast to first results, at alkaline pH levels. The addition of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer to plasma with a high pH attenuated the effects; however, the effect was most significant if added before the CO_2 escaped.

Conclusion: Modifications of plasma pH can significantly alter thrombin generation. In alkaline samples, for example, after lengthy storage in a freezer where pH can increase considerably, thrombin generation is lowered. To minimize this effect, plasma should be stored in tubes filled to the maximum volume.

KEYWORDS

alkaline plasma, carbon dioxide, endogenous thrombin potential, pH, preanalytical variation

Essentials

- Escape of CO_2 from plasma increases pH, potentially impacting the results of thrombin generation.
- The effect of an increased pH on the assay results was measured after modifying plasma pH.
- A high pH, caused by escape of CO_2 or addition of a buffer, reduced thrombin generation.
- pH changes of plasma samples should be avoided by keeping plasma in filled, closed tubes.

1 | INTRODUCTION

Thrombin generation (TG) assays have become important for determining the global coagulation capacity in plasma. A TG assay was described in 1953,[1] but a more reproducible method was developed and standardized by Hemker et al.[2–4] Calibrated automated thrombin generation (CAT) is widely used to assess TG, but other methods are available with minor differences.[5] With this assay, it is possible to demonstrate a lower capacity in patients prone to bleeding and an increased capacity in patients with a higher risk of thrombosis,[4,6,7] and it has been widely used in research to investigate coagulation in various patient groups.[6,7] One drawback of the method has been a rather high imprecision, especially a high interlaboratory variation.[6,7]

In vivo plasma has a meticulously regulated pH of approximately 7.4. However, when plasma is used for analysis, CO_2 may escape from it due to a higher partial pressure in the fluid than in atmospheric air, and this results in an increase in pH. In assays, the reagents contain buffers to maintain a neutral pH, but the buffer capacity may not always be strong enough, especially if the fraction of plasma for an assay is relatively high. In the CAT analysis, the reagents contain 20 mmol/L of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer,[4] but the fraction of plasma in the final mixture is quite high (usually 80 µL of 120 µL).

Although the importance of various preanalytical conditions have been investigated,[8,9] the effect of alkalinization of plasma caused by loss of CO_2 has not been evaluated.

The aim of this study was to determine the potential importance of pH modifications of plasma on thrombin generation.

2 | MATERIALS AND METHODS

Blood samples were from healthy volunteers without signs of any illness after informed consent. Ethical approval was obtained from the local ethical committee (N-20110068). Blood was collected with a 21-gauge needle in 6-mL citrated (0.105 mol/L) siliconized glass tubes (BD Vacutainer, BD Biosciences, Franklin Lakes, NJ, USA) using minimal stasis. Samples were centrifuged within half an hour (2 × 15 min) at 2500 g, as recommended by Lacroix et al,[10] to obtain platelet-free plasma, which for each participant was assembled and used either fresh or after freezing at −80°C (filled tubes). The number n in each experiment refers to the number of experiments using different individuals.

2.1 | Thrombin generation

TG was measured by means of CAT (Thrombinoscope BV, Maastricht, The Netherlands), according to the manufacturer's instructions: 20 µL of trigger reagent and 80 µL of sample material were mixed, followed by the addition of 20 µL of FLuCa (containing the fluorogenic substrate Z-Gly-Gly-Arg-AMC) to a final volume of 120 µL. Fluorescence was read for 60 minutes with a 390/460 nm filter set, and TG curves were calculated using Thrombinoscope software version 5.0 (Thrombinoscope BV). The CAT analyses were performed with final concentrations of 5 pmol/L TF and 4 µmol/L phospholipids (PPP reagent, Thrombinoscope BV) or 1 pmol/L TF and 4 µmol/L phospholipid (PPP-low reagent, Thrombinoscope BV) as indicated in the text. The analyses were run in duplicate, and the results were measured as endogenous thrombin potential (ETP), peak, lagtime, and time-to-peak (ttPeak).

2.2 | Experiments

To measure the pH changes of the plasma, 2-mL Sarstedt microtubes without or with a cork were kept at ambient temperature for 0 to 6 hours or for 2 weeks at −80°C (closed vials). pH was measured on an ABL 837 (Radiometer, Copenhagen, Denmark) at 37°C. To measure pH changes during the TG procedure, pH was measured in plasma samples or relevant mixtures added to the microplate.

For experiments with a high and a normalized pH in samples for TG, the alkalinization was induced in fresh blood samples, where plasma was kept in a beaker (large surface) without a cork for 30 to 60 minutes to allow for the escape of CO_2. To normalize pH again, the plasma was then kept in an atmosphere with 5% CO_2 (blown into the tube for a few minutes and mixed).

To investigate the effect of pH during the CAT procedure, plasma was mixed 1 + 1 with a 50-mmol/L imidazole buffer (Amplicon, Odense, Denmark) or a 20-mmol/L HEPES buffer (containing 140 mmol/L NaCl and bovine serum albumin 5 g/L) adjusted to different pH values between approximately 7 and 8. Due to variance between the experiments, the results were assembled in groups with pH values within 0.2 pH units (Figure 2).

In the final experiments with normalization of pH in the plasma samples using a HEPES buffer, a 300-mmol/L HEPES buffer (containing 5 g/L bovine serum albumin, pH 7.35) was used.

2.3 | Statistical methods

The results within the different experiments were normalized using the applicable sample as the reference (ratio: sample/reference sample) in order to calculate the mean and standard error of the mean (SEM). Differences, where appropriate, were tested with Student's *t*-test and were considered significant at $P < 0.05$.

3 | RESULTS AND DISCUSSION

The effect of loss of CO_2 in plasma samples is shown in Table 1. Two-milliliter tubes containing different volumes of plasma were left open or closed with a cork for 1 to 6 hours at ambient temperature or 2 weeks in a freezer. Baseline pH of citrate plasma was 7.2 (standard deviation, 0.025). pH increases much more in open tubes, but the

changes are highly dependent on the volume in the tube. The process is slowed down in a freezer, but with a low volume of plasma in the vial, pH does increase slowly, and from personal experience, samples older than 1 year can have pH values of 7.7 to 7.8 or even higher.

First, the effect of an increased pH on TG was demonstrated. An increase in pH to almost 8.0 after CO_2 was allowed to escape from the plasma had a distinct effect on TG, especially on ETP, and the effect was slightly more pronounced using PPP-low (1 pmol/L TF; Table 2). ETP decreased significantly (12%-15%) as pH increased. If the CO_2 content was restored to a pH of approximately 7.3, TG increased again and was actually higher than in fresh plasma. ETP increased to 2% to 12% above baseline, but the peak increased even more. Figure 1 shows all these results.

TABLE 1 pH changes in open or closed tubes with different volumes of plasma

		Open vials	Closed vials
	Time	Δ pH mean ± SD	Δ pH mean ± SD
2000 µL	1 h	0.08 ± 0.03	0.00 ± 0.01
	2 h	0.15 ± 0.03	0.01 ± 0.01
	4 h	0.28 ± 0.04	0.03 ± 0.02
	6 h	0.39 ± 0.05	0.03 ± 0.02
	2 wk		0.03 ± 0.02
1000 µL	1 h	0.13 ± 0.04	0.07 ± 0.02
	2 h	0.22 ± 0.03	0.06 ± 0.04
	4 h	0.37 ± 0.05	0.06 ± 0.03
	6 h	0.50 ± 0.05	0.09 ± 0.03
	2 wk		0.11 ± 0.03
500 µL	1 h	0.24 ± 0.04	0.15 ± 0.05
	2 h	0.37 ± 0.06	0.16 ± 0.03
	4 h	0.58 ± 0.06	0.18 ± 0.04
	6 h	0.77 ± 0.09	0.21 ± 0.02
	2 wk		0.25 ± 0.04

Note: Plasma samples (n = 5) were filled (volume as indicated) into 2-mL tubes and left open or closed for the time indicated, 1-6 h at ambient temperature, or 2 wk in a freezer. The table depicts the differences from baseline pH (mean ± SD).

Investigation of pH changes during the analysis procedure showed that pH of plasma samples pipetted into the microwells (80 µL) increased 0.20 to 0.25 units after 10 minutes, 0.3 to 0.4 units after 20 minutes and 0.4 to 0.5 units after 30 minutes, since the surface area is quite high in relation to the sample volume. The initial procedure pipetting the samples usually lasts 10 to 20 minutes, followed by a 10-minute incubation at 37°C. The reagents for the CAT procedure, the trigger reagent and FluCa, contain 20 mmol/L HEPES buffer with pH 7.3.[4] Addition of the trigger reagent to the plasma lowers the pH 0.4 to 0.5 units, thus normalizing pH when using a fresh sample; however, in thawed samples with pH near 8 or higher, it will not be normalized. FluCa reagent also contains HEPES buffer, and this buffer will tend to normalize pH (not possible to measure because coagulation takes place). However, in the mixtures, pH will increase further. Thus, in samples with an initial pH that is normal, pH will increase during the handling procedures, but the reagents ensure a normalized pH. However, in thawed samples with a high pH and in samples for which the measurement takes some time after pipetting into the plates, pH will not be normalized during the measurements, potentially affecting the results, as demonstrated in Table 2. In frozen samples, a pH effect may accentuate a potential decrease of factor activity caused by freezing.

To investigate the effect of pH, plasma samples were mixed with HEPES or imidazole buffers with pH adjusted to different levels between approximately 7 and 8. Figure 2 depicts the effect on TG from the average of 8 series of determinations, the results of

TABLE 2 Effect of alkaline pH on thrombin generation

		Fresh plasma	pH 7.9-8.0	pH normalized (+CO_2)
Reagent		TG value, M ± SD	Ratio (mean ± SEM)	Ratio (mean ± SEM)
PPP	ETP	1170 ± 99	0.88 ± 0.02***	1.02 ± 0.04
	Peak	203 ± 19	0.85 ± 0.02***	1.19 ± 0.07*
	Lagtime	2.3 ± 0.2	1.04 ± 0.02	1.00 ± 0.02
	ttPeak	5.5 ± 0.2	1.07 ± 0.02**	0.93 ± 0.03*
PPP-low	ETP	1010 ± 61	0.85 ± 0.03***	1.12 ± 0.02***
	Peak	100 ± 14	0.78 ± 0.03***	1.86 ± 0.15***
	Lagtime	4.4 ± 0.2	1.05 ± 0.02	0.96 ± 0.03
	ttPeak	9.8 ± 0.6	1.04 ± 0.02	0.81 ± 0.04***

Note: Plasma samples (n = 12) were left open with a large surface in beakers without a cork to increase pH to close to 8. Subsequently, the samples were incubated in a CO_2 atmosphere to normalize pH. The results were normalized relative to the results in the fresh plasma column, in which the original results (mean ± SD) of TG are described. The reagent PPP contains 4 pmol/L tissue factor and 4 µmol/L phospholipids, and PPP-low contains 1 pmol/L tissue factor and 4 µmol/L phospholipids.

*P < .05; **P < .01; ***P < .001.

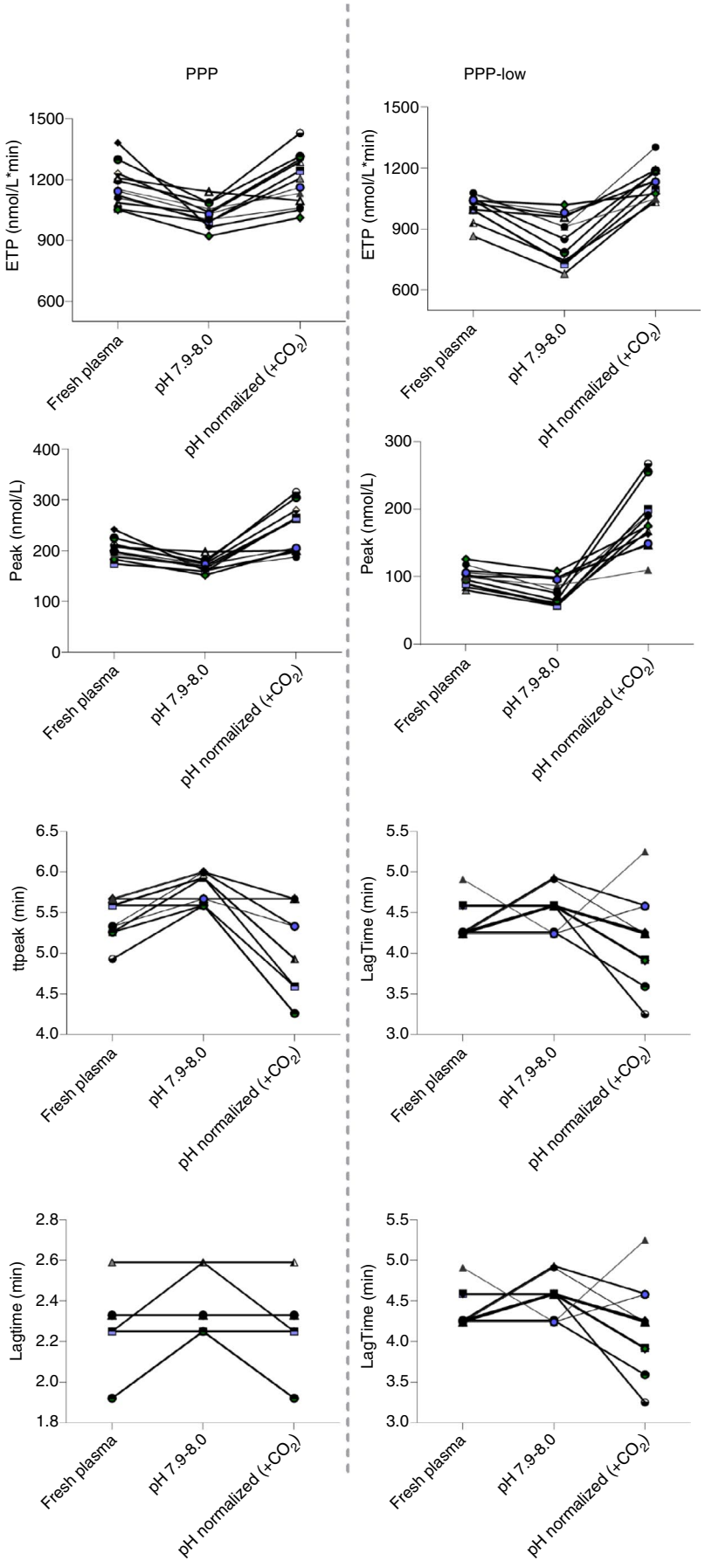

FIGURE 1 Individual results (from top to bottom: ETP, peak, time-to-peak, and lagtime) from each of the 12 plasma samples in Table 2 using the reagent PPP (left figures) or PPP-low (right figures). ETP, endogenous thrombin potential

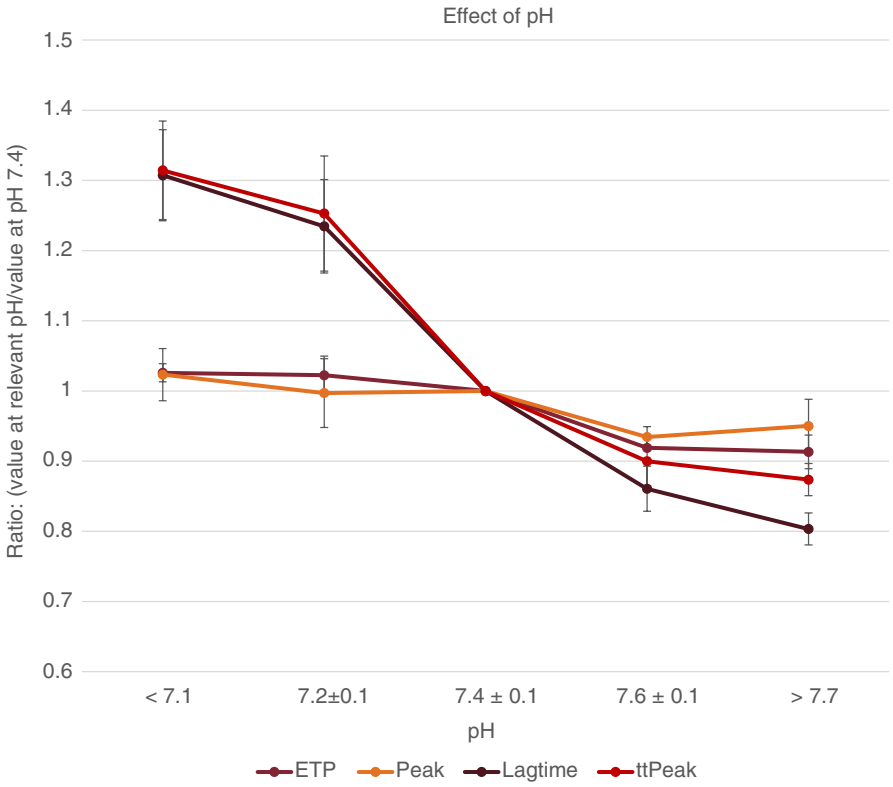

FIGURE 2 TG (using PPP-low reagent) from the average of 8 series of determinations of which the results have been cumulated in 0.2 pH unit intervals of pH levels normalized to the measurements around 7.4 (7.4 ± 0.1). Samples with pH < 7.1 ranged from 6.98-7.09, and samples > 7.7 ranged from 7.73-8.00. The points and bars indicate mean and standard error of the mean. TG, thrombin generation

TABLE 3 Effect of alkaline pH and addition of HEPES buffer on thrombin generation

N = 8	Plasma fresh, TG value	Plasma, pH 7.7-7.9	Plasma, HEPES added	Plasma, pH 7.7-7.9, HEPES added	
	mean ± SD	Ratio (mean ± SEM)	Ratio (mean ± SEM)	Ratio (mean ± SEM)	Ratio (mean ± SEM)
ETP	1694 ± 377	0.92 ± 0.02**	1.16 ± 0.03	1.13 ± 0.02	
Peak	250 ± 72	0.93 ± 0.02*	1.19 ± 0.04	1.19 ± 0.04	
Lagtime	6.2 ± 1.3	0.98 ± 0.02	0.79 ± 0.03	0.74 ± 0.02	
ttPeak	10,0 ± 1.8	0.98 ± 0.02	0.82 ± 0.02	0.80 ± 0.02	
N = 8	**Plasma fresh**	**Plasma, pH > 8.0**	**Plasma, HEPES added**	**Plasma, pH > 8.0, HEPES added**	**Same, HEPES added before pH increased**
ETP	2020 ± 324	0.74 ± 0.03***	1.11 ± 0.02	0.89 ± 0.04***	1.04 ± 0.02*
Peak	304 ± 43	0.82 ± 0.02***	1.04 ± 0.01	0.95 ± 0.02**	1.02 ± 0.01
Lagtime	5.7 ± 0.6	1.04 ± 0.02	0.97 ± 0.02	0.96 ± 0.01	0.94 ± 0.01
ttPeak	9.0 ± 0.7	1.06 ± 0.03	0.97 ± 0.01	0.94 ± 0.02	0.91 ± 0.02*

Note: Plasma samples were left open with a large surface in beakers without a cork to increase pH to between 7.7 and 7.9 (upper part) (n = 8) or to > 8.0 (lower part) (n = 8). To these samples and the fresh plasma a 300 mmol/L HEPES buffer was added 1 + 9 (HEPES buffer + plasma), and in last column HEPES was added before CO_2 was removed. TG was measured in all the samples using PPP-low reagent. The results were normalized relative to the results in the fresh plasma column, in which the original results (mean ± SD) of TG are described. Statistical comparison: Plasma with increased pH is compared with fresh plasma, whereas plasma with normalized pH after the addition of HEPES is compared with fresh plasma with HEPES added.

*P < .05; **P < .01; ***P < .001.

which have been cumulated in 0.2-unit intervals of pH levels and normalized to the measurements around 7.4 (7.4 ± 0.1). Despite differences between experiments and subjects, the general impression was that ETP and peak were unaffected at acidic levels but were lowered at alkaline pH, whereas lagtime and ttPeak were considerably and significantly prolonged at acidic pH but decreased at alkaline pH. Similar findings were observed when using an imidazole buffer, but the differences between the acid and the alkaline part were slightly more marked (the buffer had a higher buffer capacity). This finding indicates a relevant importance of pH in the sample material, potentially inducing variation in the assay results. Mitrophanov et al[11] reported a mechanistic modeling of the effect

of acidosis and found that lagtime and ttPeak increased at acid pH, and ETP was almost unchanged, but the peak decreased. The first 3 observations are in accordance with the present findings.

Since an increased pH, for example, in stored samples, is problematic, it would be advantageous to be able to normalize pH, thereby reducing the variation. Normalization using 5% CO_2 may be a quite unstable and inconvenient procedure. Furthermore, TG actually increased after normalization (Table 2). We attempted, therefore, to stabilize the samples using a 300-mmol/L HEPES buffer, pH 7.35, added to the samples 1 + 9 (HEPES buffer + plasma sample). This addition resulted in a final concentration of 30 mmol/L HEPES, and only 10% dilution of plasma without changing the osmolality, and the pH was reduced to 7.3 to 7.4. The effect of this was first tested in samples before and after removal of CO_2, which increased pH to 7.7 to 7.9 (n = 8). Table 3 (top) shows that TG measured in the mixtures with HEPES buffer was significantly higher than that in the fresh plasma samples (ETP and peak were higher, lagtime and ttPeak shorter; $P < 0.002$). It is not an effect of the dilution 1 + 9, since the same dilution with saline had no effect. In plasma with high pH, ETP and peak were significantly lower than in fresh plasma, whereas lagtime and ttPeak were almost unchanged. Normalization of pH with HEPES also normalized TG close to that in fresh plasma with HEPES added. When pH increased to >8.0, the ETP and peak were more pronouncedly affected, and it was not possible to normalize with HEPES (constituted only 85% and 87% of plasma with HEPES added). Therefore, HEPES was added to the plasma before the removal of CO_2 (Table 3, bottom). This addition almost normalized TG, although ETP, lagtime and ttPeak were slightly lower than in plasma with HEPES added.

Thus, the addition of HEPES to samples with pH increased to <7.9 seems to be able to normalize TG, but in samples with pH >8, sample normalization is not possible. However, adding HEPES before CO_2 was allowed to escape avoided an increase in pH, reducing this effect considerably. Interestingly, lagtime and ttPeak tend to increase at a high pH, but when they are neutralized, they actually tend to decrease. In partial contrast and for unknown reasons, lagtime and ttPeak were lowered at alkaline pH in the experiments in Figure 2, but here, the added buffer probably reduced the effect of the buffer in the reagents, and the plasma was diluted 1 + 1, which may have an effect.[12]

The importance of pH changes in samples used for measuring TG has not been recognized before, but in old samples in a freezer or in fresh samples without a cork, where pH may be considerably increased, TG can potentially be lowered, adding to the preanalytical variation. The addition of a small volume of a strong buffer may normalize pH, reducing this variation, but only to some extent if pH has increased considerably. For unknown reasons, TG was always higher after the addition of a strong HEPES buffer than it was in the fresh plasma, and therefore, if these samples are compared to fresh samples, then HEPES must also be added to the fresh samples. Thus, it is not an ideal solution.

In conclusion, this study shows that samples that are alkaline, for example, after storage in a freezer, will have a reduced TG, and this will potentially increase variation between samples with different pH levels. Plasma samples should be kept in filled tubes with corks to avoid pH changes.

RELATIONSHIP DISCLOSURE

The authors report nothing to disclose.

AUTHOR CONTRIBUTIONS

SRK designed the study, analyzed the data, and wrote the manuscript. JN contributed to the design and performed the analyses. SP contributed to the design and interpretation. All authors read and revised the manuscript.

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