

# Stability of thromboxane in blood samples

This article was published in the following Dove Press journal:  
*Vascular Health and Risk Management*

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**Introduction:** Conventional venous blood collection requires a puncture with a needle through the endothelium of a vessel. The endothelial injury causes activation of circulating platelets and the release of thromboxane A<sub>2</sub>. The aim of the study was to investigate if platelets continue to form thromboxane A<sub>2</sub> in the blood tube after sample collection, but such synthesis would give false information about the actual circulating thromboxane A<sub>2</sub> value.

**Methods:** Thromboxane B<sub>2</sub> is a biologically inactive but stable metabolite of thromboxane A<sub>2</sub> and can be measured in blood samples by a standard enzyme immunoassay. Thromboxane B<sub>2</sub> measurements reflect thromboxane A<sub>2</sub> concentration. Blood samples were collected in 3.2% sodium citrate vials and EDTA vials from ten individuals and centrifuged and frozen at different time points (0, 30, and 120 minutes). Plasma aliquots were transferred to and frozen in 1.8 mL polypropylene tubes and the citrate samples were also transferred to and frozen in propylene tubes containing indomethacin.

**Results:** Concentrations of thromboxane B<sub>2</sub> in plasma samples collected in citrate vials and stored in propylene tubes increased very rapidly as the samples were left for longer after sampling and allowed to stand at room temperature. After 120 minutes, the amount of thromboxane B<sub>2</sub> was 400% higher than in the reference sample at time zero. In comparison, thromboxane B<sub>2</sub> concentration was about 200% higher in the 120-minute samples compared to the reference in samples collected in citrate vials but stored in indomethacin tubes. In samples collected in EDTA vials, a 10% reduction in thromboxane B<sub>2</sub> concentration in the 120-minute samples was observed.

**Conclusion:** Storage conditions, type of sampling vial and time from sampling until sample processing (centrifuging) has a major impact on thromboxane B<sub>2</sub> stability.

**Keywords:** thromboxane A<sub>2</sub>, thromboxane B<sub>2</sub>, stability, platelet function

## Introduction

Platelets or thrombocytes are disk-shaped cells circulating in the blood stream with a lifespan of about 10 days. They have no cell nucleus and are produced by megakaryocytes in the bone marrow.<sup>1</sup> A normal platelet count ranges from 150 to 400×10<sup>9</sup>/L<sup>1,2</sup> and they are involved in the initial cellular response to endothelial damage and repairing the vessel. Platelet function that depends on platelet thromboxane production can be determined by measuring platelet thromboxane A<sub>2</sub> (TxA<sub>2</sub>) release.<sup>3</sup> Activated platelets convert arachidonic acid to TxA<sub>2</sub> by the enzyme cyclooxygenase (COX-1).<sup>4</sup> TxA<sub>2</sub> stimulates platelet aggregation as well as smooth muscle contraction.<sup>5</sup> Consequently, TxA<sub>2</sub> has both prothrombic properties as well as being a potent vasoconstrictor.

Measuring thromboxane in blood samples is useful for evaluating the efficacy of acetylsalicylic acid (aspirin) since it inhibits irreversibly the platelet COX, preventing the formation of prostaglandin H<sub>2</sub> and therefore TxA<sub>2</sub>.<sup>6</sup>

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TxA<sub>2</sub> is very unstable under physiological conditions and is rapidly degraded into an inactive metabolite called thromboxane B<sub>2</sub> (TxB<sub>2</sub>).<sup>4</sup> TxA<sub>2</sub> has a half-life of about 30 seconds, where TxB<sub>2</sub> has a half-life of 5–7 minutes whereafter it is rapidly metabolized to urinary metabolites such as 11-dehydrothromboxane B<sub>2</sub>. Due to the ultra-short half-life TxA<sub>2</sub> cannot be analyzed in blood samples. TxB<sub>2</sub> and 11-dehydrothromboxane B<sub>2</sub>, however, can be measured with various methods such as gas and liquid chromatography, mass spectroscopy and enzyme immunoassays. The concentrations of TxB<sub>2</sub> or the urinary metabolite 11-dehydrothromboxane B<sub>2</sub> are used to reflect the levels of its source, TxA<sub>2</sub>.<sup>3,7</sup>

The collection of a blood sample requires a puncture through the endothelium of a vessel with a needle, resulting in that local platelets are activated and start to produce TxA<sub>2</sub>. The platelets that are collected into the tube are, therefore, in an activated state and may continue to produce TxA<sub>2</sub> inside the tube. This may give false information about the true TxA<sub>2</sub> value in the circulation.

The aim of this study was to study the stability of TxB<sub>2</sub> in blood samples by measuring possible ex-novo production of TxA<sub>2</sub> in blood samples. Samples were collected in two different blood tubes and consequently processed and frozen at different time points. TxB<sub>2</sub> concentration was then measured using standardized enzyme immunoassay. Optimizing a method for handling platelets is complicated, involving many different factors, including heat sensitivity, time-dependent differences and factors due to anticoagulants and plasma preparation.<sup>8,9</sup> The method used in this study was set up based on standard procedures recommended by the Clinical Department of the Icelandic University Hospital, Landspítali.

## Materials and methods

The study was approved by the Ethics Committee at Landspítali University Hospital in Iceland (No. 22/2016). All participants provided written informed consent after receiving information about the study in accordance with the Declaration of Helsinki.

### Blood sample collection and preparation

Ten healthy volunteers with body mass index (BMI) under 30 kg/m<sup>2</sup> participated in the study. All participants were free of any medication considered to affect the coagulation process, such as aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs) at least one week prior to participation in the study and all participants were nonsmokers. Blood samples from each participant were collected in a total of

seven blood tubes; (i) three Vacuette® blood tubes (Greiner Bio-One GmbH, Kremsmünster, Austria) containing 3.2% sodium citrate; and (ii) four tubes containing EDTA. One of the EDTA blood samples was collected to measure complete blood count using Sysmex XE-5000 hematology instrument (Sysmex XE-5000 analyzer; Sysmex, Kobe, Japan). The other six samples were centrifuged at 3,200 rpm for 14 minutes at 4°C.

After centrifugation, the plasma was aliquoted into 1.8 mL polypropylene tubes or in sampling tubes containing 10 µm indomethacin (Cayman Chemicals, Ann Arbor, MI, USA, No. 10,951). Indomethacin is a COX inhibitor that prevents ex-vivo thromboxane formation that cannot be prevented using anticoagulant alone. The samples stored in tubes containing indomethacin were stored frozen at –20°C. Samples stored in polypropylene tubes were frozen immediately in dry ice and then stored at –80°C.

The whole blood samples collected in vials containing citrate, and three of the whole samples collected in EDTA vials were centrifuged and plasma aliquoted at following times: Right after blood collection (0 minutes), 30 minutes, and 120 minutes after blood withdrawal (samples were allowed to stand at room temperature (23°C) until centrifugation).

### TxB<sub>2</sub> measurements

Concentration of TxB<sub>2</sub> was measured using commercially available reagents from Cayman Chemicals (Ann Arbor, MI, USA), No. 501020. The measuring method is a competitive enzyme immunoassay (EIA) based in the competition between TxB<sub>2</sub> and a TxB<sub>2</sub>-acetylcholinesterase (AChE) conjugate (TxB<sub>2</sub> tracer) for a limited number of TxB<sub>2</sub>-specific rabbit antiserum binding sites. The concentration of the TxB<sub>2</sub> tracer is held constant, thus the concentration of TxB<sub>2</sub> is inversely proportional to the concentration of TxB<sub>2</sub> on the well. The method can be used for quantification of TxB<sub>2</sub> in urine, serum, plasma, culture media samples, and other sample matrices. Plasma (citrate and EDTA) was used in this study. The EIA displays an IC<sub>50</sub> of approximately 60 pg/mL and has a detection limit of 5 pg/mL. Standards, buffers, washing buffer, and all other solutions supplied with the kits were used as recommended from the manufacturer.<sup>3</sup>

### Statistics

To get 80% power in the study with 5% acceptance for type 1 error, number of subjects were calculated based on statistical method for qualitative outcome.<sup>10</sup> The p<sub>1</sub> value was defined as zero (proportion of the control expected to

be more than zero), since subjects act as their own control. The  $p_2$  value was set to 50% (proportion of the study group expected to show change compared to control). Total number of subjects needed based on the calculations were eight. Since sampling, measurement and quantification of  $\text{TxB}_2$  is a very delicate procedure two more participants were added to the study, resulting in a total of ten volunteers participating in the study.

Results are expressed as percentage change from the reference (0 minutes) sample. Results are expressed as mean  $\pm$ SEM of N were N is the number of participants. Student's *t*-test was used to analyze the difference in relative concentration of  $\text{TxB}_2$  between groups and  $P < 0.05$  was considered as significant.

## Results

Total number of participants were ten, six females and four males. Table 1 summarizes the main characteristics of the

**Table 1** Characteristics of participants in the study

Characteristics	Total	Male	Female
Total number of participants, % (N)	100 (10)	40 (4)	60 (6)
Age (years), mean $\pm$ SD	27.0 $\pm$ 5.6	24.8 $\pm$ 4.2	28.5 $\pm$ 6.3
BMI, mean $\pm$ SD	23.2 $\pm$ 3.3	24.2 $\pm$ 3.1	22.6 $\pm$ 3.5
Platelets (count) [ $10^9/\text{L}$ ], mean $\pm$ SD	231.4 $\pm$ 40.8	219.3 $\pm$ 35.1	239.5 $\pm$ 45.4

**Abbreviation:** BMI, body mass index.

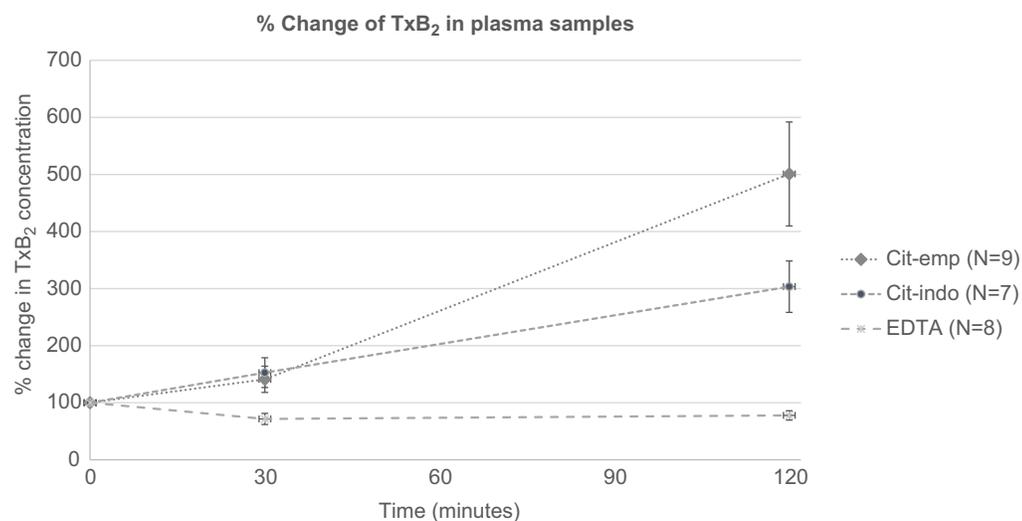
participants in the study. Blood status values of all participants were within normal range. No correlation was found between platelet count and the  $\text{TxB}_2$  levels.

Figure 1 shows that both the type of blood vials (citrate or EDTA) and the storage conditions (with or without indomethacin) of the plasma samples are important for  $\text{TxB}_2$  measurements. The individual variation was found to be high, especially for samples collected in citrate tubes and stored in the empty polyethylene tubes.

Citrate plasma samples stored in the empty polyethylene tubes (cit-emp) showed 40% increase in  $\text{TxB}_2$  levels in samples processed after 30 minutes compared to the reference sample at time zero. In the 120-minute samples, however, the increase reached an average of 400% compared to the time zero sample.

Citrate plasma samples stored in indomethacin tubes (cit-into) were more stable compared to the cit-emp samples. The increase was found to be similar (Figure 1) in the 30-minute cit-into and cit-emp samples. However, in the 120-minute samples the increase in the cit-into was about 200% compared to the reference sample. There was a statistical difference between the cit-emp and cit-into storage tubes in the 120-minute samples ( $P = 0.038$ ).

EDTA plasma samples had the least variation, where there was on average no increase in the  $\text{TxB}_2$  concentration over the studied range. On the contrary, there was about 10% decrease in the concentration detected at 30 minutes and 120 minutes compared to the zero sample. The difference at 120 minutes was statistically significant



**Figure 1** Relative change in thromboxane  $\text{B}_2$  concentration.

**Notes:** EDTA plasma samples stored in empty polypropylene tubes. Samples were processed at 0, 30, and 120 minutes after blood collection. Data are reported as mean  $\pm$  SEM.

**Abbreviations:** Cit-emp, citrate plasma samples stored in empty polypropylene tubes; Cit-into, citrate plasma samples stored in indomethacin tubes.

when compared to cit-emp ( $P<0.001$ ) and compared to cit-indo ( $P=0.001$ ) samples.

## Discussion and conclusion

The results indicate that the time from sampling to storage is an important factor for thromboxane B<sub>2</sub> stability. The type of sampling tubes as well as the storage conditions were also found to have a great impact on the true TxB<sub>2</sub> in the blood samples.

The cit-emp tubes showed up to 400% increase in TxB<sub>2</sub> concentration, indicating that there is a significant ex-novo formation of TxB<sub>2</sub> after the samples have been collected and frozen. The cit-indo tubes also showed a high increase in the TxB<sub>2</sub> formation post sampling, although the indomethacin tubes showed less TxB<sub>2</sub> formation compared to the cit-emp tubes.

Interestingly, there was no ex-novo TxB<sub>2</sub> formation in the EDTA plasma samples even though these samples were stored in polypropylene tubes containing no indomethacin. Citrate and EDTA are both anticoagulants used in vitro to prevent clot formation in blood samples. Calcium is essential for normal platelet adherence and for a wide range of enzyme reactions of the coagulation cascade. Removing it prevents blood clotting within the blood collection tube.<sup>11</sup> EDTA is a polyprotic acid containing four carboxylic acid groups and two amine groups with lone-pair electrons that chelate calcium and several other metal ions in 1:1 metal-EDTA complexes.<sup>12</sup> Sodium citrate is the sodium salt of citric acid with alkalinizing activity. Citrate also acts by removing calcium from the blood sample, but unlike EDTA, it is reversible. Residual calcium ions are not expected to be present in samples in the EDTA tube since the excess amount of EDTA irreversibly forms 1:1 complex with calcium ions in the sample. When collecting blood samples in sodium citrate tubes the ratio between the blood sample (thus calcium) and sodium citrate in the tube is important. If the ratio is shifted residual calcium ions are expected to be present. This possible imbalance was minimized by filling the tube with enough whole blood, and by trying to have the amount as equal as possible between each tube.

Previous study suggested that EDTA is capable of penetrating biological membranes such as plasma membranes to chelate intracellular calcium to prevent platelet activation.<sup>13</sup> However further studies have shown that in vitro activation does occur in EDTA-anticoagulated blood.<sup>8</sup> Thus, the extent of this factor on the overall results of this particular study is not clear.

In conclusion, these results are interesting since both citrate and EDTA are able to bind calcium ions in the blood. The different mechanism behind the calcium removal in the blood sample might play a crucial role in the stability of TxB<sub>2</sub> in the samples. It has also been shown that acidity has an effect on the coagulation, where lowering the pH reduces coagulation.<sup>14</sup> The acidity of the plasma might also be of importance. These results are important for future analysis and work with TxB<sub>2</sub>.

## Acknowledgments

This study was supported by a grant from the Icelandic research fund (Rannis). Cayman Chemicals kindly provided equipment for this study. The authors also thank Landspítali University Hospital in Iceland for the facilities used.

## Disclosure

The authors report no conflicts of interest in this work.

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