

THROMBIN GENERATION IN DIFFERENT COMMERCIAL SODIUM CITRATE BLOOD TUBES

STVARANJE TROMBINA U RAZLIČITIM KOMERCIJALNIM EPRUVETAMA ZA KRV SA NATRIJUM CITRATOM

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Summary

Background: This study aimed to verify whether blood drawn into six different commercial coagulation tubes generated comparable results of thrombin generation.

Methods: Blood was sequentially collected from 20 healthy subjects into different brand and draw volume 3.2% sodium citrate tubes (4.3 mL Sarstedt, 3.0 mL Greiner, 2.7 mL Becton Dickinson, 2.0 mL Kima, 1.8 mL Sarstedt and 1.0 mL Greiner). Thrombin generation was measured in plasma with the fully-automated ST Genesis analyzer using the weakest trigger (STG-BleedScreen).

Results: Different values of lag time (LT), time to reach thrombin peak (TP), thrombin peak height (PH) and endogenous thrombin potential (ETP) were commonly found in different tubes. Thrombin generation was the lowest in 4.3 mL Sarstedt tubes and the highest in 1.0 mL Greiner tubes. Other tubes displayed intermediate values. In multiple comparisons, LT was significantly different in 6/15 cases (40%), whilst PH, TP and ETP were significantly different in 14/15 (93%), 13/15 (87%) and 13/15 (87%) cases. The mean percent bias of LT, PH, TP and ETP ranged between -6% and +1%, -27% and +116%, -22% and +8%, and between -18% and +65%. The intra-assay imprecision of LT, PH, TP and ETP was exceeded in 0/15 (0%), 13/15 (87%), 6/15 (40%) and 13/15 (87%) comparisons. The correlation of LT, PH, TP and ETP values in different tubes ranged between 0.718–0.971, 0.570–0.966, 0.725–0.977 and 0.101–0.904.

Kratak sadržaj

Uvod: Ovo izučavanje je imalo za cilj da potvrdi da li krv uzeta u šest različitih komercijalnih epruveta za koagulaciju proizvodi uporedive rezultate stvaranja trombina.

Metode: Krv je uzastopno uizimana od 20 zdravih osoba u različite zapremine epruveta sa 3,2% natrijum citrata (4,3 mL Sarstedt, 3,0 mL Greiner, 2,7 mL Becton Dickinson, 2,0 mL Kima, 1,8 mL Sarstedt i 1,0 mL Greiner). Stvaranje trombina mereno je u plazmi na potpuno automatizovanom ST Genesis analizatoru primenom slabog okidača (STG-BleedScreen).

Rezultati: Različite vrednosti lag vremena (LT), vremena dostizanja trombinskog pika (TP), visine trombinskog pika (ETP) su nađeni u različitim epruvetama. Stvaranja trombina bilo je najniže u 4,3 mL Sarstedt epruvetama a najviše u 1,0 mL Greiner epruvetama. U drugim epruvetama nađene su srednje vrednosti. U multiplom poređenju, LT se značajno razlikovao u 6/15 slučajeva (40%), dok su se PH, TP i ETP značajno razlikovali u 15/15 (93%), 13/15 (87%) i 13/15 (87%) slučajeva. Srednji procenat odstupanja LT, PH, TP i ETP kretao se između -6% i +1%, -27%, i +116%, -22% i +8% i između -18%, i +65%. Unutrašnja nepreciznost za LT, PH, TP i ETP bila je povećana u 0/15 (0%), 13/15 (87%), 6/15 (40%) i 13/15 (87%) slučajeva. Korelacija LT, PH, TP i ETP vrednosti u različitim epruvetama kretala se između 0,718–0,971, 0,570–0,966, 0,725–0,977 i 0,101–0,904.

Zaključak: Sakupljanje krvi zahteva lokalnu standardizaciju primenom identičnih epruveta za vrstu i zapreminu i

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Conclusions: Blood collection for thrombin generation assays requires local standardization using identical tubes for brand and draw volume, and reference ranges calculated according to type of tubes.

Keywords: preanalytical variability; blood tubes; blood collection; thrombin generation

Introduction

Laboratory hemostasis represents an essential part of diagnostic reasoning and clinical decision making in patients with hemostasis disorders, either hemorrhagic or thrombotic (1). Among the various hemostasis tests, thrombin generation is now emerging as a valuable investigative tool, since it helps in obtaining essential clinical information for monitoring the global hemostasis potential of patients with inherited (i.e., hemophiliacs) or acquired (i.e., severely injured or heavily traumatized) bleeding disorders, for assessing the risk of development or recurrence of venous thromboembolism, as well as for monitoring both anti-hemorrhagic and anti-thrombotic treatments (2, 3). Although thrombin generation assays have been empirically used since early 1950s, it was only recently that a standardized calibrated automated thrombin generation (CAT) assay has been developed by Hemker et al. (4). This method allows assessing thrombin generation in both platelet-poor and platelet-rich plasma, whereby the latter sample matrix would provide results also dependent on platelet function (4). The main aspects evaluated with the standardized CAT assay include the lag time (LT; reflecting the time necessary for initial thrombin generation after adding the trigger), the time to reach the thrombin peak (TP; mirroring the speed of thrombin generation), the thrombin peak height (PH; reflecting the highest value of thrombin generated) and the endogenous thrombin potential (ETP; underscoring the total amount of thrombin generated). The combination of these different parameters contributes to accurately define the hemostatic potential in the test plasma, as reflected by the speed and amount of thrombin that can be actually generated (5).

As with any other laboratory and hemostasis tests (6–8), thrombin generation assays are highly vulnerable to the impact of many preanalytical variables, which may ultimately influence the final quality of test results. The Scientific and Standardization Committee (SSC) of the International Society of Thrombosis and Hemostasis (ISTH) has recently published an official document aimed at highlighting the many preanalytical variables directly impacting the reliability of this test (9), which encompass the procedures used for drawing blood, for transportation of samples and for their preparation and storage before testing. Notably, the SSC of the

referentne vrednosti izračunate prema tipu epruveta koje se koriste za uzimanje krvi.

Ključne reči: preanalitička varijabilnost, epruvete za krv, sakupljanje krvi, stvaranje trombina

ISTH has also highlighted that some important differences may be noted using different brands of blood tubes, thus hampering within- and between-laboratory standardization of this important measurement. Therefore, this study was essentially aimed at verifying whether or not blood drawn into different commercial coagulation blood tubes would generate comparable results of thrombin generation on a newly commercialized fully automated thrombin generation system, ST Genesis, using the weakest trigger available with it, as most differences are reported at low trigger activity (9, 11).

Materials and Methods

The study population consisted on 20 ostensibly healthy subjects recruited among the laboratory staff (mean age: 40 ± 10 years; 10 women and 10 men). Blood was sequentially collected by the same expert phlebotomist, early in the morning, by straight needle venipuncture into different brand and draw volume 3.2% sodium citrate blood tubes, as follows: 13×75 mm, 4.3 mL non-evacuated S-Monovette (Reference number: 04.1922.001; Lot number: 8034811; Sarstedt, Nümbrecht, Germany); 13×75 mm, 3.0 mL evacuated Vacuette (Reference number: 454334; Lot number: A180848F; Greiner Bio-One, Kremsmünster, Austria); 13×75 mm, 2.7 mL Vacutainer (Reference number: 363048; Lot number: 8242840; Becton Dickinson [BD], Plymouth, United Kingdom); 13×75 mm, 2.0 mL evacuated Vacutest (Reference number: 14074; Lot number: A2908; Kima, Padova, Italy); 1.8 mL non-evacuated S-Monovette (Reference number: 04.1955.001; Lot number: 9030111; Sarstedt, Nümbrecht, Germany); 13×75 mm, 1.0 mL evacuated Vacuette (Reference number: 454320; Lot number: A181039T; Greiner Bio-One, Kremsmünster, Austria). The sequence of the tubes was varied among subjects, by escalating one tube for each next subject in the sequence. Blood was drawn in both Sarstedt tubes, by manual rather than by vacuum aspiration (i.e., blood was aspirated by slowly withdrawing the plunger until complete filling). Plasma was immediately separated after blood collection (i.e., within 15 min), by centrifugation at $1500 \times g$, for 15 min, at room temperature.

Thrombin generation parameters (i.e., LT, TP, PH and ETP) were measured, immediately after plasma separation, by using the novel fully-automated

analyzer ST Genesis with STG-BleedScreen (Diagnostica Stago, Asnières sur Seine, France), which is essentially based on the reference CAT assay developed by Hemker et al. (4). This method encompasses the assessment of thrombin generation based on measurement of fluorophore amino-methylcoumarin (AMC) generation, after adding a standard amount of human recombinant tissue factor and synthetic phospholipids to the test plasma. AMC generation is monitored every 15–20 seconds at 450 nm, and mirrors the quantity of thrombin generated throughout the measuring range. All measurements were carried out in duplicate, during a single analytical session, and final results were reported as mean of the two duplicate measures. According to manufacturer's specifications, the intra-assay imprecision of thrombin generation with ST Genesis and STG-BleedScreen is 7.0% for both LT and TP, 7.5% for PH and 6.2% for ETP.

Normality of value distributions were verified with Shapiro-Wilk Test. The statistical analysis was hence performed using parametric tests and results were finally reported as mean and 95% confidence interval (95% CI). More specifically, the difference and correlation of values among all tubes were evaluated with Student's T and Pearson's tests, respectively. Due to the lack of biological variation studies for thrombin generation, results obtained using different tubes were considered significant when the percent bias of values between two tubes was larger than the intra-assay imprecision of each thrombin generation parameter. The statistical significance was set at $p < 0.05$. The statistical analysis was performed with Analyse-it (Analyse-it Software Ltd, Leeds, UK). All subjects provided a written informed consent for participating to this study, which was cleared by the local Ethics Committee (970CESC; July 20, 2016).

Results

The results of this study are shown in *Tables I and II*. The lowest thrombin generation in plasma was observed in 4.3 mL Sarstedt blood tubes, as mirrored by the longest values of LT and TP as well as by the lowest values of PH and ETP. The highest thrombin generation in plasma was instead found in 1.0 mL Greiner blood tubes, as reflected by the shortest values of LT and TP as well as by the highest values of PH and ETP. All other blood tubes exhibited intermediate values of all thrombin generation parameters. Notably, in multiple comparisons among the different blood tubes (*Table II*), LT was found to be significantly different in 6/15 cases (40%), whilst PH, TP and ETP were found to be significantly different in 14/15 (93%), 13/15 (87%) and 13/15 (87%) cases, respectively. The mean percentage bias of LT, PH, TP and ETP among the different blood tubes was comprised between -6% and +1%, -27% and +116%, -22% and +8%, and between -18% and +65%, respectively (*Table II*). Notably, although in no case did the bias among the different blood tubes exceed the intra-assay imprecision of LT (i.e., ± 7.0), the intra-assay imprecision of PH ($\pm 7.5\%$), TP ($\pm 7.0\%$) and ETP ($\pm 6.2\%$) was exceeded in 13/15 (87%), 6/15 (40%) and 13/15 (87%) comparisons, respectively. The correlation (r) of LT, PH, TP and ETP values among the different sodium citrate blood tubes was comprised between 0.718–0.971, 0.570–0.966, 0.725–0.977 and between 0.101–0.904, respectively. The multiple correlations among blood tubes were all good or acceptable and statistically significant (i.e., $p < 0.05$), except for ETP measured in 1.0 mL Greiner blood tubes compared to any other blood tube (i.e., p values comprised between 0.074 and 0.672).

In multiple linear regression analysis, where brand and draw volume of blood tubes were entered as dependent variables and the different thrombin

Table I Mean and 95% confidence interval of thrombin generation parameters in plasma collected with different blood tubes.

Parameters	Sarstedt 4.3 mL	Greiner 3.0 mL	BD 2.7 mL	Kima 2.0 mL	Sarstedt 1.8 mL	Greiner 1.0 mL
Tube name	S-Monovette	Vacurette	Vacutainer	Vacutest	S-Monovette	Vacurette
Type of tube	Non-evacuated	Evacuated	Evacuated	Evacuated	Non-evacuated	Evacuated
Lag time (min)	3.06 (2.79–3.33)	3.11 (2.86–3.35)	3.09 (2.81–3.36)	3.05 (2.80–3.29)	2.96 (2.71–3.20)	2.93 (2.73–3.12)
Peak Height (nM)	137 (111–162)	194 (170–217)	142 (118–167)	167 (144–190)	157 (130–184)	295 (274–316)
Time to peak (min)	6.68 (6.18–7.18)	6.29 (5.88–6.70)	6.82 (6.32–7.31)	6.41 (6.01–6.80)	6.55 (6.04–7.05)	5.35 (5.00–5.69)
ETP (nM*min)	994 (871–1117)	1251 (1144–1358)	1029 (910–1148)	1145 (1043–1247)	1147 (1026–1269)	1644 (1531–1757)

Table II Mean percent variation, statistical significance and correlation of thrombin generation parameters in plasma collected with different blood tubes.

Blood tubes	Greiner 3.0 mL	BD 2.7 mL	Kima 2.0 mL	Sarstedt 1.8 mL	Greiner 1.0 mL
Sarstedt 4.3 mL	LT: +1%; p=0.152; r=0.959 [§] PH: +42%; p<0.001; r=0.902 [§] TP: -6%; p=0.001; r=0.918 [§] ETP: +26%; p<0.001; r=0.852 [§]	LT: +1%; p=0.343; r=0.906 [§] PH: +4%; p=0.143; r=0.922 [§] TP: +2%; p=0.017; r=0.972 [§] ETP: +4%; p=0.122; r=0.891 [§]	LT: +1%; p=0.425; r=0.790 [§] PH: +22%; p<0.001; r=0.957 [§] TP: +4%; p=0.010; r=0.914 [§] ETP: +15%; p<0.001; r=0.898 [§]	LT: -3%; p=0.003; r=0.971 [§] PH: +15%; p<0.001; r=0.966 [§] TP: -2%; p=0.012; r=0.977 [§] ETP: +15%; p<0.001; r=0.880 [§]	LT: +4%; p=0.033; r=0.876 [§] PH: +116%; p<0.001; r=0.595 [§] TP: -20%; p<0.001; r=0.725 [§] ETP: +65%; p<0.001; r=0.185*
Greiner 3.0 mL	-	LT: -1%; p=0.377; r=0.922 [§] PH: -27%; p<0.001; r=0.894 [§] TP: +8%; p<0.001; r=0.930 [§] ETP: -18%; p<0.001; r=0.832 [§]	LT: -2%; p=0.211; r=0.834 [§] PH: -14%; p<0.001; r=0.925 [§] TP: +2%; p=0.113; r=0.906 [§] ETP: -8%; p<0.001; r=0.904 [§]	LT: -5%; p=0.001; r=0.949 [§] PH: -19%; p<0.001; r=0.901 [§] TP: +4%; p=0.014; r=0.918 [§] ETP: -8%; p=0.003; r=0.842	LT: -6%; p=0.001; r=0.914 [§] PH: +52%; p<0.001; r=0.771 [§] TP: -15%; p<0.001; r=0.924 [§] ETP: +31%; p<0.001; r=0.316*
BD 2.7 mL	-	-	LT: -1%; p=0.227; r=0.924 [§] PH: +17%; p<0.001; r=0.875 [§] TP: +6%; p=0.001; r=0.913 [§] ETP: +11%; p=0.003; r=0.793 [§]	LT: -4%; p=0.045; r=0.860 [§] PH: +10%; p=0.004; r=0.930 [§] TP: -4%; p<0.001; r=0.971 [§] ETP: +12%; p<0.001; r=0.856 [§]	LT: +5%; p=0.032; r=0.824 [§] PH: +107%; p<0.001; r=0.570 [§] TP: -22%; p<0.001; r=0.763 [§] ETP: +60%; p<0.001; r=0.101*
Kima 2.0 mL	-	-	-	LT: -3%; p=0.173; r=0.739 [§] PH: -6%; p=0.029; r=0.935 [§] TP: +2%; p=0.139; r=0.878 [§] ETP: 0%; p=0.472; r=0.837 [§]	LT: -4%; p=0.095; r=0.718 [§] PH: +77%; p<0.001; r=0.670 [§] TP: -17%; p<0.001; r=0.745 [§] ETP: +44%; p<0.001; r=0.409*
Sarstedt 1.8 mL	-	-	-	-	LT: -1%; p=0.309; r=0.860 [§] PH: +88%; p<0.001; r=0.648 [§] TP: -18%; p<0.001; r=0.734 [§] ETP: +43%; p<0.001; r=0.368*

§, p value of correlation <0.05; *, p value of correlation >0.05

LT, lag time; PH, thrombin peak height; TP, time to reach the thrombin peak; ETP; endogenous thrombin potential.

generation parameters were entered as independent variables, ETP was found to be significantly associated with both brand (p=0.042) and draw volume (p<0.001), PH with draw volume (p<0.001) but not with brand (p=0.087), TP with draw volume (p=0.001) but not with brand (p=0.296), whilst LT was not associated with either draw volume (p=0.267) or brand (p=0.493).

Discussion

Although the SSC of the ISTH has recently stated that blood drawn into different brands of coagulation tubes may be a source of bias in thrombin generation studies, limited evidence for this presumption has been published in the scientific literature to the best of our knowledge. Earlier information was published by Dargaud and Negrier

(10), who showed that the overall thrombin generation (i.e., ETP) measured in platelet-rich plasma was considerably higher when blood was drawn by forced aspiration into evacuated BD Vacutainer tubes than with slow manual aspiration in Sarstedt Monovette tubes. In a subsequent study, Loeffen et al. (11), assayed thrombin generation in seven different sodium citrate tubes (12), and also found that ETP was consistently lower in plasma collected into 4.3 mL Sarstedt Monovette tubes (i.e., 270 nM*min) than in standard evacuated blood tubes such as 2.7 mL BD Vacutainer (i.e., 465 nM*min) and 9.0 mL Greiner Vacuette (i.e., 490 nM*min), whilst the other parameters remained virtually similar.

According to our data, the direct comparison of thrombin generation measured in plasma collected into 1.8 mL Sarstedt tubes and 2.0 mL Kima tubes does not support the hypothesis that slow (manual)

aspiration of blood would result in lower pre-activation of blood coagulation, since all thrombin generation parameters were found to be non-statistically different between these two tubes, the mean difference was always comprised within the intra-assay imprecision, and the Spearman's correlation of the various parameters was also satisfactory (i.e., comprised between 0.739 and 0.935) (Table II). On the other hand, data obtained in plasma collected into the 4.3 mL Sarstedt tube were comparable to those obtained in the 2.7 mL BD tube, since in no case the percent variation calculated on these two tubes was larger than the intra-assay imprecision. This would lead us to conclude that both draw volume and tube composition may produce a larger impact on thrombin generation than the mode of blood aspiration (i.e., manual or forced by the vacuum).

Therefore, the first conclusion that can be made according to our data, is that the accurate assessment of thrombin generation would need standardized blood collection by always using identical tubes for brand and draw volume. This procedure seems actually unavoidable since specific tube-dependent reference ranges should be defined for all the different parameters, and is even more important for enabling a reliable longitudinal monitoring of patient's data. Irrespective of the highly anomalous values obtained using plasma collected into 1.0 mL Greiner tubes, the difference observed comparing the values of the other five tubes were also generally noteworthy, since the intra-assay imprecision was exceeded in 8/10 (80%) of cases for both PH and ETP (Table II). Another important aspect that emerged from this investigation is that the use of very low-draw citrate collection tubes (i.e., 1.0 mL) may be unsuitable for measuring thrombin generation, since their use may substantially increase the speed and total amount of thrombin generation, so producing values that are not at all comparable with those of other tubes, as clearly shown by the correlation coefficients reported in Table II. It is indeed conceivable that very low volume blood draw was the main source

of this variation, since the comparison of the two Greiner blood tubes (which share the same composition and additive) yielded mean differences of PH, TP and ETP as high as 116%, -20% and +65%, in all circumstances largely exceeding the intra-assay variability of the method. This also means that any studies assessing thrombin generation which may potentially use low volume versus standard volume tubes (e.g., in neonates and adults, respectively) would lead to substantial bias in the results of such study.

Conclusions

In conclusion, the results of our study further emphasize the importance of standardizing the preanalytical phase for obtaining reliable and consistent results of thrombin generation to be used in clinical practice. In particular, we suggest that blood collection needs to be locally standardized by always using identical tubes for brand and draw volume, and that any reference ranges for the different thrombin generation parameters must be locally calculated according to the type of tubes used for blood collection.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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