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# Comparison of Commercially Available Blood Collection Tubes Containing Sodium Citrate and Hirudin in Platelet Aggregation Testing

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Study Design A  
Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
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**Background:** Platelet reactivity assessment is an important tool in both the causal determination of bleeding diathesis as well as in the evaluation of the efficacy of anti-platelet therapy in patients at risk of thrombosis. Sodium citrate is the most widely used anticoagulant for hemostasis investigations. However, some doubt exists over the suitability of sodium citrate in platelet function testing. Hirudin has been suggested as a superior replacement. Nevertheless, only 1 study compared citrated and hirudin treated samples with light transmission aggregometry. Therefore, limited evidence exists to conclusively prove the supremacy of hirudin over sodium citrate in light transmission aggregometry. The aim of our study was to compare citrated and hirudin treated samples, collected in commercially available blood collection tubes, using the 5 most common agonists, with light transmission aggregometry.

**Material/Methods:** Blood was obtained from 20 healthy volunteers. Platelet counts were performed on platelet-rich plasma. Light transmission aggregometry was performed within 4 h of sample collection using ADP, collagen, arachidonic acid, epinephrine, and ristocetin as agonists.

**Results:** Platelet counts for the respective anticoagulants did not differ significantly. ADP-induced aggregation was comparable between the samples. However, among all the agonists, hirudin-treated platelets had significantly weaker aggregatory responses.

**Conclusions:** Commercially available sodium citrate should remain the anticoagulant of choice for routine platelet function testing in our setting. However, the time limitation associated with the use of sodium citrate in platelet function testing remains a concern. Thus, alternative anticoagulants should still be explored.

**MeSH Keywords:** **Anticoagulants • Hirudins • Platelet Function Tests**

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## Background

Platelets are small megakaryocyte fragments that play a vital role in hemostasis. Functional and/or quantitative platelet defects may cause excessive bleeding, which in some cases may be life-threatening. However, excessive platelet reactivity may conversely lead to potentially disabling or fatal thrombosis, dictating the use of anti-platelet agents [1]. Functional platelet defects can occur as a result of defective platelet adhesion, granule secretion, or aggregation. Light transmission aggregometry (LTA) is the most commonly used method to evaluate platelet aggregation in response to the addition of different agonists to platelet-rich plasma (PRP). Light absorbance of the PRP decreases as platelets aggregate, with the rate of the reduction dependent on platelet reactivity to the agonist. Variables such as temperature, platelet count, and mixing speed are controlled to limit interference with test results [2]. Sodium citrate is the most universally used anticoagulant for platelet function tests. It acts by chelating extracellular calcium ( $\text{Ca}^{2+}$ ). However,  $\text{Ca}^{2+}$  plays an essential role in platelet function regulation. Cytosolic  $\text{Ca}^{2+}$  is involved in the remodelling of the platelet, secretion, and aggregation [3]. Cytosolic  $\text{Ca}^{2+}$  concentration elevation is essential for platelet activation. The agonists all lead to an increase in the intracellular  $\text{Ca}^{2+}$  concentrations. This increase in  $\text{Ca}^{2+}$  concentration contributes to platelet activation, such as remodelling of the actin cytoskeleton needed for platelet shape change, and externalization of the platelet receptor integrin  $\alpha\text{IIb}\beta_3$ , which is vital for platelet aggregation [4]. Therefore, if  $\text{Ca}^{2+}$  is chelated by sodium citrate, the several roles that it plays in platelet activation may be inhibited, resulting in spuriously decreased platelet aggregation due to the artificial modification of platelet response created by the anticoagulant. However, hirudin, an anticoagulant first isolated from leeches, directly inhibits thrombin and does not affect the levels of  $\text{Ca}^{2+}$  in blood samples. Hirudin irreversibly binds to the fibrinogen recognition site of thrombin. This effectively blocks the transformation of fibrinogen to fibrin, preventing coagulation. Hirudin has been suggested as an ideal anticoagulant for diagnostic blood tests due to its highly specific interaction with a single factor of the clotting cascade [5]. However, thrombin, similarly to  $\text{Ca}^{2+}$ , has a vital role in platelet activation [6]. Thus, inhibition of thrombin by hirudin may potentially also negatively affect platelet activation and aggregation. One major limitation to platelet aggregations with citrated samples is that these samples have to be analyzed within 4 h after collection, before significant decreases in aggregation is observed. It has been detected that hirudin-treated samples had enhancement of stability of platelet function measurements compared to citrated samples when evaluated with ADP and arachidonic acid in stored samples. Thus, hirudin-treated samples do not have to be analyzed within 4 h, as is currently recommended for citrated samples. Consequently, hirudin has been recommended as

the anticoagulant of choice for platelet function testing [7]. It was also reported that both ADP and collagen caused weaker platelet impedance responses in citrated whole blood samples compared to hirudinized samples [8]. The inferiority of sodium citrate as an anticoagulant in impedance whole-blood aggregometry has thus been well documented [7–10]. Nevertheless, conclusive evidence with an extensive range of the Clinical and Laboratory Standards Institute (CLSI) Platelet Function Testing by Aggregometry Approved Guideline recommended agonists [11], using both sodium citrate and hirudin as anticoagulants in LTA, is still lacking. The CLSI guidelines also only refer to multiple electrode impedance aggregometry for hirudinized samples. The use of non-commercial blood collection tubes may pose some inhibitory challenges for diagnostic laboratories in developing countries, such as South Africa, mainly due to limited expertise, as well as a lack of personnel and resources. Thus, the use of standardized commercially available blood collection tubes can assist in overcoming some of these challenges. Hence, the aim of our study was to compare platelet aggregation results from blood collected in commercially available citrated and hirudinized tubes using ADP, collagen, arachidonic acid, epinephrine, and ristocetin as agonists. It must be emphasized that our objective was to evaluate platelet aggregation testing results done on blood collected in commercially available anticoagulant tubes according to our diagnostic laboratory's standard operating procedure (SOP), with the anticoagulant tubes as the only variable. Thus, we did not perform multiple experiments at different time points, temperatures, or agonist concentrations. For the same reason, we also only evaluated normal volunteers, as we wanted to evaluate the anticoagulants under normal conditions using our current SOP, not affected by any underlying pathological condition.

## Material and Methods

### Subjects

This study was approved by the Health Sciences Research Ethics Committee of the University of the Free State (HSREC 85/2016) in accordance with the Declaration of Helsinki (2003). We recruited 20 (minimum number recommended by the CLSI guidelines) healthy volunteers (no history of easy bruising and/or excessive bleeding) between the ages of 19 and 58 from among our friends, family, and colleagues. Informed consent was given prior to participation. Volunteers had not taken any aspirin-containing medication for 14 days prior to blood collection.

### Sample collection and preparation

Venous blood was collected by venipuncture from an antecubital vein using a 21-gauge needle. We collected 13.5 mL

of blood in 3 tubes containing 0.5 mL 3.2% trisodium citrate (BD Vacutainer®, Ref: 367714, BD South Africa, Woodmead, South Africa). Subsequently, 9 mL of blood was collected in 3 tubes containing >15 µg/mL recombinant hirudin (Hirudin Blood Tube for Multiplate analysis, Ref: 6675751001, Roche Products, Randburg, South Africa). Samples were kept at room temperature, and analyzed within 4 h as stipulated in our diagnostic laboratory SOP. Platelet-rich plasma (PRP) was obtained through centrifugation in a PDQ Platelet Function Centrifuge (Ref: 106842, Bio/Data Corporation, Horsham, USA) on the manufacturer's recommended PRP setting (2220×g for 30 s). From each volunteer, a roughly 1.5-mL sample of PRP was removed from each tube with a plastic pipette without disturbing the buffy coat or red blood cells, and was pooled together in 15 mL tubes, for each anticoagulant separately, and allowed to rest for 30 min at room temperature. The tubes were returned to the centrifuge and platelet-poor plasma (PPP) was obtained by centrifuging the samples on the manufacturer's recommended PPP setting (8880×g for 180 s). The top two-thirds of the PPP was removed and pooled together in a 15-mL tube, taking care not to disturb the buffy coat or red blood cells. The PDQ Platelet Function Centrifuge is a purpose-built instrument specifically designed for platelet aggregation PRP and PPP preparation; thus, the G-force and centrifugation times specified may differ from previously published guidelines. We sent 300 µL of the PRP to our routinely used hematology laboratory for platelet count analysis.

### Light transmission aggregometry method

LTA was performed on the PEP-8E Platelet Aggregation Profiler (Ref: 106077, Bio/Data Corporation, Horsham, USA) according to the manufacturer's recommendations. Platelet counts were not adjusted for the PRP prior to analysis. Aggregation was stimulated by the addition of the agonists ADP (Ref: 5366, Helena Laboratories, Beaumont, USA), collagen (COLtest, Ref: 06675832, Roche Products, Randburg, South Africa), arachidonic acid (Ref: 5364, Helena Laboratories, Beaumont, USA), epinephrine (Ref: 5367, Helena Laboratories, Beaumont, USA), and ristocetin (Ref: 5199, Helena Laboratories, Beaumont, USA). The final agonist concentrations were based on the CSLI guidelines [11]. The final concentration for each agonist was as follows: ADP (2 µM), collagen (2 µg/mL), arachidonic acid (500 µg/mL), epinephrine (5 µM), and ristocetin (1.2 mg/mL). Light absorbance was measured for 6 min following agonist addition. We reported the maximum aggregation (MA), primary slope (PS), and area under the curve (AUC) for each sample. The CSLI guideline recommend the use of MA and slope in reporting of LTA results; however, AUC is only reserved for reporting of impedance aggregometry results [11].

### Statistical analysis

We used the *t* test to determine significance in differences between the citrated and hirudinized samples, with  $p < 0.05$  taken as a significant difference. We also determined the mean and standard deviation (SD) for each of the parameters.

## Results

### Platelet counts

The average platelet counts for the citrated and hirudinized PRP samples were  $216 \pm 98 \times 10^9/L$  and  $206 \pm 88 \times 10^9/L$ , respectively. This gave a *p*-value of 0.35, which was not a significant difference.

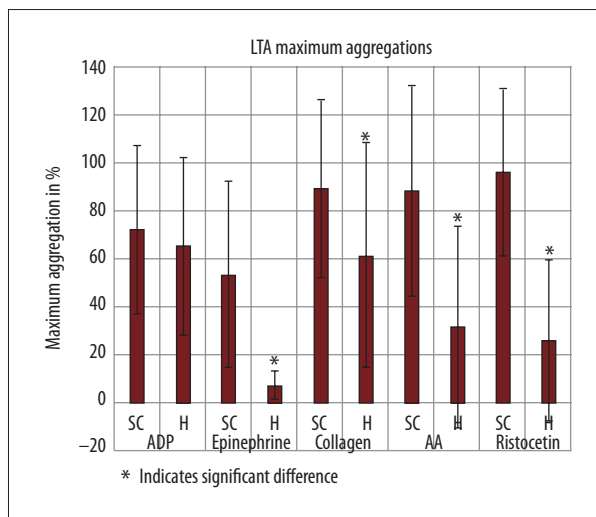
### Light transmission aggregometry results (Table 1)

Figure 1 contains the mean MA results for each of the agonists. With ADP, we found the mean MA of  $71.8 \pm 35.14$  and  $65.15 \pm 36.76$  for the citrated and hirudinized samples, respectively. The *p*-value for ADP MA was 0.58, indicating a non-significant difference. With epinephrine, we established the mean MA of  $53.15 \pm 38.92$  and  $7.2 \pm 6.3$  for the citrated and hirudinized samples, respectively. The *p*-value for epinephrine MA was  $< 0.05$ , indicating a significant difference. With collagen, we determined the mean MA at  $89.0 \pm 37.33$  and  $61.5 \pm 46.5$  for the citrated and hirudinized samples, respectively. The *p*-value for collagen MA was  $< 0.05$ , indicating a significant difference. With arachidonic acid, we found the mean MA of  $87.75 \pm 43.82$  and  $31.55 \pm 41.69$  for the citrated and hirudinized samples, respectively. The *p*-value for arachidonic acid MA was  $< 0.05$ , indicating a significant difference. The ristocetin-induced mean maximum platelet agglutination was  $96.05 \pm 34.74$  and  $25.9 \pm 33.67$  for the citrated and hirudinized samples, respectively. The *p*-value for ristocetin was  $< 0.05$ , indicating a significant difference.

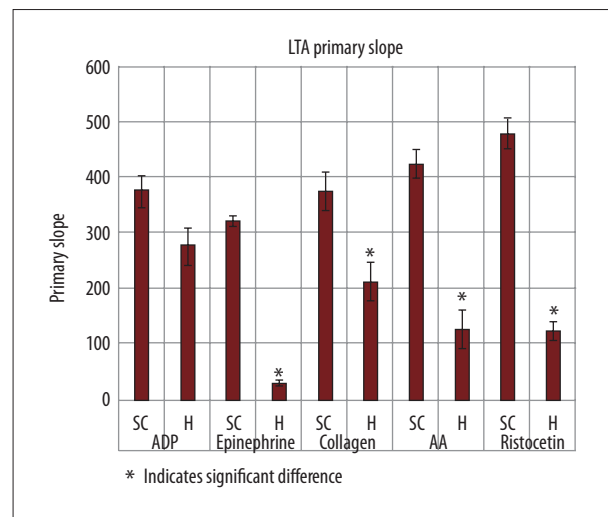
Figure 2 contains the mean PS results for each of the agonists. PS is an indication of the initial rate of aggregation [11]. With ADP, we found the mean PS of  $57.5 \pm 28.05$  and  $51.7 \pm 34.76$  for the citrated and hirudinized samples, respectively. The *p*-value for ADP PS was 0.58, indicating a non-significant difference. With epinephrine, we established the mean PS of  $18 \pm 10.52$  and  $3.15 \pm 5.18$  for the citrated and hirudinized samples, respectively. The *p*-value for epinephrine PS was  $< 0.05$ , indicating a significant difference. With collagen, we determined the mean PS at  $60.7 \pm 34.98$  and  $32.65 \pm 34.01$  for the citrated and hirudinized samples, respectively. The *p*-value for collagen PS was  $< 0.05$ , indicating a significant difference. With arachidonic acid, we found the mean PS of  $51.75 \pm 25.25$  and  $19.95 \pm 34.91$  for the citrated and hirudinized samples, respectively. The *p*-value for arachidonic acid PS was  $< 0.05$ , indicating

**Table 1.** Averages, margin of errors, and upper and lower limits of a 95% confidence interval, for each anticoagulant and agonist, for maximum aggregation, area under the curve, and primary slope, respectively.

	ADP		Epinephrine		Collagen		AA		Ristocetin	
	SC	H	SC	H	SC	H	SC	H	SC	H
<b>Maximum aggregation</b>										
Average	71.80	65.15	53.15	7.20	89.00	61.50	87.75	31.55	96.05	25.90
Margin of error (95%)	15.40	16.11	17.06	2.76	16.36	20.38	19.20	18.27	15.22	14.76
Upper limit	87.20	81.26	70.21	9.96	105.36	81.88	106.95	49.82	111.27	40.66
Lower limit	56.40	49.04	36.09	4.44	72.64	41.12	68.55	13.28	80.83	11.14
<b>Area under the curve</b>										
Average	374.55	274.6	322.25	31.1	375.75	214	424.35	126.85	480.4	123.3
Margin of error (95%)	85.77	86.88	66.55	12.73	75.84	82.47	92.10	79.80	77.64	69.70
Upper limit	460.32	361.48	388.80	43.83	451.59	296.47	516.45	206.65	558.04	193.00
Lower limit	288.78	187.72	255.70	18.37	299.91	131.53	332.25	47.05	402.76	53.60
<b>Primary slope</b>										
Average	57.5	51.7	18	3.15	60.7	32.65	51.75	19.95	60.75	13.15
Margin of error (95%)	12.29	15.23	4.61	2.27	15.33	14.90	11.07	15.30	12.25	7.86
Upper limit	69.79	66.93	22.61	5.42	76.03	47.55	62.82	35.25	73.00	21.01
Lower limit	45.21	36.47	13.39	0.88	45.37	17.75	40.68	4.65	48.50	5.29



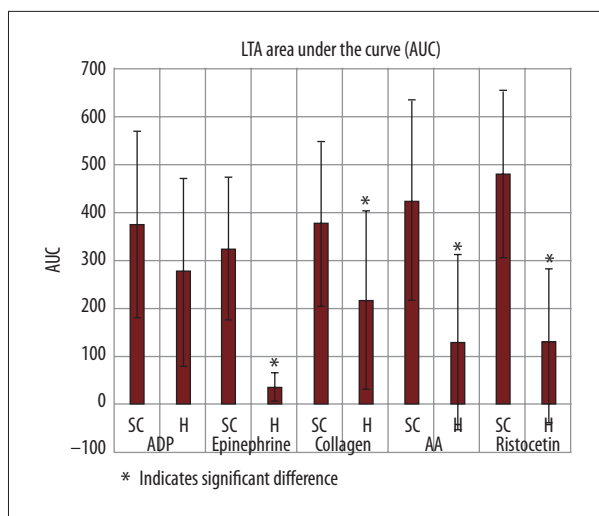
**Figure 1.** Comparison of citrated (SC) and hirudinized (H) samples relating to the maximum aggregating effects of ADP, epinephrine, collagen, arachidonic acid, and ristocetin. A significant difference is indicated with an asterisk (\*).



**Figure 2.** Comparison of citrated (SC) and hirudinized (H) samples relating to the primary slope of ADP, epinephrine, collagen, arachidonic acid, and ristocetin. A significant difference is indicated with an asterisk (\*).

a significant difference. The ristocetin-induced mean PS was  $60.75 \pm 27.95$  and  $13.15 \pm 17.94$  for the citrated and hirudinized samples, respectively. The p-value for ristocetin PS was  $<0.05$ , indicating a significant difference.

Figure 3 contains the mean AUC results for each of the agonists. With ADP, we found the mean AUC of  $374.55 \pm 195.7$  and  $274.6 \pm 198.24$  for the citrated and hirudinized samples, respectively. The p-value for ADP AUC was 0.11, indicating a



**Figure 3.** Comparison of citrated (SC) and hirudinized (H) samples relating to the area under the curve of ADP, epinephrine, collagen, arachidonic acid, and ristocetin. A significant difference is indicated with an asterisk (\*).

non-significant difference. With epinephrine, we established the mean AUC of  $322.25 \pm 151.85$  and  $31.1 \pm 29.05$  for the citrated and hirudinized samples, respectively. The p-value for epinephrine AUC was  $<0.05$ , indicating a significant difference. With collagen, we determined the mean AUC at  $375.75 \pm 173.04$  and  $214 \pm 188.18$  for the citrated and hirudinized samples, respectively. The p-value for collagen AUC was  $<0.05$ , indicating a significant difference. With arachidonic acid, we found the mean AUC of  $424.35 \pm 210.14$  and  $126.85 \pm 182.08$  for the citrated and hirudinized samples, respectively. The p-value for arachidonic acid AUC was  $<0.05$ , indicating a significant difference. The ristocetin-induced mean AUC was  $480.4 \pm 177.16$  and  $123.3 \pm 159.03$  for the citrated and hirudinized samples, respectively. The p-value for ristocetin AUC was  $<0.05$ , indicating a significant difference.

## Discussion

Platelet function testing has a central role in the management of patients with suspected platelet dysfunction [1], as well as in patients who are at risk of thrombosis who receive anti-platelet therapy [12]. Platelet reactivity, as measured by platelet aggregometry, is a very useful tool in predicting patient response to anti-platelet therapy and in predicting the risk of restenosis following surgical interventions such as percutaneous coronary interventions [12,13]. The clinical applications of platelet reactivity testing has put a focus on development of novel strategies for platelet reactivity assessment [14]. But with the development of new and improved hemostatic assessment methods, the issue of the correct pre-analytical conditions, such as which anticoagulant and concentration to

use, has been receiving increased attention from researchers [7–10,15]. Thus, the aim of our study was to clarify some of the issues regarding anticoagulant use in LTA.

Because the platelet counts for the 2 different anticoagulants were statistically comparable, we can assume that any difference between the subsequent platelet aggregation tests were functional differences, and not attributable to quantitative differences. The results with all agonists, except ADP, showed that hirudinized samples have significantly weaker aggregation compared to citrated samples with all 3 parameters (MA, PS, and AUC). This was the first reported study to compare the effect of epinephrine, collagen, and ristocetin with LTA in citrated and hirudinized samples. Comparable ADP results between citrated and hirudinized samples were previously reported. However, the significant difference we found between the arachidonic acid results was in contrast to what was previously described [7]. We used the same concentration of arachidonic acid as described in the formerly reported study. However, the current tube manufacturer's stated concentration of hirudin of  $>15 \mu\text{g/mL}$  remains a concern, as a specific concentration is not indicated; therefore, we cannot confidently assert that the hirudin concentrations within the 2 studies were the same. Nonetheless, the tubes we used were the same commercial tubes (TI-tubes<sup>®</sup>) used in the other study [7], but since the handover of the product manufacturing and distribution to a different company, the stated hirudin concentration has changed from  $25 \mu\text{g/mL}$  to  $>15 \mu\text{g/mL}$ . The current study was strictly performed according to the routine diagnostic standard operating procedure in our laboratory. The fact that hirudin-treated samples showed significantly weaker aggregation than citrated samples, when evaluated under the same conditions in our laboratory, is of concern, given that the use of hirudin instead of citrate in LTA has been previously recommended [7]. A possible reason why only ADP-induced aggregations were not significantly different may be that ADP at this concentration only induces the primary wave of platelet aggregation, where calcium and thrombin has limited roles, thus limiting the effect the anticoagulant has on aggregation.

## Conclusions

Commercial hirudin tubes are not widely available, with only 1 manufacturer supplying hirudin-containing tubes in our country, at a substantially higher cost than the sodium citrate tubes. Taken together with the non-specific hirudin concentration within the tubes, as well as the significantly weaker aggregation of hirudin-treated samples when activated by some of the most common platelet activation agonists, we conclude that the commercially available hirudin tubes do not provide a superior anticoagulant effect compared to the commercially available sodium citrate tubes for LTA studies. Therefore, we



recommend that sodium citrate remains the anticoagulant of choice for LTA studies in our setting. However, the time limitation of 4 h associated with the use of sodium citrate in platelet function testing remains a concern. Thus, more extensive investigations are needed to find a more suitable anticoagulant for platelet function testing.

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## Conflict of interest

None.

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