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Original Paper

A new anticoagulant for blood sampling tubes for platelet function tests[1)]

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Abstract

Background: The difficult pre-analytical conditions of platelet function diagnostics discourage its widespread use, e.g., to detect resistance to acetylsalicylic acid (ASA) and/or clopidogrel. Platelet activation during blood drawing and sample preparation cannot be prevented by citrate anticoagulation. Here, we tested a new blood sampling tube containing the anticoagulant benzylsulfonyl-D-Argininyl-Prolyl-4-amidinobenzylamide (Thrombovette®, Probe&go, Germany).
Methods: A total of 46 emergency patients taking ASA and clopidogrel as well as 20 healthy volunteers were included in the study. Blood was drawn into Thrombovette® and citrate S-Monovette® tubes (Sarstedt AG&Co, Germany) and transported to the laboratory via the pneumatic tube system. Whole blood impedance aggregometry (model 560Ca, Chrono-Log, Havertown, PA, USA) was performed using collagen and ADP as platelet agonist (final concentrations 1 mg/L and 5 μM, respectively).
Results: Reference ranges and cut-off values for the determination of drug resistance were obtained from the healthy cohort. Positive predictive values were 94.6% and 94.1% for inhibition of aggregation by ASA, and 68.0% and 53.3% for inhibition by clopidogrel using Thrombovette® and citrate S-Monovette®, respectively. Negative predictive values were 82.8% and 75.0% for ASA resistance and 87.8% and 83.3% for clopidogrel resistance using Thrombovette® and citrate S-Monovette®, respectively.
Conclusions: Thrombovette® is a promising new system for blood sampling and could improve the validity of platelet function diagnostics.

Keywords: anticoagulant; aspirin; clopidogrel; platelets.

Introduction

The antithrombotic medications acetylsalicylic acid (ASA) and clopidogrel are indispensable components of acute coronary syndrome therapy. Even the successes of interventional vascular medicine would not be possible without the platelet inhibition resulting from combined doses of ASA and clopidogrel. ASA irreversibly blocks cyclooxygenase I in the blood platelets and thereby inhibits the synthesis of the platelet agonist thromboxane A2, while clopidogrel prevents the ADP-induced platelet activation by irreversibly blocking the ADP receptor $P2Y_{12}$.

Nonetheless, the likely reduction of platelet function fails to occur in 10%–30% of patients, a phenomenon called ASA or clopidogrel resistance [1]. Patients in whom ASA or clopidogrel resistance can be detected by means of a platelet function test show an annual cardiovascular event rate of more than 30%, while the rate for patients with normal ASA or clopidogrel effectiveness is only 13% [2]. To be able to identify these high-risk patients, we need reliable and simple test methods that meet the requirements of day-to-day clinical practice. Since blood collection and sample processing are particularly sensitive processes in regard to platelet function diagnostics, it is necessary to first adapt pre-analytical precautions to routine conditions.

The fundamental problem of pre-analytics is the activation of platelets through thrombin formation in the blood collection tube, since the citrate solution (0.109 mol/L trisodium citrate, DIN 58905-1) customarily used as an anticoagulant does not inhibit the blood clotting factors XII, XI and prekallikrein. Thrombin, however, is a strong platelet activator that can bring about pre-activation of the platelets in the blood specimen and consequently can lead to false results in the aggregation test.

The development of benzylsulfonyl-D-argininyl-prolyl-4-amidinobenzylamid, the synthetic inhibitor of blood clotting factors Xa and IIa, and its replacement of citrate as the anticoagulant in the blood collection tube solved this problem (Thrombovette®, Probe&go GmbH, Osburg). This study aims to evaluate the applicability of the new Thrombovette® for whole blood aggregometry (WBA) in comparison to the conventional anticoagulant citrate in day-to-day clinical practice.

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Materials and methods

Patients

This study was conducted in accordance with the rules and regulations of the Local Ethics Commission and the Helsinki Declaration. Participation in the study was voluntary and had no influence on the further treatment of the patients. Included were 46 patients (33 males, 13 females; ages 37–94 years, on average 70 ± 12 years of age) with stable angina pectoris (n = 12, 26.1%), unstable angina pectoris (n = 13, 28.3%), non-ST-segment elevation myocardial infarction (n = 17, 37.0%), and ST-segment elevation myocardial infarction (n = 4, 8.7%) from cardiologic emergency admissions. For comparison, we tested the blood specimens of a healthy reference group (11 males, 9 females; 26–57 years; on average 37 ± 8 years of age).

A total of 52.2% (n = 24) of patients had taken ASA alone and 47.8% (n = 22) of patients had taken ASA in combination with clopidogrel. The test persons of the reference group had taken no ASA, clopidogrel or non-steroidal antiphlogistic agents for at least 10 days prior.

Blood collection and specimen processing

We used a butterfly system (Valu-Set BD, 21 G) to collect one venous blood specimen each with a Citrate S-Monovette® [9 mL whole blood + 1 mL 3.2% (0.109 mol/L) trisodium citrate solution, Sarstedt AG & Co, Nümbrecht, Germany] and with a Thrombovette® (4.9 mL whole blood + 0.5 mL of a 0.5 mM benzylsulfonyl-D-argininyl-prolyl-4-amidinobenzylamid solution as anticoagulant). After 15 min both tubes were transported to the laboratory via pneumatic dispatch. Following an additional 30 min of incubating the tubes at room temperature we performed impedance aggregometry on both in parallel time. Storage and transport conditions as well as laboratory analytics therefore were identical for the Thrombovette®- and the Citrate S-Monovette®.

Impedance aggregometry

With impedance aggregometry or WBA the change in electrical resistance (impedance) is measured between the two parallel platinum wires of an electrode that are spaced 1 mm apart and the electrode is immersed in the diluted whole blood specimen. Aggregation of the platelets begins following the addition of a platelet agonist, whereby platelet aggregates attach themselves to the platinum wires of the electrode while the reaction mixture is being stirred. The accompanying increase of impedance correlates with the amount of accumulated platelet aggregates and thereby with the platelet function of the tested blood specimen.

Aggregometric measurements were performed with the Chrono-Log Whole Blood-Lumi-Aggregometer Model 560Ca [3, 4]. The whole blood specimen (0.5 mL) was put into the polycarbonate cuvette of the aggregometer with 0.5 mL of a physiologic sodium chloride solution pre-warmed to 37°C and with a stirring rod (1000 U/min). The electrode was then immersed in the diluted specimen. Following a 2-min incubation at 37°C aggregation was started by adding a platelet agonist. Impedance was measured after a reaction time of 6 min as ohmic resistance. The agonists used were ADP (5 μL of a millimolar ADP solution) with a final concentration of 5 μM in the reaction mixture and collagen (1 μL of a 1 mg/mL collagen solution) with a final concentration of 1 mg/L in the reaction mixture.

Table 1 Reference ranges for whole blood aggregometry (6 min impedance) with collagen and ADP for the blood collection systems Citrate S-Monovette® and Thrombovette®.

Platelet agonist	Citrate S-Monovette®, Ohm	Thrombovette®, Ohm
Collagen (1 mg/L)	9–18	14–30
ADP (5 μM)	8–14	8–20

Statistical analysis

Normally distributed random samples were analyzed as a post-hoc test with the *t*-test and the one-way factorial analysis of variance (ANOVA) as well as the Bonferroni test. Not normally distributed random samples were compared with the Mann-Whitney U-test and with the Kruskal-Wallis H-test. Categoric variables were compared with Fisher's exact test and the χ^2-test. Differences were considered statistically significant with an error probability of $p < 0.05$.

Results

Reference ranges and decision limits

To be able to compare the two blood collection systems, we determined reference ranges and decision limits for the detection of ASA and clopidogrel resistance. As reference range we chose the 5th to the 95th percentile of the normal group (Table 1). We established decision limits by subtracting double the value of the standard deviation from the respective mean value of the normal group (Table 2).

Influence of platelet count and hemoglobin (Hb)

The influence platelet count and Hb had on the WBA was each examined by means of the Thrombovette® and the Citrate S-Monovette®. Regardless of the blood collection system and the platelet agonist used, the platelet count showed a moderate correlation with the WBA result when stimulated with collagen (Citrate S-Monovette®: $r^2 = 0.49$; $p < 0.01$), Thrombovette®: $r^2 = 0.44$; $p < 0.01$) and also when stimulated with ADP (Citrate S-Monovette®: $r^2 = 0.59$; $p < 0.01$, Thrombovette®: $r^2 = 0.62$; $p < 0.01$). The Hb values correlation with the results of the WBA following stimulation with collagen was weak negative (Citrate S-Monovette®: $r^2 = -0.29$, $p = 0.05$; Thrombovette®: $r^2 = -0.30$, $p = 0.04$). In contrast,

Table 2 Decision limits of whole blood aggregometry with collagen and ADP (6 min impedance) for the existence of an ASA and clopidogrel resistance with Citrate S-Monovette® and Thrombovette®.

Issue	Citrate S-Monovette®, Ohm	Thrombovette®, Ohm
ASA resistance (1 mg/L collagen)	≥ 8	≥ 11
Clopidogrel resistance (5 μM ADP)	≥ 6	≥ 7

there was no evidence of a correlation with the platelet agonist ADP.

Comparison of Thrombovette® and Citrate S-Monovette® to prove the effectiveness of ASA and clopidogrel through WBA

Specimen collection by means of the Thrombovette® always resulted in higher impedance values with WBA than were obtained with the Citrate S-Monovette®. With collagen stimulation this difference of the WBA values was significant in the test persons as well as in patients under ASA medication (Figure 1). With ADP stimulation the difference of the WBA values was significant in the test persons and the patients not receiving clopidogrel (Figure 2).

As expected, with patients under ASA medication WBA with collagen stimulation by use of the Thrombovette® and the Citrate S-Monovette® resulted in lower impedance values than with persons not under ASA medication (Figure 1). In the same way, the use of clopidogrel led to an inhibition of platelet function, which, when stimulated with ADP, was evidenced by lowered impedance values when the Thrombovette® was used for specimen collection. In contrast, when using the Citrate S-Monovette® impedance values were only negligibly lowered (Figure 2).

When determining the sensitivity and specificity of WBA in relation to ASA and clopidogrel resistance, we also used the specimens of patients not receiving aggregation inhibiting medication and the specimens of test persons, since a resistance in WBA presents itself as a non-usage of medication. Of the 40 patients treated with ASA the WBA results showed an inhibition of platelet function in the blood specimens of 32 patients when using the Citrate S-Monovette® and in 35 patients when using the Thrombovette® (sensitivity 80.0% or 87.5%). In 24 of 26 persons without ASA medication this was recognized by means of WBA both with the Thrombovette® as well as with the Citrate S-Monovette® (specificity 92.3%). Of the 22 patients under clopidogrel medication WBA results showed adequately lowered platelet

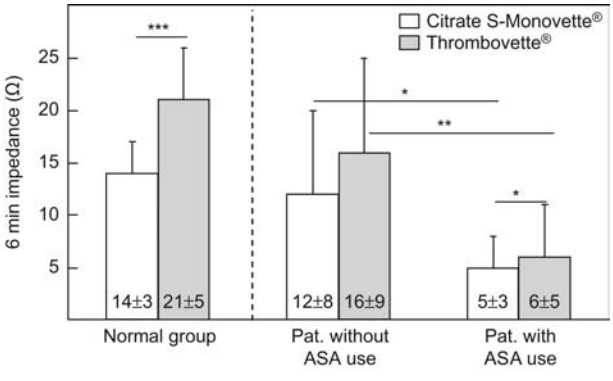

Figure 1 Results (mean value ± standard deviation) of the WBA with collagen (1 mg/L) and the blood collection systems Citrate S-Monovette® and Thrombovette®.
Stars indicate differences with significance levels of $p < 0.05$ (*), of $p < 0.01$ (**) and of $p < 0.001$ (***).

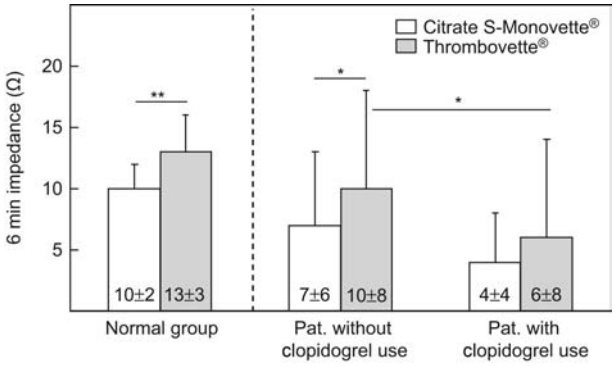

Figure 2 Results (mean value ± standard deviation) of the WBA with ADP (5 μM) and the blood collection systems Citrate S-Monovette® and Thrombovette®.
Stars indicate differences with significance levels of $p < 0.05$ (*) and of $p < 0.01$ (**).

function in 16 patients when using the Citrate S-Monovette® and in 17 patients when using the Thrombovette® (sensitivity 72.8% or 77.3%). The ineffectiveness or non-usage of clopidogrel could be confirmed through WBA results with the Citrate S-Monovette® in 30 and with the Thrombovette® in 36 of 44 persons without clopidogrel (specificity 68.2% or 81.8%). The resulting values for positive (PPV) and negative predictive values (NPV) concerning the actual use or the resistance of ASA and clopidogrel are summarized in Table 3.

Discussion

The results of platelet function diagnostics are influenced by pre-analytical factors to a much larger degree than most other laboratory analysis tests. The artificial activation of platelets through the procedures of blood collection, specimen preparation, transport and storage frequently lead to a distortion of test results. The use of citrate as anticoagulant does not completely prevent the formation of thrombin in the blood specimen, which can lead to an in vitro activation of the platelets. Additionally, citrate produces an unphysiologically low calcium concentration in the blood specimen and thereby alone changes the function of the platelets. If the direct thrombin inhibitor hirudin is used as an alternative anticoagulant, the physiological calcium concentration in the blood specimen is maintained, however, hirudin also is unable to completely prevent the formation of thrombin [5–7]. In 2007, Hellstern and colleagues were first to propose the use of benzylsulfonyl-D-argininyl-prolyl-amidinobenzylamid as an anticoagulant for platelet function diagnostics. They succeeded in showing that it completely prevents thrombin generation in the blood specimen and provides a specimen stability of 24 h at room temperature for turbidimetric platelet aggregometry, for WBA and the platelet function analyzer 100 (PFA 100) [7]. The use of blood collection tubes with benzylsulfonyl-D-argininyl-prolyl-4-amidinobenzylamid as

Table 3 Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) in dependence of blood collection system (Citrate S-Monovette® and Thrombovette®) and diagnostic issue (ASA or clopidogrel resistance). To identify ASA or clopidogrel resistance, we used collagen (1 mg/L) or ADP (5 μM) as the platelet agonist.

	ASA resistance		Clopidogrel resistance	
	Citrate S-Monovette®, %	Thrombovette®, %	Citrate S-Monovette®, %	Thrombovette®, %
Sensitivity	80.0	87.5	72.8	77.3
Specificity	92.3	92.3	68.2	81.8
PPV	94.1	94.6	53.3	68.0
NPV	75.0	82.8	83.3	87.8

anticoagulant (Thrombovette®, Probe&go GmbH, Osburg) has as yet neither been tested in the routine clinical practice, nor has it been used for the detection of ASA or clopidogrel resistance. Accordingly, no reference range of WBA has as yet been determined specifically for the Thrombovette®. These are the issues that were examined in this study. We intentionally refrained from specific instructions for obtaining specimens, but accepted the blood collection and transport conditions common in routine clinical practice. For WBA we assumed a specimen stability of 4 h at room temperature. We were able to identify signs of a decrease of the impedance values within this period of time for analyses with the Citrate S-Monovette® as well as the Thrombovette®. This decrease was lower with the Thrombovette®. Further studies will be required to determine the maximum specimen stability of the Thrombovette®.

For normal test groups as well as for patient groups we detected higher impedance values with the Thrombovette®, regardless of the platelet agonists used. This observation might be explained by the physiologic calcium concentration and a lesser in vitro activation of the platelets when using benzylsulfonyl-D-argininyl-prolyl-4-amidinobenzylamid as an anticoagulant. Hence, WBA would reflect the in vivo function of the platelets better with the Thrombovette® than WBA with the Citrate S-Monovette®.

It should be emphasized that the use of the Thrombovette® achieves a distinct increase of specificity and of PPV in regard to the use of ASA and clopidogrel. By using the Thrombovette® a larger number of right positive patients with ASA and/or clopidogrel resistance can be diagnosed through WBA. This is important because up to now and based on conventional platelet function tests from citrate blood the relatively high number of patients with ASA and/or clopidogrel resistance did not correspond to the distinctly lower number of clinical events. It is possible that with platelet function tests from citrate blood medication resistance cases are frequently recognized as false positives, which could be the consequence of an in vitro activation of platelets through thrombin being formed in the citrate blood.

This study does have limitations. Although it included a higher number of individuals than other studies for the evaluation of benzylsulfonyl-D-argininyl-prolyl-4-amidinobenzylamid as anticoagulant for platelet function tests, an even higher number of cases would further improve the statistical validity. Since as yet no independent gold standard method exists for the detection of ASA and/or clopidogrel resistance, test persons and patients not taking medications were considered as individuals with resistance when analyzing sensitivity and specificity. Furthermore, studies of patient groups from other medical specialties should be studied, in order to be able to confirm the general suitability of the Thrombovette®.

In summary, our results demonstrate that the Thrombovette® is a new and promising blood collection system for platelet function diagnostics. Through higher specificity and the higher PPV the Thrombovette® can be expected to provide improved clinical validity of platelet function diagnostics for the identification of ASA and/or clopidogrel resistance. The higher specimen stability through the use of benzylsulfonyl-D-argininyl-prolyl-4-amidinobenzylamid as anticoagulant described by Hellstern could for the first time also allow the examination of patient specimens that are sent to the laboratory from more distant locations.

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Conflicts of interest

The Thrombovette® blood collection tubes were made available for this study by courtesy of Probe & Go. The study was planned and conducted independently of Probe & Go.

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