The In Vitro Bleeding Test Standardization of the methodical procedure

Der In-Vitro-Blutungstest: Standardisierung der Methode

G. Dietrich, D. Weber, V. Kretschmer

Dept. Transfusion Medicine and Coagulation Physiology, University Hospital, Marburg, FRG

Zusammenfassung:

The In-Vitro-Bleeding-Test (IVBT) is a screening test for disorders of platelet function: Citrated blood is drawn through a capillary and the aperture of a collagen coated filter. Latter is occluded by adhering and aggregating platelets. Initial blood flow (IF), bleeding time (BT) and bleeding volume (BV) are determined. The influence of several variables on the test results were observed by systematic variation: 30 to 180 min is the optimal interval between blood sampling and start of the test. Since 1990 there are no significant differences between the filter lots influencing the sensitivity and variability of the test. A smaller interior aperture of the filter reduces BT and BV. Capillaries of larger inside diameter result in higher BV but shorter BT. Occlusion of the filter is enhanced best by ADP, least by saline. Filters proved to be stable over a period of at least 14 days after opening the package. Filters and blood samples have to be warmed to 37°C whereas the temperature of the capillary does not influence the results. Drying of the capillaries is necessary for optimal precision. In our opinion IVBT is suitable for routine use if these parameters are considered.

Schlüsselwörter:

In-vitro diagnostic – standards – coagulation – platelet function tests – platelet aggregation – platelet adhesion – bleeding time

Summary:

Der In-vitro-Blutungstest (IVBT) ist ein Suchtest für Plättchenfunktionsstörungen: Citratblut wird durch eine Kapillare und eine mit Collagen beschichtete Filterappertur gesaugt, die durch Adhärenz und Aggregation von Plättchen verschlossen wird. Initialer Blutfluß (IF), Blutungszeit (BT) und Blutungsvolumen (BV) werden bestimmt. Wir untersuchten den Einfluß verschiedener Testvariablen durch systematische Variation dieser: das optimale Intervall zwischen Blutentnahme und Testbeginn beträgt 30 bis 180 Minuten. Seit 1990 ergaben sich keine signifikanten Veränderungen der verwendeten Filterchargen hinsichtlich Sensivität und Variabilität des Tests. Eine kleinere innere Öffnung des Filters reduziert Blutungszeit und Blutungsvolumen. Ein größerer Innendurchmesser der Kapillaren resultiert im höheren Blutvolumen und kürzerer Blutungszeit. Der Verschluß der Filterappertur wird durch ADP am stärksten, durch physiologische Kochsalzlösung am geringsten beschleunigt. Die Filter erwiesen sich nach Öffnung der Verpackung für mindestens 14 Tage funktionell stabil. Filter und Blutproben müssen vor dem Test auf 37°C angewärmt werden, während die Temperatur der Kapillaren ohne Einfluß auf die Testergebnisse ist. Die Kapillaren müssen für eine optimale Präzision trocken sein. Unseres Erachtens ist der IVBT bei Berücksichtigung dieser Parameter für die routinemäßige Anwendung geeignet.

Keywords:

In-vitro-Diagnostik – Standards – Gerinnung – Plättchenfunktionstests – Plättchenaggregation – Plättchenadhäsion – Blutungszeit

Introduction

Whereas the screening of plasmatic coagulation is routinely used before surgery there is only little interest in platelet function. The reason is that up to now no easy test for platelet function has been introduced in clinical laboratories.

The In-Vitro-Bleeding-Test (IVBT, Thrombostat®, Baxter, Germany, Munich) simulates primary hemostasis in an in

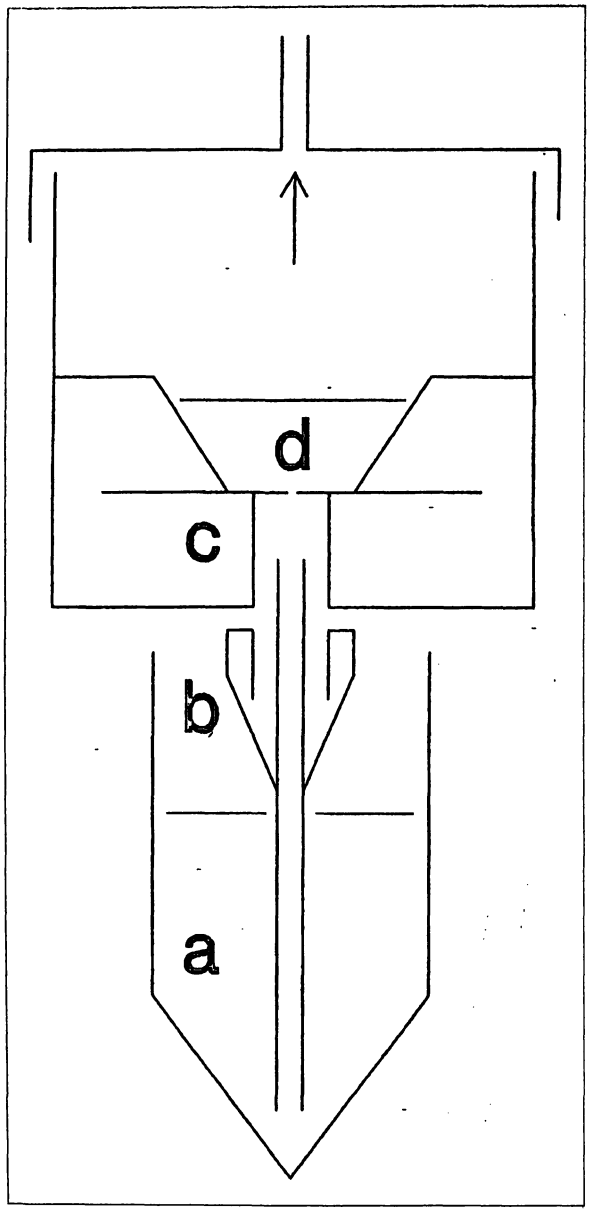

Fig. 1. The principle of the IVBT. a: blood sample, b: capillary, c: filter, d: aggregating agent.

vitro system: Citrated blood (Fig. 1, a) passes a capillary made of teflon (Fig. 1, b). Then it is sucked through the aperture of a collagen-coated cellulose-acetate filter (Fig. 1, c), where the platelets adhere. An aggregating agent (Fig. 1, d) may be used to accelerate the platelet aggregation.

The IVBT has been developed by Kratzer and Born (1, 2) in 1985. It proved to detect sensitively functional disorders of blood platelets (2–6).

Though the test is rather easy to perform, there are many methodical variables influencing the results. For its routine use standardization of IVBT has been necessary. Therefore the aim of this study was to evaluate the various methodical variables and to standardize the IVBT for the routine application.

Materials and methods

Citrated blood (1:10) of healthy blood donors was used for the IVBT. A "preliminary standard-test" was compared to modifications, always changing only one parameter.

The "preliminary standard test"

In the "preliminary standard test" blood was drawn from volunteer blood donors. It was transferred immediately into an Eppendorf cup. IVBT was started one hour later.

Collagen-coated filters (lot.-no. 7/8/88 resp. 12/8/90, interior aperture 150 µm) were used.

Filters were moistened with a solution of ADP (10 mmol/l) mixed V:V with $CaCl_2$ (2 mmol/l). Those filters have been stored at 4°C in a closed aluminium cover. Half an hour before the start of the IVBT they were taken out of the cover and allowed to rest at room temperature. Four minutes before the start they were warmed at 37°C.

Capillaries (inside diameter 200 µm) were cleaned with saline and afterwards 2×10ml air were blown through for drying. They were put onto the filters immediately before the start of the test.

After the start of the IVBT blood was aspirated with a constant pressure of –40 mbar. Initial blood flow (IF), bleeding volume (BV) and bleeding time (BT) were determined with double measurement.

The parameters changed in order to determine their influence on the test results were:

1. The interval between the drawing of blood and the start of IVBT.
The IVBTs of ten blood samples were determined at eight different points of time ranging from 30 minutes to 8 hours.

2. The different filter lots.
Three filter lots from 1988 and 1989 were compared with the same blood samples (n = 8, quadruple measurement). The same was done two years later to compare three lots in 1990 and 1991 (n = 10, double measurement).

3. The diameter of the filter's aperture.
We compared a filter lot (4/69) with an interior aperture of 120 µm to two filter lots (3/88, 8/88) with an aperture of 150 µm (n = 8, quadruple measurement).

4. The diameter of the capillaries.
The IVBTs with capillaries of an inside diameter of 150, 175, 200, 225 and 250 µm were determined with the same blood samples (n = 10).

5. The aggregating agents.
The influence of ADP (10 mM), ristocetin (15 mg/ml), adrenalin (1 mg/ml), collagen (0.2 mg/ml), destilled water,

$CaCl_2$ (25 mmol/l in aq. dest.), $CaCl_2$ (2 mmol/l in saline) and saline 0.9% was tested (n = 20).

6. The concentration of the aggregating agent.
Different concentrations of ADP (10^{-4}–10 mmol/l), ristocetin (0.5–50 mg/ml) and collagen (0.125–40 µg/ml) were used with the same blood samples (n = 3). The results were also compared to those obtained with $CaCl_2$ (2 mmol/l).

7. The time interval after opening the covers of the filters.
Ten filters each are packed in an aluminium cover by the manufacturer in order to keep them dry and stable. Covers were opened and the filters were stored at 4°C. We compared the IVBT of those filters after up to fourteen days, with filters taken out just after opening the cover (controls), using the same blood samples always for one pair.

8. The temperature of filter, capillary and blood.
Before starting the IVBT, filters were soaked with ADP and $CaCl_2$, capillaries and blood were incubated at 37°C for four minutes. We compared the results to those, obtained with the same materials at room temperature.

9. Preparation of the capillary.
We compared our "preliminary standard test" in wich the capillary is dried by inflating 10 ml air twice, with a method of drying the capillary completely by suction with an electric motor pump before starting the IVBT (quadruple test, n = 10).

Statistical Evaluation

We determined the mean values as well as the mean coefficient of variation.

For statistical evaluation we used Wilcoxon's matched pairs signed rank test (7). If more than two groups were compared Friedman's test was chosen (8).

Results

The "preliminary standard test."
The normal values obtained with the "preliminary standard test" are shown in Table 1.

Variations

The variations of this method had following influence on the test results of the IVBT:

1. The interval between drawing of blood and start of IVBT.
Within the period from 30 min to 3 hours the results did not differ significantly (Fig. 2). When the interval between the drawing of blood and IVBT was not within this limits, BT, BV and variation were higher.

Table 1: Normal values obtained with the "preliminary standard test" (n = 18, $\bar{x} \pm s$).

Initial flow (IF)	124 ± 15	µl/min
Volume (BV)	245 ± 80	µl
Time (BT)	169 ± 47	sec

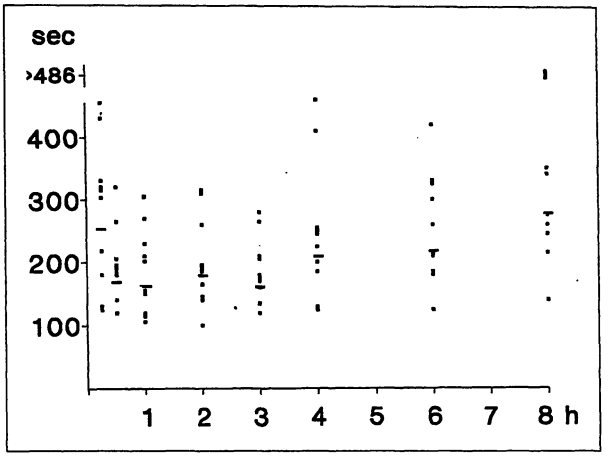

Fig. 2. The influence of the interval between drawing of the blood sample and the start of the IVBT (n = 10).

2. The different filter lots.
With the early filters (1988/89) we found considerable differences between the lots if $CaCl_2$ was used as "aggregating agent" (Tab. 2). The variation concerned BT, BV and the precision of these parameters as well (Tab. 2). Since 1990 significant differences between the different filter lots could not further be demonstrated.

3. The diameter of the filters' aperture.
Reduction of the filter's aperture from 150 to 120 µm lead to significantly shorter BT and lower BV. IF was only little reduced (Tab. 2).

4. The diameter of the capillaries.
With capillaries of larger inside diameters IF and BV were higher then with small ones. In contrast BT was shorter (Fig. 3).

5. The aggregating agents.
Bleeding time and volume were clearly dependant on the aggregating agent used. ADP had the strongest effect, saline solution the least. There was no significant change of the initial flow (Fig. 4).

6. The concentration of the aggregating agent.
The concentration of ADP influenced the results obtained by the IVBT considerably. High concentrations caused quicker platelet plot formation (Fig. 5). The optimum was reached with 10 mmol/l. For ristocetin (Fig. 6) the dose-effect was less pronounced. The concentrations tested for collagen (Fig. 7) were all equally effective.

7. The time interval after opening the covers of the filters.
All filters proved to be stable over a period of at least fourteen days after opening the package if refrigerated (Tab. 3).

8. The temperature of filter, capillary and blood.
The temperature (25 or 37°C) of the capillary had no influence whereas cold filters or blood led to longer BT and higher BV (Tab. 4).

9. Preparation of the capillary.
When the capillary was dried by suction of a motor pump instead of blowing it through with a syringe, BT was

Tab. 2: Filter lots and interior apertures.
A: ADP 4 mmol/l, A/C: ADP 5mmol/l + $CaCl_2$ 1mmol/l, C: $CaCl_2$ 2 mmol/l, BT: bleeding time, BV: bleeding volume, $\bar{x}$: mean, s: standard deviation, ns: not significant, *: $p < 0.05$, **: $p < 0.01$ (Wilcoxon matched pairs signed rank test), n: number.

Aperture(µm)		150	150	150	150	150	120	150	150	150	150	150	150
Lot-No.		4/89	3/88	8/88	3/88	8/88	4/89	8/90	12/90	3/91	8/90	12/90	3/91
Inductor		A/C	A/C	A/C	C	C	C	C	C	C	A	A	A
BT (sec)	$\bar{x}$	140	124	144	178	212	149	157	130	142	90	109	81
	s	20	19	32	25	23	26	47	29	30	15	16	11
BV (µl)	$\bar{x}$	168	145	176	240	296	191	197	144	143	122	112	101
	s	22	17	42	42	42	32	55	23	33	24	18	20
n		8	8	8	8	8	8	10	10	10	10	10	10

slightly shorter, BV smaller and the precision especially concerning the initial flow was higher (Tab. 5).

Discussion

Platelet counts in thrombocytopenic patients do not always correlate with the bleeding risk. On the other hand normal platelet counts do not exclude a bleeding risk because of a possible impairment of platelet function.

Platelet function was up to now estimated by global tests like thrombelastography, resonance thrombography or orbitometry. These three methods also include the plasmatic coagulation. The IVBT is more specific because plasmatic coagulation is of no influence. Additionally the IVBT is considerably more sensitive (4, 9).

There are a number of special tests for platelet function e.g. adherence, aggregation or secretion etc. which are useful for further diagnosis. Most of them are not easy to perform. Each examines only one aspect of platelet function. Whereas by the IVBT one investigates platelet function in a global test of primary hemostasis.

For routine use of the IVBT it is necessary to standardize its performance and to exclude variables that impair precision or reproducibility.

Following variables examined in this study had no influence: The filter lot (since 1990), the time interval after opening the covers for at least two weeks and the temperature of the capillary.

On the other hand, the interior diameters of the filter's aperture and of the capillary, kind and concentration of the inductor, temperature of blood and filter or the preparation of the capillary are of importance.

In order to get precise and reproducible results, most of the parameters that were investigated in this study have to be considered:

Blood has to be drawn thoroughly. The blood should rest at least half an hour. This seems to be the time, the plate-

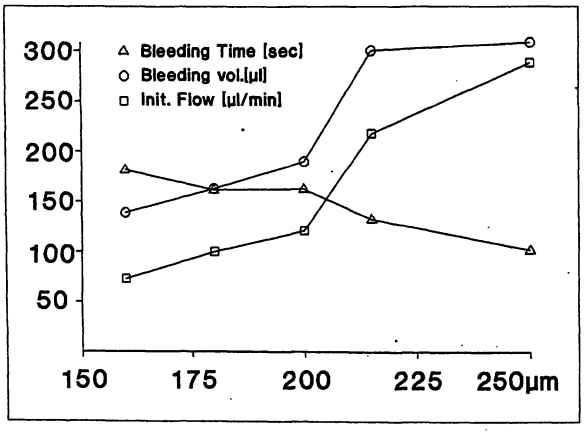

Fig. 3. The influence of the inside diameter of the capillary on the IVBT, means (n = 10).

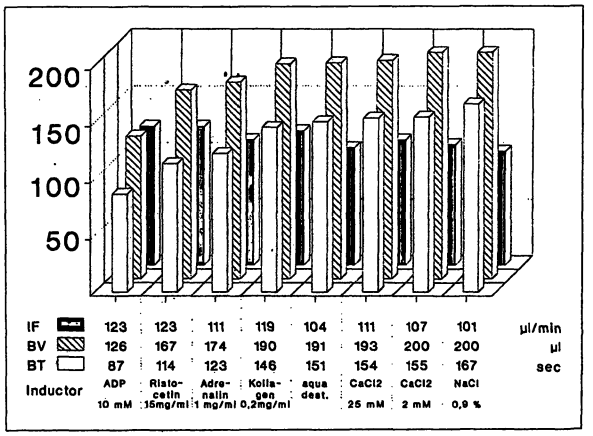

Fig. 4. Comparison of different aggregating agents. IF: initial blood flow, BV: bleeding volume, BT: bleeding time.

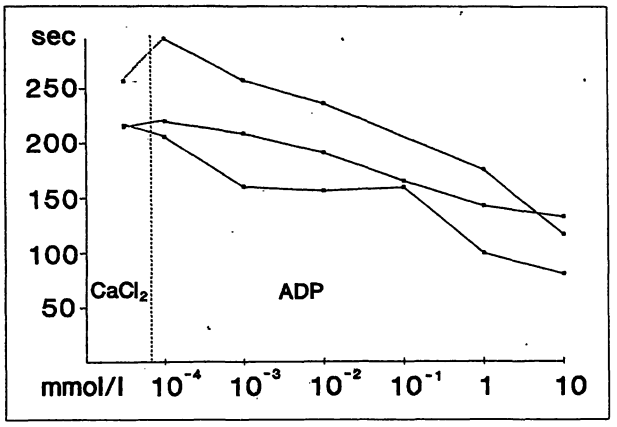

Fig. 5. The influence of the concentration of ADP on the IVBT (bleeding time). Reference: $CaCl_2$ 2 mmol/l (left).

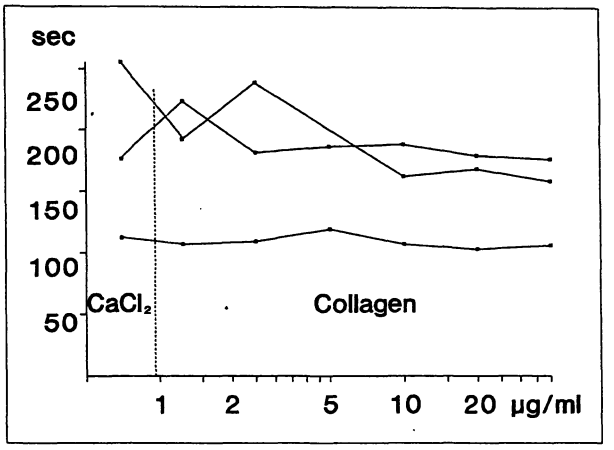

Fig. 7. The influence of the concentration of collagen on the IVBT (bleeding time). Reference: $CaCl_2$ 2 mmol/l (left).

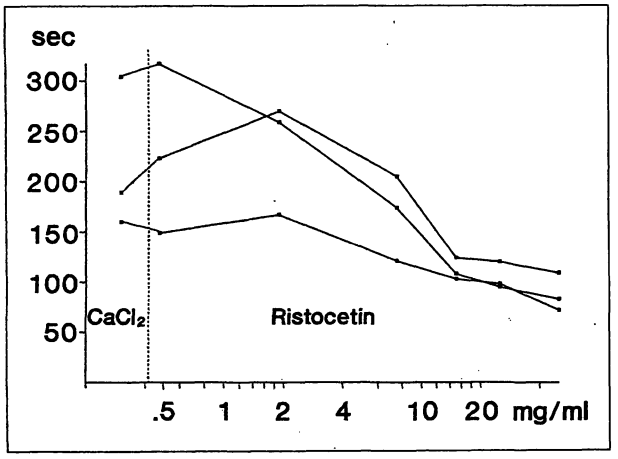

Fig. 6. The influence of the concentration of Ristocetin on the IVBT (bleeding time). Reference: $CaCl_2$ 2 mmol/l (left).

lets need to gain a normal level of activation again. This period was known from platelet aggregation tests before. The measurement should be finished within three hours. Platelets seem too loose to much activity in the IVBT then. Perhaps this time could be prolonged if a nutricient would be added to the citrate solution.

For screening tests the inside diameter of the capillary should be 200 µm, the one of the filter aperture 150 µm. The Thrombostat®4000 puts a limit of 850 µl to the BV and 486 sec to the BT. With the parameters described above also large deviations from normal values can be measured without reaching the limits of the device.

Screening tests should be as sensitive as possible. That is why we consider $CaCl_2$ or even saline solution to be the best "aggregating agent" for this aim. The comparison with ADP (4 mmol/l)-induced IVBT is suitable for the differentiation between the effect of cyclooxygenase inhibitors and other defects of platelet adhesion and aggregation. Latter method is not as sensitive but results in shorter BT and smaller BV with higher precision.

The filter has to be incubated at 37°C for four minutes before the measurement. Otherwise the expansion of heated air pretends a higher BV. Drying the capillary completely by suction with an effective pump leads to shorter BT, lower BV and to a higher precision.

We could not find significant changes of the filter lots since september 1990 concerning the test sensitivity and variability. This is the point of time, when the manufacturer developed a method that optimized the collagen-coating of the filters. Neither seems it to be of importance when the aluminium-cover is opened.

With this knowledge we designed a new standard test for future use: Blood is carefully drawn through a steel needle (at least 19 G) into a syringe prefilled 1:10 with citrate solution. After carefully mixing 1 ml each is pipetted into three Eppendorf cups.

We use disposable collagen-coated filters with an aperture of 150 µm. Filters are stored in the refrigerator. The aluminium-cover of the filters can be opened at least 14 days before use. Filters are taken out of the refrigerator half an hour before the start of the test.

Half an hour to three hours after blood drawing dry teflon capillaries (0.2×32 mm) are fixed at the filter. The filters are moistened with 40 µl ADP 4 mmol/l. Blood, filters and capillaries are warmed at 37°C for four minutes. The IVBT is run with a suction of 40 mbar.

After the tests, capillaries are immediately cleaned with destilled water and dried with the aid of an electric motor pump.

In the modification described the IVBT can easily be used for the preoperative determination of the platelet function (9). This seems to be indicated in patients with bleeding

Table 3: Preliminary standard test applied on filters stored for 1 to 14 days after opening the cover (A) in comparison to unstored filters of the same lot (B).

Storage	0	1	2	3	7	10	14	days
Initial flow (IF)								
A	99	91	110	107	104	119	106	µl/min
B	99	114	112	104	106	116	106	µl/min
Volume (BV)								
A	133	175	201	204	165	246	170	µl
B	123	188	202	206	180	251	132	µl
Time (BT)								
A	119	139	146	171	135	181	122	sec
B	111	136	150	173	144	175	123	sec

Table 4: The influence of the temperature of filter (including aggregating agent), capillary or blood sample. Std. test: preliminary standard test, signifi.: statistical significance, n.s.: not significant.

Blood	Filter	Capillary	Time (sec)	Std. test	Signifi.
37°C	37°C	37°C	195±77	182±52	<n.s.
25°C	25°C	25°C	225±50	196±27	<0.005
25°C	37°C	25°C	209±58	171±38	<0.01
37°C	25°C	25°C	205±56	171±33	<0.01

Table 5: Drying of the capillary with the aid of a motor pump compared to the "preliminary standard test" (quadruple test, n = 10).

		Motor pump	Preliminary standard test		Significance
Initial flow	$\bar{x}\pm s$	109±15	88±12	µl/min	$p \leq 0.01$
	$\bar{v}$[1]	0.14	0.17		$p \leq 0.01$
Volume	$\bar{x}\pm s$	122±19	144±21	µl	$p \leq 0.05$
	$\bar{v}$	0.16	0.27		n.s.
Time	$\bar{x}\pm s$	91±12	118±14	sec	$p \leq 0.05$
	$\bar{v}$	0.15	0.19		n.s.

[1] mean coefficient of variation $\bar{v} = \sum_{i=1}^{n} \frac{s_i}{x_i}$.

history or undergoing operations with high bleeding risks.

In cases of unclear hemorrhage the IVBT helps to distinguish platelet functional impairment from other bleeding disorders because of its specifity (9). In those cases we recommend to use it in the first diagnostic steps beside the global tests of plasmatic coagulation (PT, APTT) and platelet counting.

In our experience pathological values with the standard test (ADP) described before, always correlate to clinically relevant disturbances of the primary haemostasis with significant bleeding risks mostly due to platelet disorders. Since the hematocrit closely correlates to BV and BT as it does in primary haemostasis in vivo low haematocrit values prolong BT and increase BV, too. The IVBT with $CaCl_2$ does not necessarily correlate to a significantly increased bleeding risk because of its high sensitivity. It is especially sensitive concerning the effect of acetyl-salicylic acid (4). The clinical relevance of abnormal values of different extent in the $CaCl_2$-induced IVBT has still to be demonstrated by correlation to in vivo bleeding time and clinical bleeding signs. On the other hand, normal IVBT ($CaCl_2$) excludes disturbance of the primary haemostasis with high probability (10).

In addition, the IVBT ($CaCl_2$) could also be an instrument to control the therapeutic anticoagulant effect of acetyl-salicylic acid (4). But it seems that the filters we use today do not allow to detect the effect of very low doses of acetyl-salicylic acid (<50 mg) on platelets as sensitive as those we used in 1988 (4). We explain the differences by the enhanced collagen-coating-technique.

Additionally, in thrombocytopenia the IVBT could become valuable to determine the bleeding risk of the patients. The standard IVBT (ADP) mostly gives no measurable values when the platelet counts are below 50000/µl. The so-called "thrombocytopenia-adapted-IVBT" allows the evaluation of the platelet function of patients with a platelet range of 10–50000/µl. A detailed description of this modification has been published before (11). By this modification of the IVBT it is possible to estimate the bleeding risk of thrombocytopenic patients and to control the therapeutical efficiency of the platelets transfused (11). Additionally, the efficiency of supplementary treatment (e.g. phospholipids (12)) and the influence of inhibiting drugs can be evaluated.

In addition, recently Glaser et al. (13) reported that another modification of the IVBT is suitable for the quality assurance of plateletpheresis concentrates during storage.

Nevertheless, there are still some problems to be solved: The Thrombostat®4000 is a device that works only half-automatically. One still needs at least double-measurements which take five to ten minutes. The capillaries have to be cleaned from remaining blood afterwards. This means a risk of infection for the personnel. Additionally this procedure impairs the precision of the test. Therefore an automatic device should be developed, that works with disposable materials only.

The precision of the test also highly depends on the collagen-coating of the filters. As this problem seems to be solved, single measurements might be sufficient in future. This would also reduce the costs.

In our opinion the IVBT now is suitable for broad application if the parameters we described are considered. In our clinics the standard test described is used routinely since about 2 years to detect or exclude relevant platelet disorders. From our experience a normal $CaCl_2$ induced IVBT excludes any significant bleeding risk during surgery due to platelet disorders and a pathological ADP induced IVBT recognizes significant platelet disorders.

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Anschrift des Verfassers:

Prof. Dr. Volker Kretschmer, M.D. PhD
Abteilung für Transfusionsmedizin und Gerinnungsphysiologie
Universitätsklinikum
Conradistraße
35043 Marburg