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Monitoring the effects of platelet glycoprotein IIb/IIIa antagonists with a microtiter plate method for detection of platelet aggregation

Siegfried Krause, Thomas Scholz, Uta Temmler, Wolfgang Lösche

Measurement of platelet aggregation in platelet-rich plasma (PRP) is a fundamental tool in platelet studies, despite the fact that the technique required for this is time-consuming, may need large volumes of blood, and require particular skill and special equipment. The use of a microplate reader seems useful to perform platelet aggregation more rapidly and with less material. So, the aim of the present study was to validate a simple and rapid method which enables performance of kinetic measurements of platelet aggregation directly in a microtiter plate reader. Platelet aggregation was carried out in 96-well, flat-bottomed microtiter plates. Samples of PRP (140 μl/well) were placed in a microtiter plate. Agonists (10 μl/well) were added using an electronic multichannel dispenser directly before the reading was started. Measurements of the optical density were performed at 650 nm using a THERMOmax™ microplate reader (Molecular Devices, Sunnyvale, USA). During the run time the plate was incubated at 37°C and was mixed with the automix function of the reader. The technique was verified by comparing dose-response curves of platelet agonists and glycoprotein IIb/IIIa antagonists, obtained with the standard aggregometer and with the microtiter plate reader. Platelet aggregation in microtiter plates is simple and rapid. It offers the advantages of lowering the test volumes and the possibility to perform about 90 tests simultaneously. The method was successfully applied to measure platelet inhibition by glycoprotein IIb/IIIa antagonists.

Introduction

Aggregometry in platelet-rich plasma (PRP) is the classical method for assessing platelet activation. The method is based on the monitoring of light transmission through a stirring PRP or platelet suspension which is continuously recorded.[1,2] In a standard aggregometer, platelet aggregation is measured in cuvettes in which a stirring bar mixes the platelet suspension on the bottom.[3] The number of samples which can be analyzed simultaneously using an aggregometer is limited to a maximum of four channels, and the limited stability of platelet preparations after blood collection does not allow analysis of a large number of samples. The problem of proper interpretation occurs very often. Moreover, novel classes of anti-platelet drugs, such as adenosine diphosphate (ADP) receptor and glycoprotein IIb/IIIa receptor antagonists, are being developed and introduced into routine clinical practice, so that the need for monitoring of platelet function arises.[4]

As an alternative to measurement using an aggregometer, the aggregation of isolated platelets may be studied in microtiter plates based upon the difference in optical density between non-aggregated and aggregated platelets.[5,6] Advantages of the use of the aggregation in microtiter plates are: rapid and simple performance of the assay, the simultaneous performance of up to about 90 tests, reduction of blood and PRP volumes, and lower volumes of agonists and inhibitors.

Siegfried Krause, Thomas Scholz, Uta Temmler, Wolfgang Lösche, Centre of Vascular Biology and Medicine, Friedrich-Schiller-University of Jena, Erfurt, Germany.

Correspondence to: Siegfried Krause, Friedrich-Schiller-University of Jena, Centre of Vascular Biology and Medicine, Nordhäuser Str. 78, D-99089 Erfurt, Germany. Tel.: +49-361-741-1457; Fax: +49-361-741-1202; E-mail: krause@zmkh.ef.uni-jena.de

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The purpose of this study was to compare the standard platelet aggregation methodology with aggregometry performed in microtiter plates, and to study the inhibition of platelet aggregation by different antagonists (EMD76334, Eptifibatide) with ADP and collagen as agonists.

Materials and methods

Sample preparation

Venous blood was obtained from healthy volunteers who had not taken any drugs for at least 2 weeks. Blood was anticoagulated with sodium citrate (3.13%, 1:10, v/v) or with recombinant hirudin (Refludan, Aventis Pharma) at a final concentration of 20 μg/ml[7] and centrifuged at 240 × *g* for 10 min for female donors and for 13 min for male donors in capped plastic tubes to prepare platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was prepared by subsequent centrifugation of PRP (17 000 × *g*, 8 min). PRP were kept in tightly stoppered plastic tubes at room temperature during the experimentation.

The following antagonists were used: Eptifibatide (Essex Pharma, Munich, Germany) and EMD76334 (Merck, Darmstadt, Germany); the stock solutions were both 2 mg/ml. Solutions were prepared daily by dilution with saline. Solutions of inhibitors were added prior to the aggregation measurements to the PRP samples. Final concentrations in PRP were for eptifibatide 0.0625, 0.125, 0.25 and 0.5 μg/ml and for EMD76334 0.01, 0.02, 0.03 and 0.04 μg/ml

Performance of aggregometry in aggregometers

Platelet aggregation was measured in a four-channel aggregometer PAP-4C (Bio/Data, Horsham, USA) and in a dual-channel LA220 laser aggregometer (Biola, Obninsk, Russia). The LA220 was used in this study for standard aggregometry, despite the fact that this device is also able to detect microaggregate formation using laser-light scattering.

The aggregometers were calibrated with PPP and the stirring speed was set at 1000 rpm. Aliquots of PRP (480 μl for the PAP-4, 288 μl for the LA220) were placed in cuvettes containing magnetic stirring bars and were prewarmed at 37°C for 1 min, then stirred for 1 min to obtain a stable baseline. The agonists (PAP-4, 20 μl; LA220, 12 μl) were then added, and changes in light transmission were recorded for at least 4 min.

Inducers were ADP (Sigma-Aldrich, Germany), collagen (Nycomed, Germany) and the sodium salt of arachidonic acid (Sigma-Aldrich, Germany). The maximum extent and rate of aggregation were analyzed by monitoring the amount of light transmitted and the maximum slope of the rate of change of light transmittance. The aggregation data were partly analyzed by the corresponding software programs: 'Platelet aggregation profiler data interface', version 2.2. (Bio/Data) or 'Aggregation analyzer program', version 2.16 (Biola).

Performance of aggregometry in a microplate reader

The procedure of platelet aggregation in microtiter plates was carried out using a THERMOmax microplate reader (Molecular Devices) in combination with the software SOFTmax for Windows, version 2.35. This instrument enables kinetic measurements at 37°C. The device may read the optical density in 96 wells of microtiter plates in one operation every 9 s, with mixing of the plate between the readings.

Aggregation of platelets was performed in 96-well, flat-bottomed microtiter plates as follows: samples of 140 μl of PRP were pipetted per well of the microplate; for calibration, 140 μl of PPP were placed in some wells (in general three wells). Reverse pipetting is recommended to avoid the formation of air-bubbles on the surface of the liquids. The plate was preincubated at 37°C for 3 min and an initial reading was taken in the 'endpoint' mode of the instrument.

The agonists were added to the appropiate wells (10 μl per well) with an electronic eight-channel Eppendorf Response pipette in the dispensing mode (Eppendorf, Hamburg, Germany). This enables the aspirated amount of agonist to be dispensed in several steps, and therefore the aggregation can be induced very rapidly in the various platelet samples of the microtiter plate. Dilutions of agonists were prepared in microtube racks (with 1.3-ml microtubes, 8.5 mm diameter × 44 mm) or in deep well plates (Greiner, Frickenhausen, Germany) to aspirate the different agonist samples with the multi-channel pipette at the same time.

After the addition of agonists, kinetic reading of the plate was immediately started. Measurements of the optical density were performed at 650 nm for 10 min. During the run time the plate was incubated at 37°C and was mixed using the automix function of the reader. This option enables a shaking of the plate for 10 s prior to the initial kinetic reading and for 3 s between the readings.

Data analysis

Raw data files of SOFTmax software were converted into Microsoft Excel spreadsheets, and calculation of extent (maximum) and rate (slope) of aggregation were done using macro-commands in Excel. Statistical analyses were carried out with WinSTAT for Excel (version 1999.1). Percentage of aggregation was calculated using the formula 100 × [OD of PRP – OD of sample]/[OD of PRP – OD of PPP]. For estimation of slope, the increase over six points of measurement, i.e. 1 min, was determined. Data are presented as mean ± standard deviations, unless stated otherwise. A value of $P < 0.05$ was considered to indicate statistical significance for all tests.

Results

In order to adapt the platelet aggregation to the microplate reader, we compared platelet aggregation

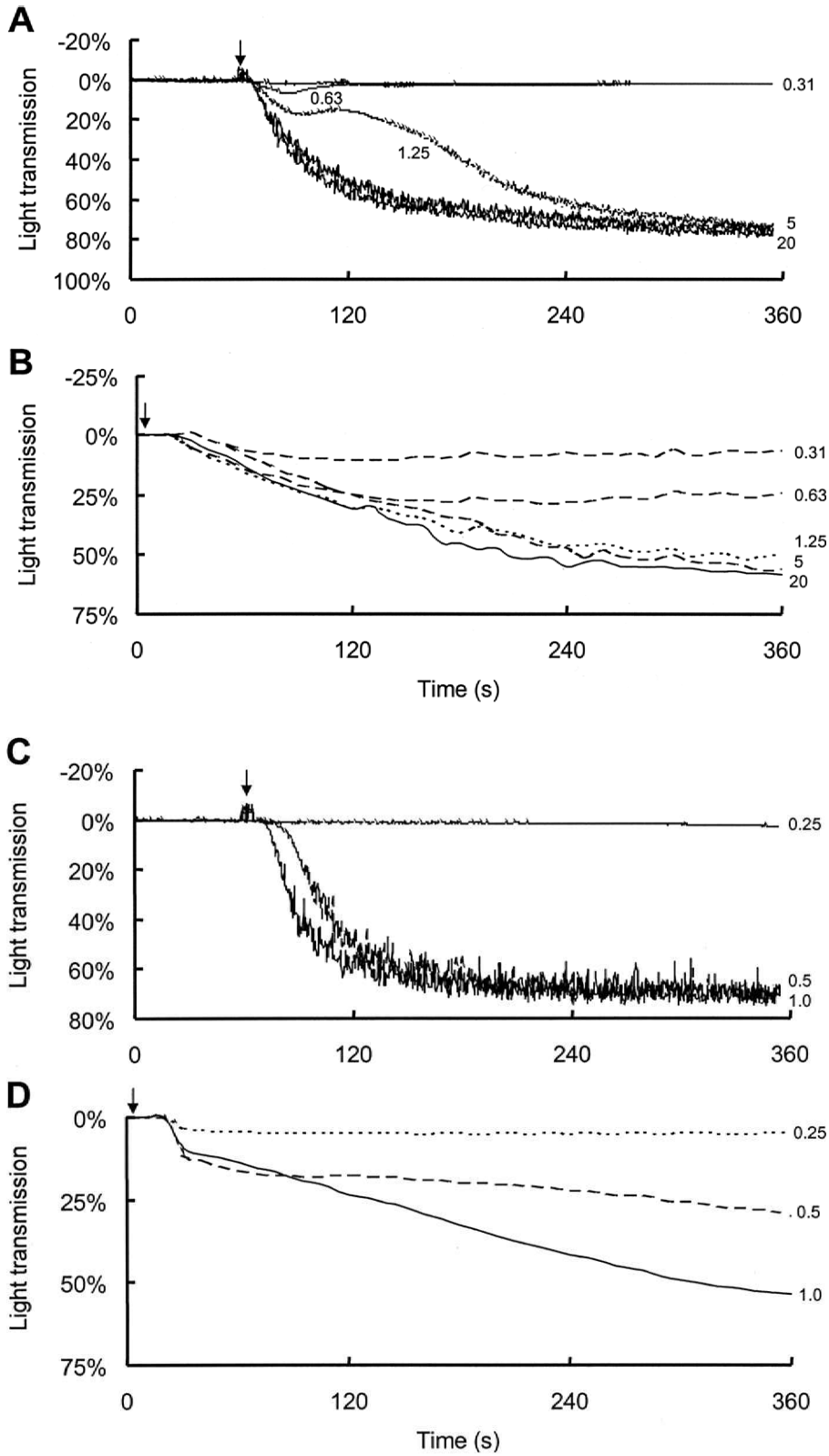

Figure 1. Comparison of aggregation induced by ADP (A,B) or by arachidonic acid (C,D) obtained with an aggregometer (PAP-4C) (A,C) and with the microplate reader (B,D). The arrow indicates the addition of the agonist.

curves measured in a standard aggregometer with that in the microplate reader. Figure 1 shows typical tracings of ADP- or arachidonic acid-induced aggregation in citrated PRP.

There are some differences between the aggregation curves produced by standard aggregometry and those in the microplate, particularly the slope and extent at lower agonist concentrations (Figure 1): the extent of platelet

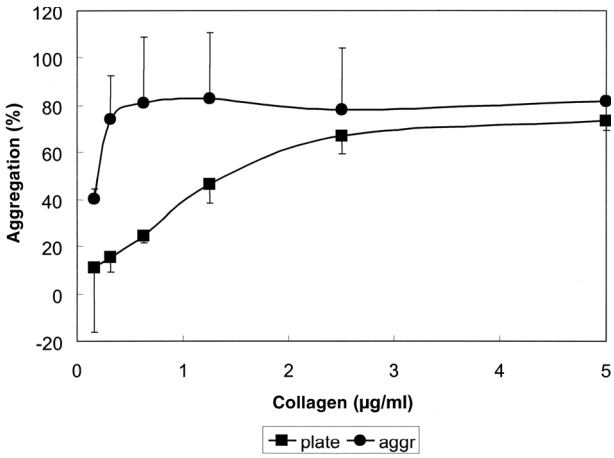

Figure 2. Dose-response of platelet aggregation induced by collagen in citrated PRP. The aggregation extent measured with the aggregometer PAP-4C (aggr) and with the microplate reader (plate) are shown (means ± S.D., $n = 4$).

aggregation induced by low concentrations of collagen was less in the microplate than in standard aggregation (Figure 2). For inhibitor studies we used concentrations of 5 and 20 µM ADP and 5 µg/ml collagen, with an aggregation time of more than 5 min, which produced comparable results by both methods.

An important advantage of the use of the microplate reader for aggregation is the possibility to perform aggregation as multiple tests. A separate study of reproducibility was not done; however, the intra-assay variation coefficients were calculated (Table 1). When we carried out multiple parallel tests of ADP-induced aggregation in the microplate, the variation coefficients amounted to 6.3–12.4% for the rate (slope) and 7.2–14.0% for the extent of aggregation, dependent on the ADP concentration used. In comparison with those assessed by standard aggregometry, the values using the plate are corresponding or lower, especially using low ADP concentrations (Table 1).

Standard aggregometry is a time-consuming procedure but, because of the variability of platelet activation and deactivation, only a limited time window is useful for assessment of platelet aggregation. An advantage of the proposed technique is that in microtiter plates several samples can be rapidly aggregated in parallel, which therefore helps to overcome this problem. In order to investigate the time dependency of standard aggregometry, we compared the aggregation response in a conventional aggregometer from citrated and hirudinized PRP 30, 60 and 90 min after blood sampling. As shown in Table 2, some parameters of platelet aggregation (extent and slope) already decrease after 60 min in hirudinized PRP and after 90 min in citrated PRP.

As expected, the tested GPIIb/IIIa antagonists inhibited platelet aggregation in a dose-dependent manner, as measured by standard aggregometry as well as in the microplate. Figure 3 illustrates the part of the data display on screen during platelet aggregation in the microplate, showing inhibition of platelet aggregation by EMD76334. Thus, continuous control of the multiple traces during aggregation is allowed. The inhibition of platelet aggregation by GPIIb/IIIa antagonists was measured by standard aggregometry and compared with data obtained in the microplate reader (Figure 4). Mean inhibition of platelet aggregation by EMD76334 and Eptifibatide, measured as extent and slope of ADP-induced aggregation (20 µM) in citrated PRP, is represented in Figure 4. As shown, the inhibition occurs with identical efficacy in the different assay systems.

The mean IC_{50} values determined by ADP-induced aggregation (20 µM) varied between 22.4 and 27.4 ng/ml for EMD76334 and 0.11 and 0.14 µg/ml for eptifibatide (Figure 5). In contrast, in hirudinized PRP, IC_{50} values for eptifibatide but not for EMD76334 were about 2–3-fold higher than those obtained with citrated PRP (Figures 5 and 6). There were no differences between the IC_{50} values for inhibition of ADP- and collagen-induced aggregation by eptifibatide and EMD76334 determined by either the microplate method or standard aggregometry (Figures 5 and 6).

Discussion

Platelet function and inhibition are important methods in clinical pharmacological studies. There is a variety of platelet-testing procedures for characterizing drug effects on platelet function, most of them showing the influence of drugs on platelet aggregation induced *in vitro* by

Table 1. Intra-assay variation of ADP-induced aggregation, expressed as CV (%), in citrated PRP analyzed by standard aggregometry in PAP-4C (agg 1) and LA220 (agg 2) or in the THERMOmax microplate reader (plate) (n.d., not detected)

Instrument	Extent			Slope		
	Agg 1 ($n = 10$)	Agg 2 ($n = 8$)	Plate ($n = 20$)	Agg 1 ($n = 10$)	Agg 2 ($n = 8$)	Plate ($n = 20$)
Agonist						
20 µM ADP	4.1	9.2	7.2	7.7	12.4	6.3
5 µM ADP	3.4	14.0	9.0	10.7	21.4	4.7
1 µM ADP	133.0	n.d.	13.8	20.1	n.d.	6.7
0.5 µM ADP	316.2	n.d.	14.0	14.3	n.d.	12.4

Table 2. Dependency of ADP-induced aggregation in citrated and hirudinized PRP on storage time after cell separation, mean (± S.D.)

	Time after PRP preparation (min)					
	Citrated PRP			Hirudinized PRP		
	30	60	90	30	60	90
20 μM ADP						
Extent	80.0 (7.8)	81.4 (10.6)	81.5 (4.1)	76.4 (10.2)	76.4 (9.4)	69.8 (11.5)
30′ versus 60′ *p*<		n.s.			n.s.	
30′ versus 90′ *p*<			n.s.			n.s.
60′ versus 90′ *p*<			n.s.			0.03
Slope	47.8 (4.4)	46.8 (6.7)	40.0 (3.2)	50.8 (4.3)	48.2 (4.1)	36.8 (1.1)
30′ versus 60′ *p*<		n.s.			0.05	
30′ versus 90′ *p*<			0.005			0.005
60′ versus 90′ *p*<			0.005			0.01
5 μM ADP						
Extent	82.0 (3.4)	80.0 (3.1)	76.8 (6.6)	71.4 (11.1)	66.0 (10.8)	52.0 (20.9)
30′ versus 60′ *p*<		n.s.			0.02	
30′ versus 90′ *p*<			n.s.			0.02
60′ versus 90′ *p*<			n.s.			0.05
Slope	40.8 (8.0)	40.2 (2.8)	31.3 (5.4)	49.8 (7.3)	41.2 (2.0)	28.0 (3.1)
30′ versus 60′ *p*<		n.s.			n.s.	
30′ versus 90′ *p*<			0.02			0.005
60′ versus 90′ *p*<			n.s.			0.005

different agonists.[8–11] The measurement of platelet aggregation remains the most widely used method of assaying platelet function, despite its disadvantages, including sample processing time and changes of the platelet milieu by platelet isolation.[12] For example, it is proposed that aggregometry should be carried out within 60–100 min after blood sampling.[13] However, a decrease in platelet aggregability was found already after 60 and 90 min, depending on the anticoagulant. The sample instability could be overcome using a method which enables a more rapid assessment of aggregation measurements. We have presented here a simple method for measurement of platelet aggregation using a 96-well microplate reader, which allows rapid multiple assays without the need for special equipment and skills necessary for standard aggregometry. A further advantage of this method is the reduction of material requirements: e.g., this method needs only a 2–3-fold lower amount of isolated platelets as a standard aggregometer, so it can also be effectively used for study of platelets obtained from small experimental animals.[6]

The optical properties of a microplate reader differ from that of an aggregometer: the optical density is measured in the vertical direction and not horizontally as in aggregometers. This requires precise sample dosage; however, the tests performed in the microplate exhibit a high reproducibility. Another consequence could be that large aggregates formed during the aggregation process might be dropped in the cuvette and leave the light path in the standard aggregometer; however, they would be further detected in the microplate. Despite this fact, the aggregation determined in the microplate may produce useful aggregation curves and comparable results in inhibitor studies with those estimated by standard aggregometry.

Previous reports measuring platelet aggregation with the microplate reader described endpoint methods:

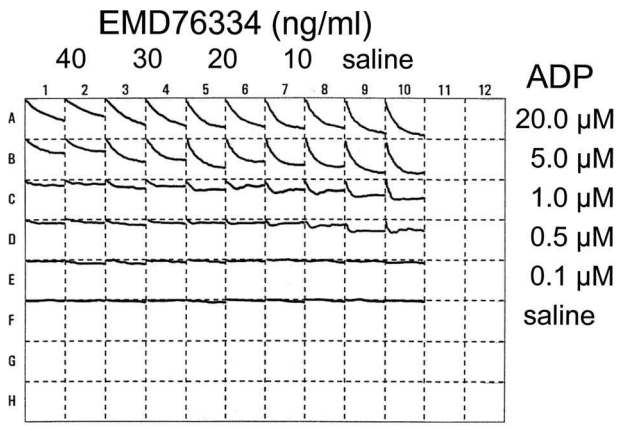

Figure 3. Kinetic data plots as shown by the SOFTmax software during aggregation in the microplate. The effects of the GPIIb/IIIa antagonist EMD76334 on ADP-induced platelet aggregation are demonstrated. Aggregation was measured in duplicate in the wells from columns 1 to 10 and rows A to F. Citrated PRP were preincubated with various concentrations of EMD76334 (10–40 ng/ml) and aggregation was induced by different concentrations of ADP (0.1–20.0 μmol/l). Optical density (OD) is shown from 0 to –0.2. Final concentrations of ADP: row A, 20; row B, 5; row C, 1; row D, 0.5; row E, 0.1 μM; and row F, saline. Inhibitor concentrations (ng/ml EMD76334): columns 1+2, 40; column 3+4, 30; columns 5+6, 20; columns 7+8, 10. The wells in columns 9+10 were not treated with the inhibitor and were used as control. The representative results of four identical experiments are shown.

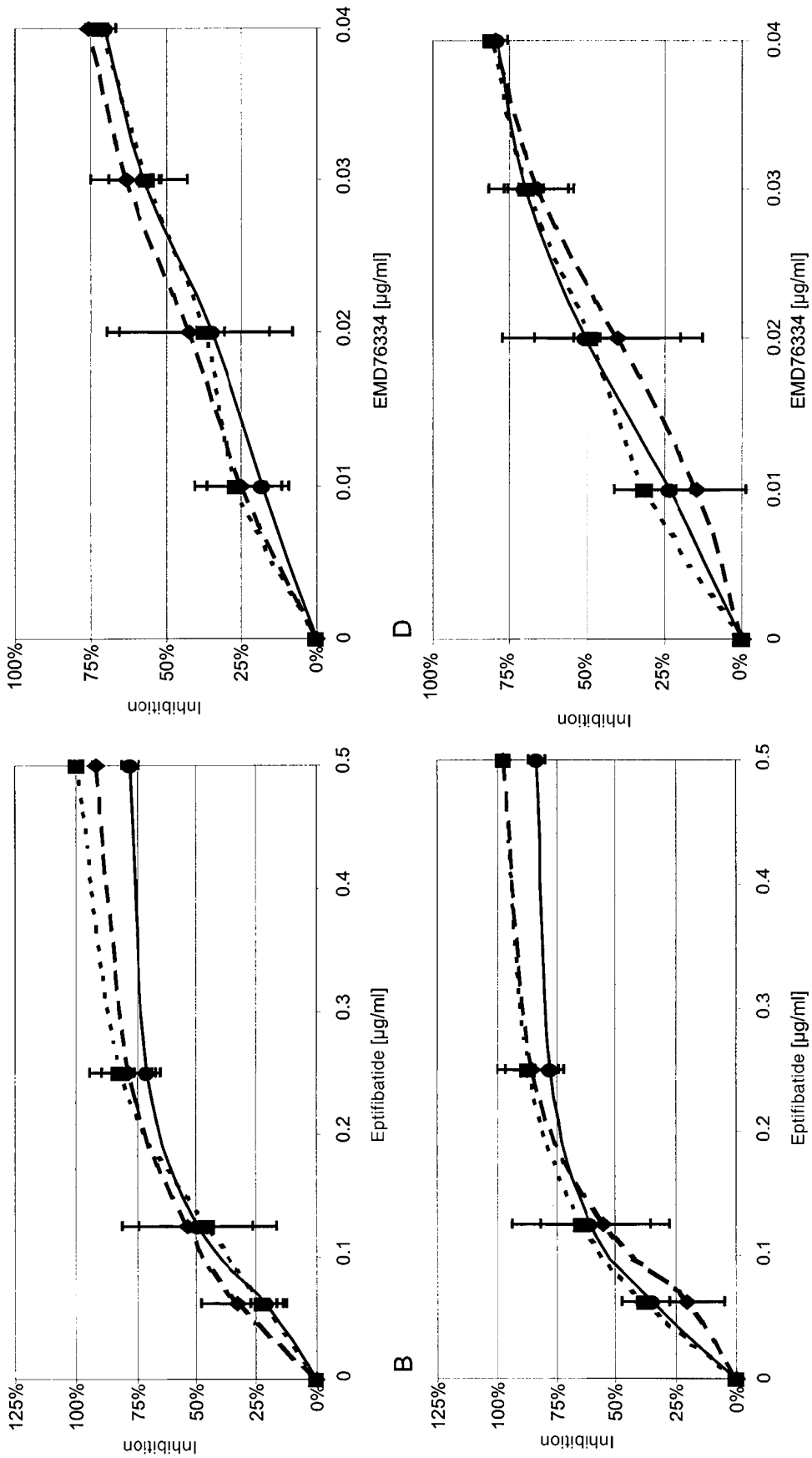

Figure 4. Effects of GPIIb/IIIa antagonists on ADP-induced platelet aggregation. Citrated PRP were preincubated with various concentrations of the GPIIb/IIIa antagonists eptifibatide (A,B) and EMD76334 (C,D). Aggregation was induced through addition of 20 µM ADP and was performed in parallel using the standard aggregometers and the microplate reader. The dose-response curves were calculated from the maximum extent (A,C) and the slope of aggregation (B,D). Instruments: PAP-4C (agg1, ♦), LA220 (agg2, ■), THERMOmax microplate reader (plate, ●). Means (± S.D.) of four independent experiments are shown.

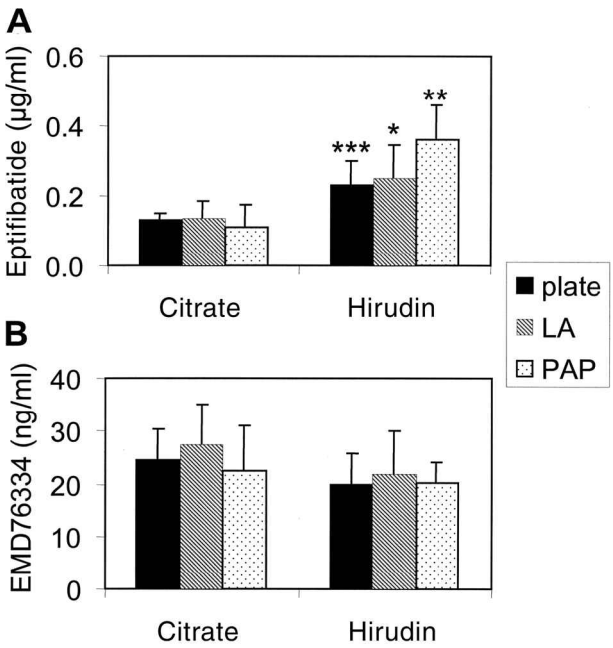

Figure 5. Mean inhibition of ADP-induced aggregation (20 µM) by eptifibatide (A) and EMD76334 (B) in citrated and hirudinized PRP. Comparison of the extent of aggregation determined by standard aggregometry or by the aggregation assay in microplates. Instruments: THERMOmax microplate reader (plate), LA220 (LA), PAP-4C (PAP), means ± S.D., n = 4–11. Significance between different anticoagulants: $^{*}P < 0.02$; $^{**}P < 0.005$; $^{***}P < 0.001$. No significant differences were obtained between inhibition measured with different instruments.

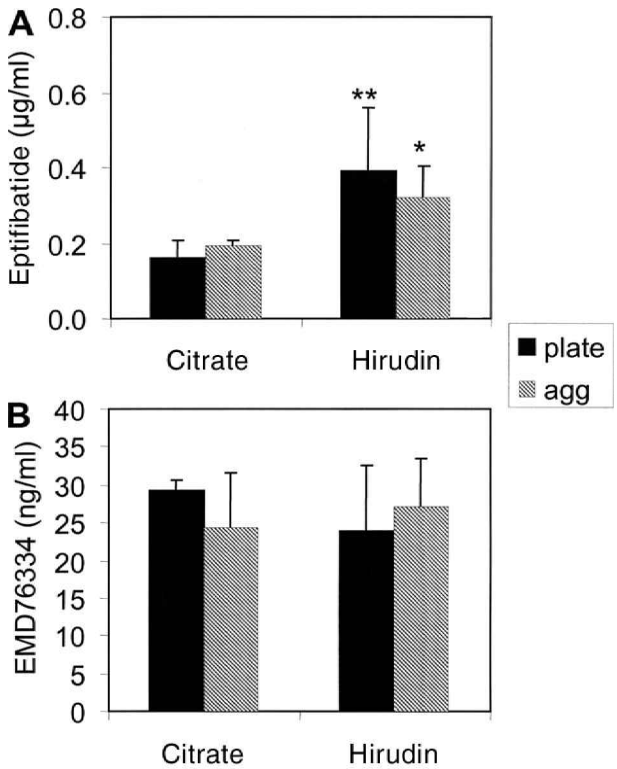

Figure 6. Inhibition of the extent of aggregation, induced by 5 µg/ml collagen. The IC_{50} values of eptifibatide (A) and EMD76334 (B) in citrated and hirudinized PRP are shown. Comparison of standard aggregometry, assessed using the aggregometer LA220 (agg) with the aggregation assay in microplates (plate), means ± S.D., n = 4. Significance between different anticoagulants: $^{*}P < 0.05$ $^{**}P < 0.005$. No significant differences were obtained between inhibition measured with the different instruments.

aggregations were performed in external agitators and the samples were quantified at different timepoints during aggregation or at the end, after fixation.[5,6,14] Other authors were able to show the usefulness of the mixing function of a microplate reader for maintaining the aggregation process, but did not perform inhibitor studies.[15]

There are, however, limitations to this technique: the platelet shape change is hardly detectable, because the readings start only after a delay of about 30 s, and the agitation frequency is fixed by the manufacturer. The possibility of adjustment of agitation frequency could improve the performance of aggregation in the microplate reader.

The data concerning *in vitro* dose-response of platelet aggregation to different GPIIb/IIIa antagonists show that this micromethod may be a meaningful tool for characterizing the pharmacodynamic properties of antiplatelet agents. The found IC_{50} values correspond well to those determined in a standard aggregometer. Higher IC_{50} values for eptifibatide in hirudinized PRP confirm previous findings that reduced Ca^{2+} plasma concentrations affect eptifibatide binding to GPIIb/IIIa and the pharmacodynamic properties of this drug.[11,12,16]

In conclusion, the described method is a suitable tool for reproducibly studying platelet aggregation. It allows estimation of a large number of agonists and antagonists with a low amount of PRP in a very short period of time. Incorporation of new software and further development of the microplate readers (especially the agitation function) would overcome some limitations of this method and would facilitate the methodology of platelet aggregation to characterize the pharmacodynamic properties of antiplatelet agents in clinical trials as well as perhaps in clinical practice.

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